CONSERVATION OF EUROPEAN FRESHWATER CRAYFISH

EDITED BY: Kathrin Theissinger, Javier Dieguez-Uribeondo, Lennart Edsman, Ivana Maguire and Japo Jussila

PUBLISHED IN: Frontiers in Ecology and Evolution, Frontiers in Genetics and Frontiers in Environmental Science

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88974-097-0
DOI 10.3389/978-2-88974-097-0

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

CONSERVATION OF EUROPEAN FRESHWATER CRAYFISH

Topic Editors:
Kathrin Theissinger, Centre for Translational Biodiversity Genomics (LOEWE-TBG), Germany
Javier Dieguez-Uribeondo, Royal Botanical Garden, Spanish National Research Council (CSIC), Spain
Lennart Edsman, Swedish University of Agricultural Sciences, Sweden
Ivana Maguire, University of Zagreb, Croatia
Japo Jussila, University of Eastern Finland, Finland

Citation: Theissinger, K., Dieguez-Uribeondo, J., Edsman, L., Maguire, I., Jussila, J. eds. (2022). Conservation of European Freshwater Crayfish.
Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-097-0

Table of Contents

frontiers in Ecology and Evolution

EDITORIAL
published: 30 November 2021
doi: 10.3389/fevo.2021.804629

Editorial: Conservation of European Freshwater Crayfish

Kathrin Theissinger[1]*, *Lennart Edsman*[2], *Ivana Maguire*[3], *Javier Diéguez-Uribeondo*[4] and *Japo Jussila*[5]

[1] Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz Centre for Translational Biodiversity Genomics, Senckenberg Biodiversity and Climate Research Centre, Frankfurt, Germany, [2] Department of Aquatic Resources, Institute of Freshwater Research, Swedish University of Agricultural Sciences, Drottningholm, Sweden, [3] Department of Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia, [4] Department of Mycology, Real Jardín Botánico–Consejo Superior de Investigaciones Científicas, Madrid, Spain, [5] Department of Environmental and Biological Sciences, University of Eastern Finland, Kuopio, Finland

Keywords: crayfish ecology, invasive species management, disease control, aquaculture, conservation, restocking

Editorial on the Research Topic

Conservation of European Freshwater Crayfish

Freshwater ecosystem functioning is often thought to be dominated by fish, determining the community structure *via* top-down control and ecosystem engineering. However, freshwater crayfish can have an even stronger effect on food web and ecosystem functioning, operating as keystone species in a water body (Longshaw and Stebbing, 2016). As environmental engineers, crayfish have a significant impact on the biodiversity within their habitat (Souty-Grosset et al., 2006). Yet over the past 150 years freshwater crayfish in Europe have faced a novel challenge in the form of a lethal disease caused by the oomycete *Aphanomyces astaci* Schikora 1906, introduced by alien crayfish species of North American origin. Today, the European native crayfish population trends are in decline nearing extinction in several cases (Souty-Grosset et al., 2006; Jussila et al., 2014).

The introduction of different *A. astaci* strains in Europe and the repeated introductions of their North American host species are a classic example of a man-made ecological disaster (Jussila et al.), stemming from the naive belief that the manipulation of an ecosystem would be straightforward. The alien crayfish species, which were supposed to replace the eradicated native stocks, not only transfer the deadly disease but in many parts of Europe also outcompete their native crayfish counterparts, because they are more aggressive giving them additional advantage regarding habitat competition in addition to higher fecundity (Alonso and Martínez, 2006). Introductions of new alien crayfish stocks and thus new *A. astaci* strains will inevitably lead to the total eradication of the remaining native European crayfish stocks.

In this Research Topic we collected scientific work on crayfish conservation from multiple scales, ranging from molecular to species and ecosystem levels, to address the consequences of invasive crayfish and host-parasite interactions on European freshwater biodiversity and ecosystem functioning, aiding conservation and management of European freshwater crayfish to prevent them from extinction.

CRAYFISH MANAGEMENT AND RE-INTRODUCTIONS

Effective management of both endangered native and invasive alien crayfish requires knowledge about distribution, monitoring of existing and early detection of newly established populations. Krieg et al. provided an overview on various management practices to control invasive crayfish in

Edited and reviewed by:
Alison G. Nazareno, Federal University of Minas Gerais, Brazil

***Correspondence:**
Kathrin Theissinger
kathrin.theissinger@senckenberg.de

Specialty section:
This article was submitted to Evolutionary and Population Genetics, a section of the journal Frontiers in Ecology and Evolution

Received: *29 October 2021*
Accepted: *10 November 2021*
Published: *30 November 2021*

Citation:
Theissinger K, Edsman L, Maguire I, Diéguez-Uribeondo J and Jussila J (2021) Editorial: Conservation of European Freshwater Crayfish. Front. Ecol. Evol. 9:804629. doi: 10.3389/fevo.2021.804629

Switzerland. They highlight the great challenges and limitations of such measures like eradication, suppression and containment, and give valuable recommendations for effective invasive crayfish control. Dams are often regarded as effective planning measures to prevent invasive aquatic species spread and to protect the native aquatic fauna, but the effects of dams on crayfish populations have not been thoroughly evaluated. The review by Barnett and Adams outlines that dams can have both beneficial and detrimental effects on crayfish population dynamics.

The underlying mechanisms of native crayfish recovery following biological invasions or their coexistence with invasive species are not clearly determined (Nyström et al., 2001; Kats and Ferrer, 2003; Rodriguez, 2006). It was suggested that the success of invasive over native crayfish represents the synergic interaction of multiple factors. One aspect is the superior competition for resources of the invasive vs. native crayfish (Pacioglu et al., 2020). The study by Parvulescu et al. showed that in interspecific agonistic interactions, the behavior strategy of the invasive crayfish species is based on sheer physical superiority, whereas the native narrow-clawed crayfish relies on intimidation display.

Over the last decades, genetic tools have proven highly beneficial as supplementary tools in biodiversity monitoring. Chucholl et al. provided a novel set of specific eDNA-assays for all native and the most relevant invasive crayfish species in Central Europe, and the efficiency of these assays was assessed regarding the influence of spatio-temporal variables such as distance to upstream population, season and stream size. They show that eDNA-detection is a highly suitable complementary monitoring tool for crayfish, particularly for a large-scale screening of data-deficient catchments or a year-round monitoring. Johnsen et al. proved that eDNA is highly reliable for presence-absence monitoring of noble crayfish, while it cannot substitute the traditional catch per unit effort data, especially for low density populations.

In a study on genetic populations by Martín-Torrijos, Correa-Villalona, Pradillo, et al. new patterns of genetic diversity of the native white-clawed crayfish of the Iberian Peninsula showed the potential effect of paleogeographic barriers on its population structure. The authors suggest that current conservation and management programs should consider three phylogeographic groups as essential management units in order to preserve the remaining genetic diversity, which is critically threatened by crayfish plague (Martín-Torrijos et al., 2019). It has been shown for many crayfish species that there are significant differences regarding the genetic diversity across Europe, and that restocking programs should always consider the natural genetic make-up of the populations (e.g., Schrimpf et al., 2014, 2017; Lovrenčić et al., 2020; Dannewitz et al., 2021). For restocking purposes, conservationists rely on efficient crayfish breeding systems. Regarding the endangered white-clawed crayfish, Nightingale et al. present the optimal diet for rearing endangered white-clawed crayfish. They showed that live food is optimal for high survival and growth in hatchlings as a diet based on plankton and vegetable matter is most beneficial. These results are important for captive breeding success prior to reintroductions.

Manenti et al. outlined an impressive example of a successful reintroduction of the endangered white-clawed crayfish in Italy, after a crayfish plague population collapse caused by the presence of alien crayfish. They showcase the importance of public awareness and stakeholder involvement to enhance the success of such reintroductions. But not only invasive alien crayfish pose a risk to native crayfish fauna. In a study by Tricarico et al. it is highlighted for the first time that invasive raccoons prey on crayfish and pose a severe risk of extinction for the endangered native white-clawed crayfish in Central Italy.

The study by Boštjančić et al. showcase that the high content and diversity of repetitive elements in the genome of the native narrow-clawed crayfish may have provided a driving force for the genome evolution of this native European crayfish. This cytogenomic approach could enhance future studies of other native crayfish species by revealing their evolutionary history and phylogenetic relations.

CRAYFISH PLAGUE MANAGEMENT

The detection of the crayfish plague disease agent *A. astaci* within water bodies is of extreme importance for successful freshwater crayfish management, in addition to the presence or absence detection of invasive crayfish. This oomycete shows a high variability in its virulence among different haplogroups (Makkonen et al., 2012, 2014, 2018; Martín-Torrijos et al., 2021). Thus, it is crucial to monitor not only the distribution of the pathogen, but also to determine its genetic group to infer its virulence and thus the consequences to the affected crayfish populations. The study by Di Domenico et al. now provides a novel set of sensitive and highly specific qPCR assays as a robust tool for fast genotyping of *A. astaci* genotype groups common in Europe.

The controlled infection experiment by Francesconi et al. provided additional evidence of how drastically strains of *A. astaci* differ in their virulence. This study confirmed the adaptation of one specific *A. astaci* haplogroup to their novel European hosts, supposedly due to ongoing coevolution (Jussila et al., 2021) as confirmed by transcriptomic data analyses (Boštjančić et al., 2021). Furthermore, Francesconi et al. experimentally showed that invasive marbled crayfish are remarkably resistant against the crayfish plague disease and could potentially be latently infected, acting as carriers of highly virulent *A. astaci* strains.

In addition to differing virulence levels among *A. astaci* strains, Martín-Torrijos, Correa-Villalona, Azofeifa-Solano, et al. confirmed the presence of the red swamp crayfish associated *A. astaci* strain in coastal habitats, while so far it has been assumed that *A. astaci* is not viable in brackish waters. This finding is of high concern for the conservation of European native freshwater crayfish and highlights once more the risk of introducing invasive alien crustaceans. The crayfish plague pandemic is not only threatening the European freshwaters, as *A. astaci* is spreading worldwide due to globalization and pet trade. Martín-Torrijos, Buckley, et al. report the first-time detection of *A. astaci* in Costa Rica, and give valuable recommendations of invasive red swamp

crayfish management in line with experiences from the European continent, to intervene the detrimental impacts of *A. astaci* on native decapod species in Central America.

MONEY KILLS NATIVE ECOSYSTEMS: EUROPEAN CRAYFISH AS AN EXAMPLE

For the past 150 years, the European crayfish fauna has been devastated by the crayfish plague. As an attempt to economically compensate for this decline, alien North American crayfish such as signal crayfish, have been deliberately released into European water courses. However, the mass mortality of native crayfish has accelerated rather than slowed down as a result. Jussila et al. explain in their Policy and Practice review how this has happened, and which lessons can be learned from this. The case of the European crayfish serves as a particularly evident example of how the introduction of non-native species to replace lost native populations ended up doing more harm than good. Science-based warnings about alien species damage to native ecosystems and native crayfish must be taken seriously and with utmost caution. Protection of native European crayfish is the core issue but not the commercial activities. Finally, Jussila et al. summarize main threats and actions needed to protect remaining native freshwater crayfish fauna in Europe, with emphasis on the halt of alien species spread by stronger EU regulations and law enforcements as well as public awareness initiatives to connect people back to nature.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

ACKNOWLEDGMENTS

We would like to thank the whole freshwater crayfish network in Europe and beyond for the great science, support, comments, and reviews to make this Research Topic on Conservation of European Freshwater Crayfish happen.

REFERENCES

Alonso, F., and Martínez, R. (2006). Shelter competition between two invasive crayfish species: a laboratory study. *Bull. Franç. Pêche Piscicult.* 380–381, 1121–1132. doi: 10.1051/kmae:2006015

Boštjančić, L. L., Francesconi, C., Rutz, C., Hoffbeck, L., Poidevin, L., Kress, A., et al. (2021). Comparative transcriptomics analysis of noble crayfish and marbled crayfish immune response to crayfish plague disease. *bioRxiv*. doi: 10.1101/2021.05.25.445163

Dannewitz, J., Palm, S., and Edsman, L. (2021): Colonization history and human translocations explain the population genetic structure of the noble crayfish (*Astacus astacus*) in Fennoscandia: implications for the management of a critically endangered species. *Aquat. Conserv. Mar. Freshw. Ecosyst.* 31, 1970–1982. doi: 10.1002/aqc.3632

Jussila, J., Francesconi, C., Theissinger, K., Kokko, H., and Makkonen, J. (2021). Is *Aphanomyces astaci* losing its stamina: a latent crayfish plague disease agent from lake Venesjärvi, Finland. *Freshw. Crayfish.* [in print].

Jussila, J., Makkonen, J., Vainikka, A., Kortet, R., and Kokko, H. (2014). Crayfish plague dilemma: how to be a courteous killer? *Boreal Environ. Res.* 19, 235–244.

Kats, L. B., and Ferrer, R. P. (2003). Alien predators and amphibian declines: review of two decades of science and the transition to conservation. *Diver. Distribut.* 9, 99–110. doi: 10.1046/j.1472-4642.2003.00013.x

Longshaw, M., and Stebbing, P. O. (2016). *Biology and Ecology of Crayfish*. New York, NY: CRC Press. doi: 10.1201/b20073

Lovrenčić, L., Bonassin, L., Boštjančić, L., Podnar, M., Jelić, M., Klobučar, G., et al. (2020). New insights into the genetic diversity of the stone crayfish: taxonomic and conservation implications. *BMC Evolut. Biol.* 20:146. doi: 10.1186/s12862-020-01709-1

Makkonen, J., Jussila, J., Kortet, R., Vainikka, A., and Kokko, H. (2012). Differing virulence of *Aphanomyces astaci* isolates and elevated resistance of noble crayfish *Astacus astacus* against crayfish plague. *Dis. Aquat. Organ.* 102, 129–136. doi: 10.3354/dao02547

Makkonen, J., Jussila, J., Panteleit, J., Keller, N. S., Schrimpf, A., Theissinger, K., et al. (2018). MtDNA allows the sensitive detection and haplotyping of the crayfish plague disease agent *Aphanomyces astaci* showing clues about its origin and migration. *Parasitology* 145, 1210–1218. doi: 10.1017/S0031182018000227

Makkonen, J., Kokko, H., Vainikka, A., Kortet, R., and Jussila, J. (2014). Dose-dependent mortality of noble crayfish *(Astacus astacus)* to different strains of the crayfish plague *(Aphanomyces astaci)*. *J. Invertebr. Pathol.* 115, 86–91. doi: 10.1016/j.jip.2013.10.009

Martín-Torrijos, L., Kokko, H., Makkonen, J., Jussila, J., and Diéguez-Uribeondo, J. (2019). Mapping 15 years of crayfish plague in the Iberian Peninsula: the impact of two invasive species on the endangered native crayfish. *PLOS ONE* 14:e0219223. doi: 10.1371/journal.pone.0219223

Martín-Torrijos, L., Martínez-Ríos, M., Casabella-Herrero, G., Adams, S. B., Jackson, C. R., and Diéguez-Uribeondo, J. (2021). Tracing the origin of the crayfish plague pathogen, *Aphanomyces astaci*, to the Southeastern United States. *Sci. Rep.* 11:9332. doi: 10.1038/s41598-021-88704-8

Nyström, P., Svensson, O., Lardner, B., Brönmark, C., and Granéli, W. (2001). The influence of multiple introduced predators on a littoral pond community. *Ecology* 81, 1023–1039. doi: 10.1890/0012-9658(2001)0821023:TIOMIP2.0.CO;2

Pacioglu, O., Theissinger, K., Alexa, A., Samoila, C., Schulz, R., Schrimpf, A., et al. (2020). Multifaceted implications behind the competition of native and invasive crayfish: a glimmer of hope for the native's long-term survival? *Biol. Invasions* 22, 827–842. doi: 10.1007/s10530-019-02136-0

Rodriguez, L. F. (2006). Can invasive species facilitate native species? Evidence of how, when, and why these impacts occur. *Biol. Invasions* 8, 927–939. doi: 10.1007/s10530-005-5103-3

Schrimpf, A., Piscione, M., Cammaerts, R., Herman, D., Collas, M., Jung, A., et al. (2017). Genetic characterization of Western European noble crayfish populations (*Astacus astacus*) for advanced conservation management strategies. *Conserv. Genet.* 18, 1299–1315. doi: 10.1007/s10592-017-0981-3

Schrimpf, A., Theissinger, K., Dahlem, J., Maguire, I., Parvulescu, L., Schulz, H. K., et al. (2014). Phylogeography of noble crayfish (*Astacus astacus*) reveals multiple refugia. *Freshw. Biol.* 59, 761–776. doi: 10.1111/fwb.12302

Souty-Grosset, C., Holdich, D. M., Noël, P. Y., Reynolds, J., and Haffner, P. (Eds.). (2006). *Atlas of Crayfish in Europe.* Paris: Muséum national d'Historie naturelle.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Environmental Science

ORIGINAL RESEARCH
published: 07 December 2020
doi: 10.3389/fenvs.2020.612253

Environmental DNA (eDNA) Monitoring of Noble Crayfish *Astacus astacus* in Lentic Environments Offers Reliable Presence-Absence Surveillance – But Fails to Predict Population Density

Stein I. Johnsen[1†], David A. Strand[2†], Johannes C. Rusch[2,3] and Trude Vrålstad[2]*

[1] Norwegian Institute for Nature Research, Lillehammer, Norway, [2] Norwegian Veterinary Institute, Oslo, Norway, [3] Department of Biosciences, University of Oslo, Oslo, Norway

OPEN ACCESS

Edited by:
Ivana Maguire,
University of Zagreb, Croatia

Reviewed by:
Michael Sweet,
University of Derby, United Kingdom
Chloe Victoria Robinson,
University of Guelph, Canada

***Correspondence:**
David A. Strand
david.strand@vetinst.no

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Environmental Science

Received: *30 September 2020*
Accepted: *17 November 2020*
Published: *07 December 2020*

Citation:
Johnsen SI, Strand DA, Rusch JC and Vrålstad T (2020) Environmental DNA (eDNA) Monitoring of Noble Crayfish Astacus astacus in Lentic Environments Offers Reliable Presence-Absence Surveillance – But Fails to Predict Population Density. Front. Environ. Sci. 8:612253. doi: 10.3389/fenvs.2020.612253

Noble crayfish is the most widespread native freshwater crayfish species in Europe. It is threatened in its entire distribution range and listed on the International Union for Concervation Nature- and national red lists. Reliable monitoring data is a prerequisite for implementing conservation measures, and population trends are traditionally obtained from catch per unit effort (CPUE) data. Recently developed environmental DNA (eDNA) tools can potentially improve the effort. In the past decade, eDNA monitoring has emerged as a promising tool for species surveillance, and some studies have established that eDNA methods yield adequate presence-absence data for crayfish. There are also high expectations that eDNA concentrations in the water can predict biomass or relative density. However, eDNA studies for crayfish have not yet been able to establish a convincing relationship between eDNA concentrations and crayfish density. This study compared eDNA and CPUE data obtained the same day and with high sampling effort, and evaluated whether eDNA concentrations can predict relative density of crayfish. We also compared two analytical methods [Quantitative real-time PCR (qPCR) and digital droplet PCR (ddPCR)], and estimated the detection probability for eDNA monitoring compared to trapping using occupancy modeling. In all lakes investigated, we detected eDNA from noble crayfish, even in lakes with very low densities. The eDNA method is reliable for presence-absence monitoring of noble crayfish, and the probability of detecting noble crayfish from eDNA samples increased with increasing relative crayfish densities. However, the crayfish eDNA concentrations were consistently low and mostly below the limit of quantification, even in lakes with very high crayfish densities. The hypothesis that eDNA concentrations can predict relative crayfish density was consequently not supported. Our study underlines the importance of intensified sampling effort for successful detection of very low-density populations, and for substantiating presumed absence, inferred from negative results. Surprisingly, we found a higher likelihood of eDNA detection using qPCR compared to ddPCR. We conclude that eDNA monitoring cannot substitute CPUE data, but is a reliable

supplement for rapid presence-absence overviews. Combined with eDNA analyses of alien crayfish species and diseases such as crayfish plague, this is a cost-efficient supplement offering a more holistic monitoring approach for aquatic environments and native crayfish conservation.

Keywords: noble crayfish *Astacus astacus*, eDNA, occupancy modeling, qPCR, ddPCR, CPUE, detection frequency, relative density

INTRODUCTION

Freshwater crayfish are regarded as keystone species and shape the littoral zone in both lotic and lentic environments (Creed, 1994; Momot, 1995). Their presence in aquatic environments, influencing sediment dynamics and benefiting other animals, has also led freshwater crayfish to be characterized as ecosystem engineers and umbrella species (Usio and Townsend, 2001; Reynolds et al., 2013). Furthermore, they are regarded as indicator species for water quality (Sylvestre et al., 2002). In addition, some species of freshwater crayfish are harvested and regarded as delicacies, obtaining high prices in the market (Ackefors, 1998). One of these prized species is the noble crayfish, *Astacus astacus*, which is indigenous to Europe and the only indigenous species of freshwater crayfish in Norway (Souty-Grosset et al., 2006; Kouba et al., 2014). There are currently about 470 registered Norwegian populations of noble crayfish (Johnsen and Vrålstad, 2017). Along with populations of other native freshwater crayfish species indigenous to Europe, the number of noble crayfish populations has declined dramatically in the last decades. This is mostly due to introduced North-American crayfish species that carry and transmit the crayfish plague pathogen *Aphanomyces astaci*, but also due to anthropogenic influences such as pollution and habitat loss (Holdich et al., 2009; Kouba et al., 2014). Hence, the noble crayfish is both on the international (Edsman et al., 2010) and the national red list (Henriksen and Hilmo, 2015). The red list status and its importance in freshwater ecosystems has led to the development of surveillance programs aiming to monitor distribution and relative density of noble crayfish (Johnsen et al., 2019). In Norway, as in other countries, estimates of relative density are obtained by trapping crayfish with baited traps (Johnsen et al., 2014). This is relatively time consuming, and in order to increase the number of monitored populations, environmental DNA (eDNA) methodology has recently been included in the Norwegian surveillance programs for both crayfish plague and freshwater crayfish (Johnsen et al., 2019; Strand et al., 2020). The methods are also used in crayfish monitoring studies in Europe (Robinson et al., 2018; Mauvisseau et al., 2019b; Rusch et al., 2020; Troth et al., 2020).

During the past decade, eDNA methods have been increasingly used as monitoring tool for freshwater organisms (Leese et al., 2016; Bylemans et al., 2019; Strand et al., 2019; Goutte et al., 2020). These methods utilize DNA traces in the environment originating from single-celled microorganisms or cells shed from complex organisms in the form of propagules, mucus, abraded epithelial cells and body fluids (Thomsen and Willerslev, 2015). These sources of eDNA are easily caught on a filter or pelleted from a water sample, from which DNA can be extracted and analyzed. Thus, from a water sample, it is possible to detect specific species or even whole communities (Deiner et al., 2017; Bylemans et al., 2019; McElroy et al., 2020). Quantitative real-time PCR (qPCR) or digital droplet PCR (ddPCR) are commonly used for species-specific detection (Rusch et al., 2018; Capo et al., 2019; Mauvisseau et al., 2019a; Strand et al., 2019), and for relative or absolute quantification of target DNA, respectively (Demeke and Dobnik, 2018; Quan et al., 2018), while high-throughput sequencing and metagenomics is used to study whole communities (Thomsen et al., 2012; Hänfling et al., 2016; McElroy et al., 2020). While qPCR is currently the most common platform to analyze eDNA samples using species-specific assays, recent studies suggest that the detection rate of eDNA in environmental samples is higher when using ddPCR compared to qPCR technology (Doi et al., 2015a; Mauvisseau et al., 2019a; Wood et al., 2019; Brys et al., 2020). With ddPCR there is no need for standard curves and ddPCR allows for absolute quantification, even at low levels of DNA copies. Additionally, ddPCR appears to be more robust against PCR inhibition (Doi et al., 2015a; McKee et al., 2015; Mauvisseau et al., 2019a; Brys et al., 2020).

Environmental DNA methods allow for rapid detection of targeted species and are considered a promising monitoring tool for aquatic species surveillance and inventories (Lodge et al., 2012; Thomsen and Willerslev, 2015), including monitoring and discovery of endangered and invasive species (Dejean et al., 2012; Laramie et al., 2015; Strand et al., 2019). Additionally, eDNA methodology is non-invasive compared to more traditional methods where the species itself is caught (Thomsen and Willerslev, 2015). An increasing number of studies show that eDNA monitoring is suitable for acquiring presence/absence information of targeted species (Hempel et al., 2020; Mason et al., 2020; Villacorta-Rath et al., 2020), although "not-detected" data cannot be taken as absolute proof of absence (Rusch et al., 2020). For marine and freshwater fish species, it has also been suggested that eDNA concentrations can be used to estimate population density or biomass of a species (Takahara et al., 2012; Thomsen et al., 2012; Lacoursière-Roussel et al., 2016). For freshwater crayfish, several studies demonstrate the use of eDNA to determine the presence or absence of species (Tréguier et al., 2014; Agersnap et al., 2017; Harper et al., 2018; Strand et al., 2019; Rusch et al., 2020), which is very useful for verification of species presence and species distribution. A few studies have investigated the relationship between population density of targeted crayfish species and eDNA concentration, but so far no or only weak correlations have been reported (Dougherty et al., 2016; Cai et al., 2017; Larson et al., 2017; Rice et al., 2018; Troth et al., 2020). More

studies are thus needed to evaluate whether eDNA concentrations can somehow reflect the relative population density of freshwater crayfish species.

Goal of Study

We aimed to make a direct comparison between the traditional monitoring of noble crayfish using baited traps, and targeted eDNA monitoring by means of species-specific qPCR and ddPCR. This will help assess if eDNA yields valid presence/absence data for noble crayfish. We further use occupancy modeling to estimate the probability to detect eDNA of noble crayfish at various population densities, ranging from very low to high density populations. We also explore whether eDNA concentrations in the water correlates with observed relative density of noble crayfish. Finally, we compared the qPCR and ddPCR results and efficiency for eDNA detection of noble crayfish.

MATERIALS AND METHODS

Study Sites

In total eight lakes in the south-eastern part of Norway were included in this study (**Figure 1A** and **Table 1**). These study sites were selected according to an expected range of crayfish abundance, based on results from other projects such as the *National surveillance program for noble crayfish* (Johnsen et al., 2019). Here, we have available data from several years of trap catches, including estimates of relative density from catch per unit effort data (CPUE). To avoid larger molting periods, which would influence both the catchability of crayfish (Westman and Pursiainen, 1982) and possibly the eDNA concentrations in the water (Laurendz, 2017), six of the locations were sampled after mid-August. Due to the high fishing pressure in Lake Einafjorden and Steinsfjorden, we surveyed these two localities from the 7 to 9th of August 2016.

Crayfish Trapping

In each lake, 50 funnel LiNi traps with two entrances and 14 mm mesh size were set along the shoreline in the depth interval 0.5–5 m (**Table 1** and **Figure 1B**), in accordance with the methods used in the national monitoring program for noble crayfish (Johnsen et al., 2019). The traps were baited with raw chicken, set in the evening and emptied the next morning. The relative density or CPUE is given as the number of crayfish per trapnight. The number of crayfish caught per trapnight is regarded as a reliable measure of crayfish density with sufficient effort, and even at efforts as low as 15 trapnights (Zimmerman and Palo, 2011; Johnsen et al., 2014).

Water Samples

A total of 8–12 water samples were collected for eDNA analysis from each lake, along the same transect in which the traps were placed (**Figure 1B**). We filtered up to 5 L per sample, but in some cases less if the filter clogged. In order to avoid any possible contamination or influence of eDNA results from the trapping activity, the water samples were collected and filtered prior to setting the traps, but still on the same day. The samples were filtered directly from the boat using a battery driven peristaltic pump (ES portable sampler, Masterflex, Cole-parmer, Vernon Hills, United States) with tygon tubing (Masterflex) and a 47 mm inline filter-holder (Millipore, Billerica, United States) (Strand et al., 2019). Glass-fiber filters (AP2504700, Millipore) with an effective pore size of 2 μm were used, as in previous studies (Agersnap et al., 2017; Strand et al., 2019; Rusch et al., 2020). The inlet of the tube was attached to a small plastic box weighed down with lead in order to collect water ∼5 cm above the bottom, between 1 and 3 meters depth depending on the shoreline and lake. After filtration, each filter was transferred to a 15 ml falcon tube and stored on ice until return to the laboratory where the filter samples were frozen awaiting DNA extraction.

Extraction of gDNA and eDNA

In order to obtain reference DNA for positive controls and standard dilution series, genomic DNA (gDNA) was extracted from noble crayfish tissue (abdominal muscle) using QIAmp® DNA mini kit with the QIAcube automated DNA extractor (Qiagen) following the manufacturer's protocol. The DNA concentration was measured using the Qubit 1x dsDNA HS Assay Kit and Qubit 4 Fluorometer (Invitrogen, life technologies) according to the manufacturer's protocol. Environmental DNA was extracted from the glass fiber filters using an extraction protocol as described in Strand et al. (2019). In short, eDNA samples were lysed in cetyltrimethylammonium buffer (CTAB) and proteinase K at 65°C for 60 min, cleaned and separated using chloroform, precipitated using isopropanol and re-suspended in TE-buffer (pH 8). Due to the large volume of eluate (4 ml) from each sample, the eluates were divided into two subsamples to bypass the volume restrictions caused by centrifuge size. These subsamples were then merged after re-suspension in TE-buffer. During the extraction of DNA, an environmental blank control and an extraction blank control were incorporated in order to measure potential contamination during the DNA extraction step.

qPCR and ddPCR Protocols

gDNA and eDNA extracts were analyzed with a species-specific assay targeting the cytochrome c oxidase subunit I (COI) of noble crayfish published in Rusch et al. (2020): forward primer 5′-CCC CTT TRG CAT CAG CTA TTG-3′, reverse primer 5′-CGA AGA TAC ACC TGC CAA GTG T-3′ and probe FAM-5′ CTC ATG CAG GCG CAT-MGBFNQ. This assay was specifically designed and optimized for both the qPCR and ddPCR platforms. A total of six technical replicates (three undiluted and three 5x diluted) were analyzed from each sample on both platforms (qPCR and ddPCR). The qPCR analysis was performed on a Bio-Rad CFX96 Touch (Bio-Rad, Hercules, CA, United States) in a 25 μL reaction volume. Each reaction consisted of 12.5 μL TaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific, Waltham, United States), 500 nM of each primer, 250 nM probe, nuclease free water and 5 μl DNA sample. The thermocycling protocol consisted of an initial warming at 95°C for 10 min followed by 50 cycles of 95°C for

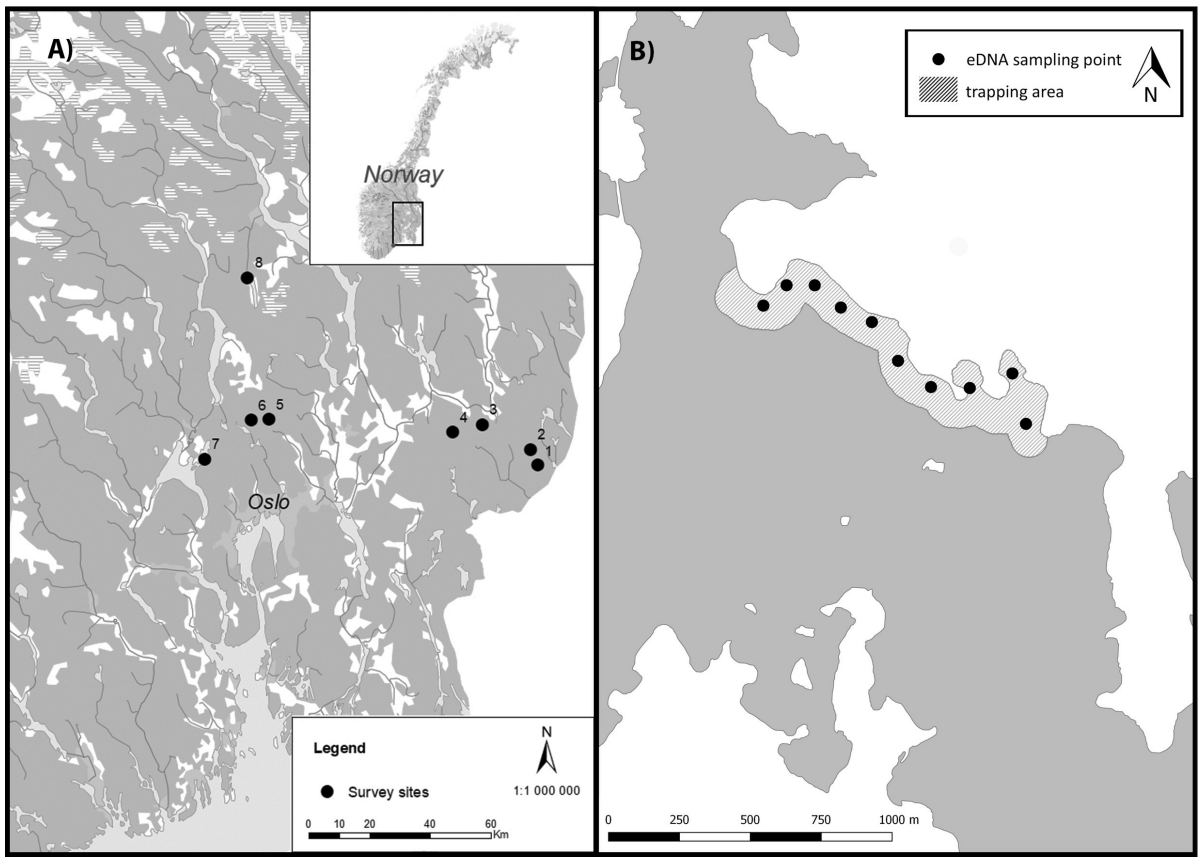

FIGURE 1 | (A) Location of the monitored lakes in Norway. Black dots indicate the lakes and numbers identify the lakes, referring to **Table 1**. **(B)** Illustration of sampling transect of crayfish trapping and eDNA sampling at location 8. Here, 50 traps were set along the shoreline in the gray shaded area, and 8–10 filtrated water samples (black dots) were collected from each lake.

TABLE 1 | Lake and lake size with sample dates and effort of eDNA samples and traps.

Lake (number)	Lake size (ha)	Sample date	# eDNA samples	Trap effort (trapnights)
Baereia (3)	138	01.09.2015	12	50
Baereia (3)	138	23.08.2017	10	50
N. Billingen (2)	176	02.09.2015	9	50
N. Billingen (2)	176	24.08.2017	10	50
S. Billingen (1)	136	02.09.2015	10	50
S. Billingen (1)	136	25.08.2017	10	50
Skårillen (4)	46	03.09.2015	10	50
Skårillen (4)	46	22.08.2017	8	50
Harestuvatnet (5)	206	10.09.2015	9	50
Gjerdingen (6)	300	09.09.2015	9	50
Steinsfjorden (7)	1384	09.08.2016	8	48
Einafjorden (8)	1382	07.08.2016	10	50

*Lake number (within parenthesis is related to the number seen in **Figure 1**).*

15 s and 60°C for 60 s. Four known concentrations of 10-fold diluted genomic DNA (noble crayfish) were run in duplicates as a positive control and standard with known DNA copy numbers (based on absolute quantification of ddPCR) in order to calculate the DNA copies in each reaction (relative quantification) using the manufactures software (CFX Manager v. 3.1.1517.0823).

The ddPCR analysis was performed on a QX200 AutoDG Droplet Digital PCR System (Bio-Rad) in a 22 µl reaction volume. Each reaction consisted of 11 µl ddPCR supermix for probes (no dUTP, Bio-Rad), 900 nM of each primer, 300 nM of probe, 0.6 µl bovine serum albumin (BSA), nuclease free water and 5 µl DNA. Droplets were generated using AutoDG

instrument (Bio-Rad), where an emulsion is created with 20 μl of the 22 μl reaction volume, resulting in a 10% loss of DNA template and supermix. After droplet generation, the plates were transferred to a TM100 thermocycler (Bio-Rad) with the following cycling conditions: an initial warming at 95°C for 10 min followed by 45 cycles of 94°C for 30 s and 60°C for 60 s and a final step at 98°C for 10 min to inactivate the enzyme. Ramp rate was set to 2°C/s. The plate was thereafter transferred to the QX200 droplet reader (Bio-Rad) for final analysis. A known concentration of genomic DNA (noble crayfish) was run in duplicate as a positive control. The DNA copies in each reaction were calculated (absolute quantification) using the manufactures software (Quantasoft v.1.7.4.0917).

The environmental blank control and extraction black control from each batch of extracted DNA were included in both the qPCR and ddPCR analysis. Negative blank controls (MilliQ water) were also included on each plate analyzed.

Limits of Detection and Quantification

For the qPCR approach, the limit of detection (LOD) had been established by Rusch et al. (2020) as five copies/qPCR reaction, and followed the criteria that LOD is the lowest concentration that yields a detection probability of 95%, ensuring <5% false negatives (Berdal et al., 2008; Vrålstad et al., 2009). Likewise, the limit of quantification (LOQ) for the qPCR assay has been established as 10 DNA copies/qPCR reaction (Rusch et al., 2020), using the same acceptance level as set for qPCR quantification of the crayfish plague pathogen *A. astaci* (Vrålstad et al., 2009), with observed standard deviation <0.5 for the Ct-values. Following previous recommendations, a cut-off was set at Ct 41 for the qPCR assay (Agersnap et al., 2017; Strand et al., 2019; Rusch et al., 2020), implying that amplification at or above this value was not considered a positive detection. For each eDNA sample, the following criteria for a positive sample were used: if three or more of the six technical qPCR replicates for a sample were positive below Ct 41, the sample was considered positive.

The same standard dilutions used to estimate the LOQ and LOD for the qPCR assay in Rusch et al. (2020) were analyzed with the ddPCR assay in order to estimate the absolute limit of quantification (aLOQ) and LOD. The aLOQ for ddPCR can be estimated as the lowest copy number of a target within the dynamic range with a relative standard deviation (RSD) of the measured copy number ≤25% (Dobnik et al., 2015). The theoretical LOD, i.e., the lowest concentration that yields a detection probability of 95%, is estimated to be 0.29 copies/μl for ddPCR in the case 17,000 accepted droplets and corresponds to ∼5.8 copies in a 20 μl reaction (Pecoraro et al., 2019). The standard used had a starter concentration of 50 ng/μl of noble crayfish gDNA, and was four-fold diluted to create a dilution series ranging from 12.5 ng/μl (4^{-1}) to 0.00000075 ng/μl (4^{-13}). As ddPCR offers absolute quantification, the ddPCR results from the 3rd fourfold-dilution of gDNA were used as a baseline for the qPCR standard in order to estimate the relative DNA copies in each qPCR reaction. There is no consensus on the number of positive droplets that are required to score a ddPCR replicate as positive, but the threshold is commonly set at 2, 3, or 5 droplets (Dobnik et al., 2015). Since we analyzed six replicates for each sample, and all analyzed blank controls were negative (N = 81), we scored a sample positive when three or more of the six technical ddPCR replicates of the sample had one or more positive droplets. Reactions with total droplet count below 8,000 were excluded.

We estimated the DNA copy numbers per liter water from each sample for both qPCR and ddPCR according to Agersnap et al. (2017) using the equation: $C_L = [C_r * (V_e/V_r)]/V_w$. Here C_L = copies of eDNA per liter lake water, C_r = copies of eDNA in reaction volume, V_e = total elution volume after extraction, V_r = volume of eluded extract used in the qPCR/ddPCR reaction, V_w = volume of filtered lake water.

Statistical Analysis

The statistical analyses were performed using RStudio (v.1.3.1073) and R (v.4.0.2). We compared the qPCR and ddPCR reaction estimates of DNA copies from gDNA using a spearman correlation test, and used a generalized linear model (GLM) with the binominal family with logit link logistic to compare detection (TRUE/FALSE) against method (qPCR/ddPCR) and dilution series. We compared the detection frequency of qPCR and ddPCR of DNA from the field samples using a GLM with the binominal family with logit link logistic to compare detection (TRUE/FALSE) against method (qPCR/ddPCR) and CPUE. We used the R package eDNA occupancy to run multiscale occupancy modeling in order to estimate the detection probability of crayfish using the targeted eDNA approach (Dorazio and Erickson, 2018). The multiscale occupancy model estimates (1) the probability of species occurrence at the location (psi or ψ), (2) the conditional probability of occurrence in a water sample given that the species is present at that location (theta or θ), and (3) the conditional probability of detection in a PCR reaction given that the species is present in the sample (ρ) (Dorazio and Erickson, 2018). The models were run separately for the qPCR and ddPCR data sets. For samples with less than three positive technical replicates all replicates were scored as negative, as described above. We first tested the occupancy models with assumed constant parameters. Then we included the factor CPUE into the occupancy modeling in order to investigate how relative crayfish densities influence the detection probability in a water sample (θ). We assumed constant parameters for eDNA occurrence in lakes (psi) and in PCR replicate (p) while the conditional probability of eDNA occurrence in water samples (theta) was assumed to be a function of CPUE. The models were run for a total of 11,000 iterations. We used the equation $1 - (1 - \theta)^n \geq 0.95$ to calculate the number of water samples to achieve a 95% detection probability, where θ is the estimated probability to detect crayfish eDNA in a water sample. We also compared (spearman correlation) the mean DNA copies from each field sample from both qPCR and ddPCR with CPUE.

RESULTS

Comparisons of qPCR and ddPCR Assay

Both the qPCR and ddPCR amplified the gDNA standard and there is a significant correlation (R^2 = 0.9, p < 2.2e-16) between

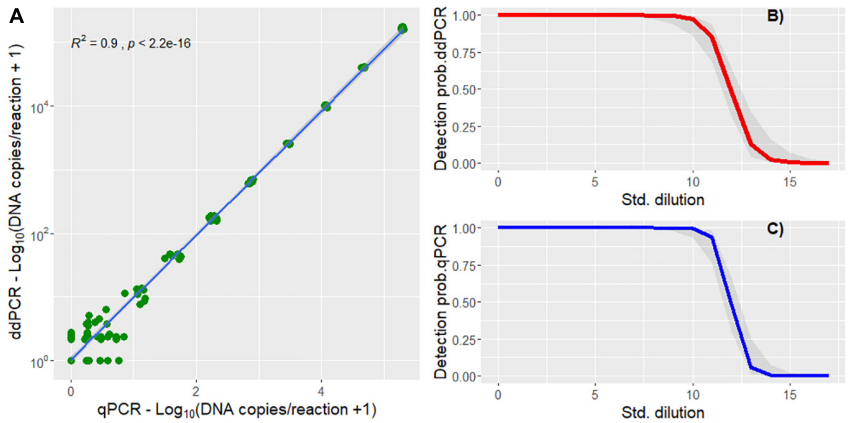

FIGURE 2 | (A) Correlation plot of the estimated DNA copies per reaction of qPCR and ddPCR analysis of gDNA from crayfish tissue using spearman correlation (R^2 = 0.9, p < 2.2e-16). **(B)** Logistic regression curve showing probability of gDNA detection against the dilution series number using ddPCR. **(C)** Logistic regression curve showing probability of detection against the gDNA dilution series number using qPCR.

TABLE 2 | The qPCR and ddPCR results for the analysis of the standard dilution used to estimate LOQ and LOD for qPCR (Rusch et al., 2020) and ddPCR (this study) using the same assay.

		qPCR							ddPCR					
Standard	**DNA (ng/µl)**	***N***	**Cq**	**Cq Std**	**DNA copies**	**SD****	**Detection**	**RSD*****	***N***	**Pos. Drops***	**DNA copies**	**SD****	**Detection**	**RSD*****
Undiluted	5.00E + 01													
S4^1	1.25E + 01	6	18.00	0.07	3046534.65	139026.71	100%	5%	6	17101.3	>200000	NA	NA	NA
S4^2	3.13E + 00	8	19.95	0.06	798995.10	34118.09	100%	4%	8	18460.0	>200000	NA	NA	NA
S4^3	7.81E-01	8	22.01	0.04	194510.60	5600.27	100%	3%	8	18258.1	167000.00	7618.77	100%	5%
S4^4	1.95E-01	8	24.08	0.07	47080.87	2210.02	100%	5%	8	15028.3	40677.50	736.98	100%	5%
S4^5	4.88E-02	8	26.11	0.05	11668.85	377.37	100%	3%	8	6326.5	10017.50	226.89	100%	2%
S4^6	1.22E-02	8	28.09	0.07	3006.44	138.10	100%	5%	8	1890.1	2548.00	50.55	100%	2%
S4^7	3.05E-03	8	30.13	0.08	740.64	38.82	100%	5%	8	486.6	650.00	33.87	100%	5%
S4^8	7.63E-04	8	32.14	0.17	186.55	21.18	100%	11%	8	123.1	171.50	13.26	100%	8%
S4^9	1.91E-04	20	34.20	0.32	46.23	9.18	100%	20%	20	33.9	43.25	3.01	100%	7%
S4^10	4.77E-05	20	36.22	0.39	11.62	2.62	100%	23%	20	6.7	9.54	3.43	100%	36%
S4^11	1.19E-05	20	38.83	1.01	2.35	1.55	100%	66%	20	1.7	1.98	1.42	85%	72%
S4^12	2.98E-06	20	39.71	0.56	0.39	0.60	35%	156%	20	1.0	0.69	0.80	45%	115%
S4^13	7.45E-07	20	39.96	0.03	0.17	0.36	20%	205%	12	1.0	0.25	0.59	17%	234%

**Average number of positive droplets, negative droplets excluded. **Standard deviation. ***Relative standard deviation.*

the estimated DNA copies from the two methods (**Figure 2A**). There was no significant difference (z = 0.492, p = 0.623, logistic regression) in detection rate between the two methods on the dilution series (**Figures 2B,C**). The aLOQ for the ddPCR was estimated to be ∼ 40 copies, the last standard dilution where the RSD was ≤25% (**Table 2**). In the initial ddPCR run several of the positive field samples showed signs of inhibition and lower amplitude of the positive droplets compared to the positive control. Adding BSA to the reaction or diluting the sample (1:5) appeared to improve this issue by increasing the amplitude enough to separate negative from positive droplets. A total of 690 reactions were run both for qPCR and for ddPCR. Here, 33 ddPCR reactions were excluded due to total droplet count below 8,000. Of the remaining 657 ddPCR reactions, 28.5% of the reactions had one or more positive droplets, while 42.6% of the 690 qPCR reactions had a positive amplification below the set cut-off (Cq 41). Both for the qPCR and ddPCR results, most of the reactions were below the LOQ (**Figure 3**) indicating low concentration of noble crayfish eDNA. None of the environmental blank controls or extraction blank controls displayed any amplification or produced positive droplets. Neither did the negative blank controls display amplification or produce positive droplets.

Relative Density of Crayfish Compared to eDNA Presence/Absence Data

The CPUE of noble crayfish in the different localities ranged in mean values between 0.08 and 17.52 (**Figure 4** and **Supplementary Table 1**). According to the classification system

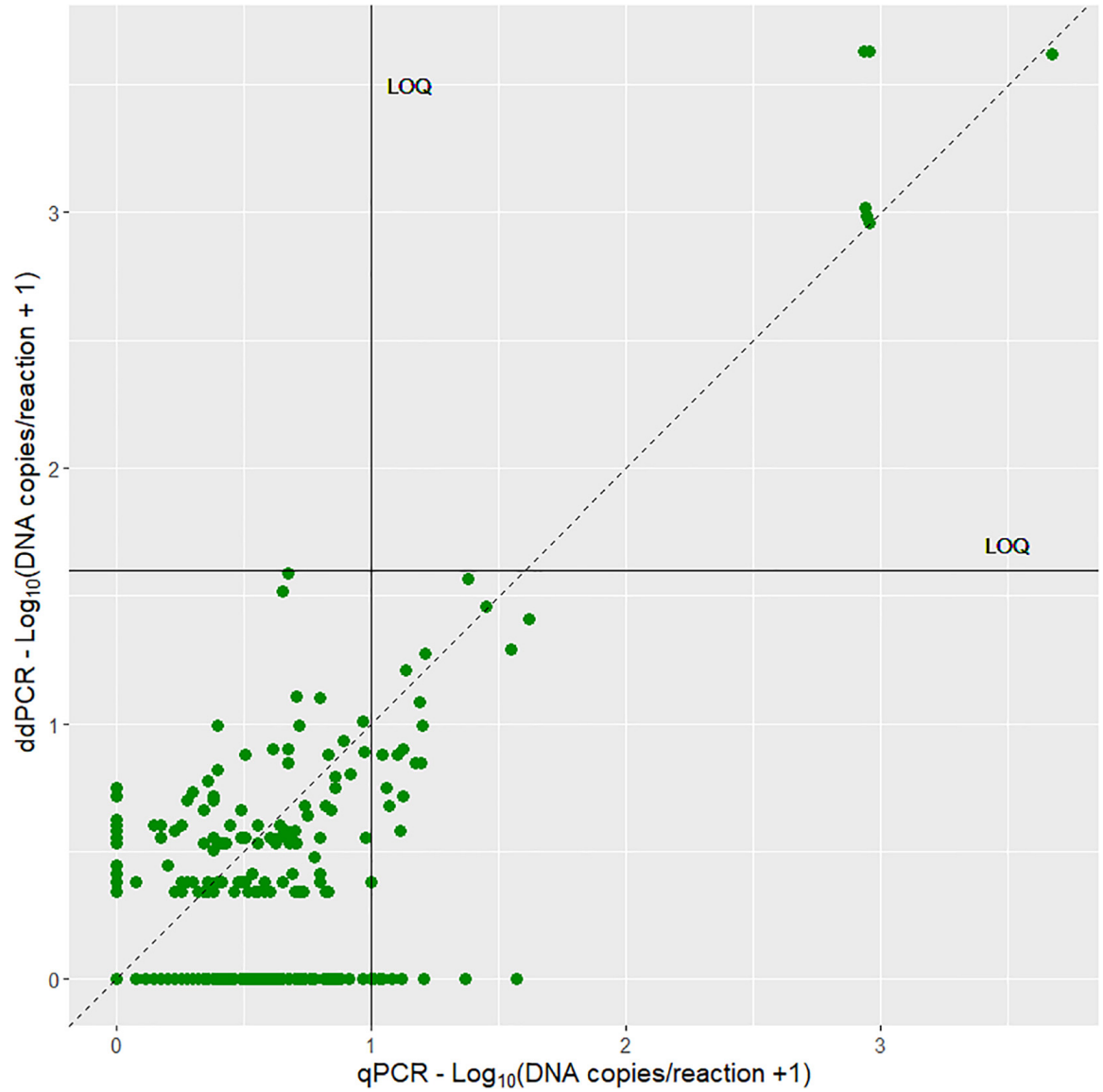

FIGURE 3 | A scatterplot of the estimated DNA copies per reaction of qPCR and ddPCR analysis of eDNA. The stippled line indicates a 1:1 ratio, while the horizontal line and vertical line indicates the LOQ for ddPCR and qPCR, respectively.

of the national surveillance program of noble crayfish in Norway (Johnsen et al., 2019), the crayfish populations cover all categories, ranging from "very low density" populations to "very high density" populations (**Figure 4**). We detected eDNA from noble crayfish in all lakes, but in 2015 we failed to detect noble crayfish eDNA in one of the sampled lakes (Lake Skårillen). In this case, there was very heavy rainfall before and during the sampling. Noble crayfish eDNA was detected in this lake in 2017 (**Figure 4**). Even in lakes with very low crayfish densities (**Figures 4**, **5**), noble crayfish eDNA had been reliably detected with a qPCR detection frequency ranging from 0.3 to 0.7. The detection frequency of noble crayfish eDNA was significantly higher (z = 4.27, p = 1.93e-05, logistic regression) for qPCR compared to ddPCR (**Figure 5A**), and the detection frequency increased (z = 4.55, p = 5.46e-06, logistic regression) for both qPCR and ddPCR with increased CPUE (**Figure 5B**).

Occupancy Modeling

The occupancy models with constant parameters (**Table 3**) also showed that it is more likely to detect eDNA of crayfish using qPCR compared to ddPCR. Including the CPUE as a factor in the occupancy models gives an estimate of detection likelihood in water samples at different relative densities of crayfish, and the likelihood to detect eDNA of noble crayfish in water samples increases with relative densities, both for qPCR and for ddPCR (**Figure 6**). At all but the highest relative density (CPUE ∼17.52) estimated occurrence of eDNA in a

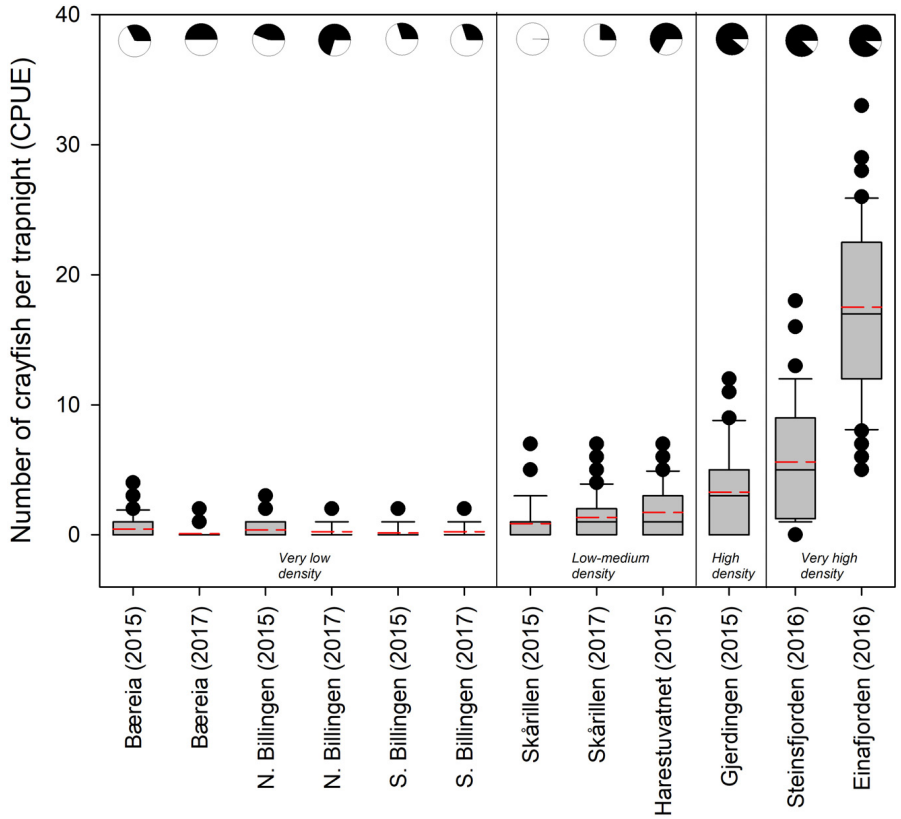

FIGURE 4 | Boxplot showing the number of noble crayfish per trapnight (CPUE) in the eight localities. The plots are based on the number of crayfish caught in the individual traps within the lake. The box contains 50% of the individual trap values, the median and the average values are shown as black and stippled red lines, respectively. The whiskers indicate the 5 and 95 percentiles and the dots (•) show values outside this interval. The pie charts depict the qPCR detection frequency (black part being positive) from the water samples. ddPCR detection frequency is not included. Exact values are given in **Supplementary Table 1**.

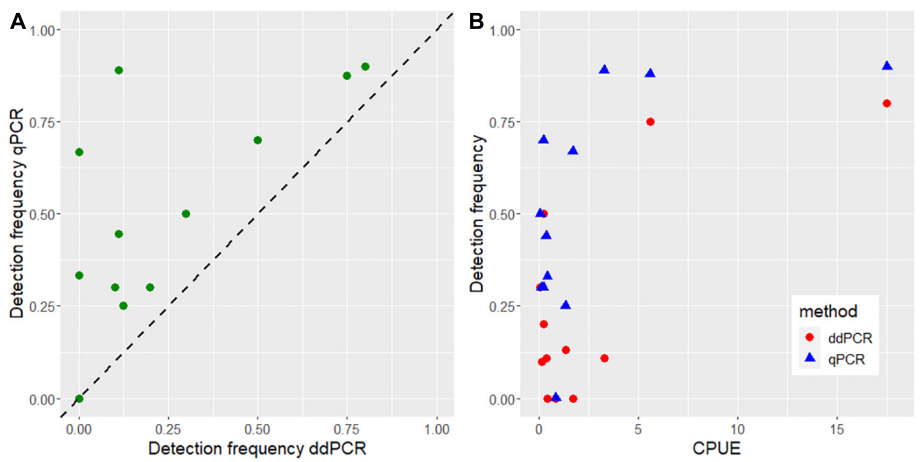

FIGURE 5 | (A) A comparison of the observed detection frequency (proportion of positive eDNA samples in a lake) between qPCR and ddPCR. The dotted line shows the 1:1 ratio. **(B)** A comparison of the observed detection frequency between CPUE against ddPCR and qPCR.

water sample is lower using ddPCR analysis compared to qPCR (**Figure 6**). For qPCR, the probability of eDNA occurrence is relatively high (around 0.5) even at the CPUE estimates below 1 (**Figure 6**). At very low to low densities five water samples are sufficient to detect eDNA of noble crayfish with 95% likelihood using qPCR, while 11 to 14 water samples are needed to detect noble crayfish with 95% likelihood using ddPCR (**Figure 7**).

TABLE 3 | The estimated likelihood of occurrence at the location (ψ), in the water sample (θ) or PCR replicate (*p*) for qPCR and ddPCR using fixed parameters.

	psi (ψ)	theta (θ)	*P*
qPCR	0.876 (0.645–0.981)	0.542 (0.447–0.634)	0.706 (0.655–0.753)
ddPCR	0.814 (0.563–0.965)	0.318 (0.225–0.419)	0.737 (0.671–0.799)

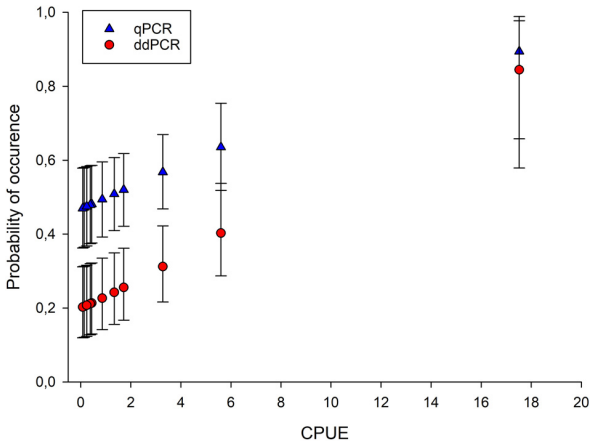

FIGURE 6 | The estimated probability of eDNA occurrence in water samples (θ) using occupancy models where the probability of eDNA occurrence in lakes was assumed to be constant, the conditional probability of eDNA occurrence in water samples was assumed to be a function of CPUE and the conditional probability of eDNA detection in PCR replicate was assumed to be constant.

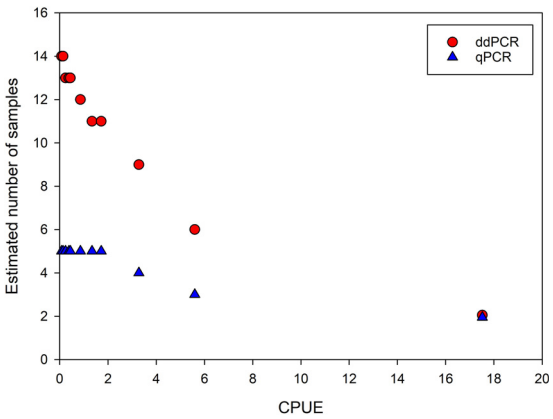

FIGURE 7 | Estimated number of water samples needed to achieve 95% detection likelihood at different relative densities of crayfish (CPUE) for qPCR and ddPCR.

Relative Density of Crayfish Compared to eDNA Copy Numbers

The copy numbers estimated from the qPCR and ddPCR reactions in our study were very low and most of the numbers were below the LOQ (**Figure 3**). The estimated DNA copy numbers that are below LOQ are unreliable due to high variation when the copy numbers in a sample are low, and thus not suitable for statistical analysis. However, it is not uncommon to see studies where eDNA copy numbers calculated below LOQ are correlated with density (or biomass) estimates. We did a correlation test (spearman) in order to compare DNA concentration in water (DNA copies per liter) to relative density of crayfish (CUPE), and the test showed a very weak positive correlation between eDNA concentrations and CPUE data, both for qPCR and ddPCR results (**Figure 8**). This result is highly uncertain and interpreted with great caution.

DISCUSSION

Populations of native European noble crayfish are currently being lost at an alarming rate, largely because of North-American invasive crayfish that carry and transmit the crayfish plague pathogen (Holdich et al., 2009; Kouba et al., 2014). There is therefore an urgent need for better, powerful and dedicated conservation and management strategies. This requires solutions

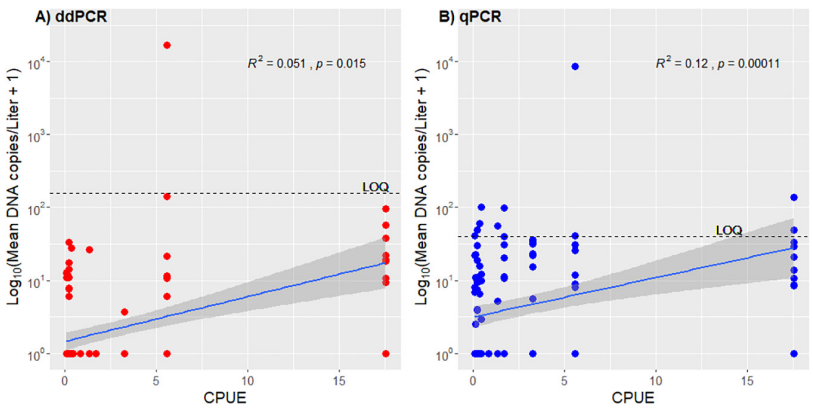

FIGURE 8 | (A) A scatterplot with correlation (Spearman) of the mean DNA copies per liter for each sample from ddPCR plotted against CPUE of crayfish. The horizontal line indicates the LOQ of ddPCR adapted to fit the copies per liter values. **(B)** A scatter plot with correlation (Spearman) of the mean DNA copies per liter for each sample from qPCR plotted against CPUE of crayfish. The horizontal line indicates the LOQ of qPCR adapted to fit the copies per liter values.

derived from understanding, knowledge and measures related to biology, socioeconomics and legal frameworks. Here, efficient monitoring strategies play a key role. In the past decade, eDNA monitoring has emerged as a promising, cost-effective tool for monitoring of endangered and invasive species (Jerde et al., 2011; Lodge et al., 2012) and disease pathogens (Strand et al., 2011, 2014; Gomes et al., 2017) with the potential to simplify and streamline species surveillance (Kelly et al., 2014; Leese et al., 2016). High expectations have been connected to eDNA data as predictors for species distribution, relative density and biomass estimates (Bohmann et al., 2014; Kelly et al., 2014; Doi et al., 2017). However, with the growing body of reports and research, the first optimistic prospects regarding the applicability of eDNA prediction have encountered reality, turning out to vary for different groups of organisms, habitats and choice of methods. Several proof-of-concept studies have shown that eDNA monitoring yields adequate presence-absence and distribution data for crayfish, with a high potential for crayfish surveillance. But, in contrast to some studies focusing on fish (Lacoursière-Roussel et al., 2016; Doi et al., 2017), there are so far few if any studies on crayfish that have established a convincing quantitative relationship between eDNA concentrations in the water and crayfish biomass or density. We have systematically compared traditional trapping methods and eDNA monitoring with a thorough sampling effort. In concordance with other studies (Dougherty et al., 2016; Cai et al., 2017; Larson et al., 2017; Strand et al., 2019; Troth et al., 2020), our results support the assumption that eDNA yields valid presence-absence data, even at very low population densities. However, lakes with low-density crayfish populations require higher sampling effort than commonly used in comparable studies and monitoring programs. We found that the probability of detecting noble crayfish from eDNA samples increased with increasing relative crayfish densities, corroborating similar studies (Dougherty et al., 2016; Larson et al., 2017). However, no significant correlation between noble crayfish eDNA concentrations in the water and relative crayfish density could be established. Thus, our eDNA data does not support the hypothesis that eDNA concentrations can predict relative crayfish density.

Efficiency for eDNA Detection of Noble Crayfish – qPCR Versus ddPCR

It is commonly reported that ddPCR overcomes the challenges connected to inhibitory substances to a higher extent than qPCR, and displays both higher sensitivity and higher quantification accuracy (Dingle et al., 2013; Rački et al., 2014; Zhao et al., 2016), and several studies have highlighted the benefits of ddPCR for eDNA detection (e.g., Doi et al., 2015a; Hunter et al., 2017; Hamaguchi et al., 2018; Mauvisseau et al., 2019a; Brys et al., 2020). However, while ddPCR offers absolute quantification, the variation in estimated copy numbers increases at low numbers, therefore the LOQ of ddPCR is often within the same range as LOQ for qPCR (Dobnik et al., 2015). In our study, the estimated LOQ for qPCR was lower than for ddPCR applying a RSD of 25% for estimated copy numbers. While we observed similar detection sensitivity for qPCR and ddPCR on DNA extracted from crayfish tissue, we had lower detection sensitivity for ddPCR compared to qPCR for the eDNA samples. This contrasts other studies where ddPCR appears to be more sensitive than qPCR for eDNA detection (e.g., Doi et al., 2015b; Hamaguchi et al., 2018; Mauvisseau et al., 2019a; Wood et al., 2019; Brys et al., 2020). In some eDNA samples we observed a reduced amplitude for the ddPCR indicating some inhibition. Although ddPCR is robust against inhibition, high concentrations of PCR inhibitors like humic acids may also inhibit ddPCR. Several qPCR mastermixes are robust against inhibition, including Taqman Environmental Mastermix 2.0 (Strand et al., 2011; Uchii et al., 2019). Thus, the type of qPCR mastermix and its ability to deal with inhibition will highly influence the results in a comparison to ddPCR. Strand et al. (2011) found that Taqman Environmental Mastermix removed close to 100% of the observed inhibition in DNA extracts for different natural water samples including water with high content of humic substances. For ddPCR, we experienced that the relevant ddPCR chemistry (Bio-Rad) had no specific mastermix developed to deal with inhibitory substances in the ddPCR other than the inherent property of ddPCR (partitioning the samples into droplets), making it less sensitive to inhibition. Since noble crayfish habitats in the Northern countries commonly are within or in close vicinity to boreal coniferous forests, the water is commonly rich in humic substances. We believe this could have negatively influenced the detectability for ddPCR in this study. Though we did not include an internal PCR control (IPC), it would be beneficial to use in this type of comparison (Goldberg et al., 2016).

eDNA Detection Probability Compared to Crayfish Population Density

While we reliably detected noble crayfish eDNA in all localities with very low crayfish densities, the detection frequency was often very low. To achieve a 95% detection likelihood, low-density lakes required an estimated five filter samples, corresponding to ∼25 L of water. Thus, the anecdotal "cup of water" (Lodge et al., 2012) or rapid, low-volume sample effort seems insufficient for monitoring low-density crayfish populations. Many other studies have also reported successful eDNA detection of freshwater crayfish at very low densities (Dougherty et al., 2016; Larson et al., 2017; Strand et al., 2019), but often with low detection frequency. Poor detection efficiency can in some studies also be associated with very small water volumes per sample (Tréguier et al., 2014; Dougherty et al., 2016) or the use of ethanol precipitation instead of filtration, which has been shown to be less efficient (Spens et al., 2016; Hinlo et al., 2017; Troth et al., 2020). For several crayfish eDNA studies, consistent comparisons to CPUE data are also deficient or missing (Agersnap et al., 2017; Harper et al., 2018; Mauvisseau et al., 2018; Rusch et al., 2020).

Environmental DNA detection frequency compared to CPUE data, and occupancy modeling, clearly shows a correlation between the eDNA detection probability and crayfish population densities. Very low densities required high sampling effort while the few monitored high-density lakes had a very high detection frequency and required only two samples for a 95% detection probability. Dougherty et al. (2016) also observed that the eDNA

detection probability increased with crayfish population density. However, they also found that a cumulative probability of 95% varies with water clarity, where an increase in Secchi disk values drastically lowered the detection probability of *Faxonius rusticus* eDNA. Rice et al. (2018) found poor relationship between *Faxonius eupunctus* eDNA-detection probability and crayfish density in a large river system. Instead, they showed a strong relationship between eDNA detection probability and upstream river distance, implying that the probability of detecting *F. eupunctus* eDNA increased downstream. Downstream eDNA transport may therefore increase the detection probability more than the crayfish population density at the sampling site.

eDNA Concentrations Compared to Observed Relative Density of Noble Crayfish

Stewart (2019) emphasizes that the factors influencing both the eDNA sources (amount of eDNA released from focal taxa) and eDNA sinks (removal of eDNA from the environment) for individual species and aquatic habitats, are manifold and complex, both in space and time. Meaningful comparisons between eDNA concentrations and species density will therefore depend on in-depth knowledge of species biology and habitat, combined with complex modeling (Stewart, 2019). A prerequisite is nevertheless a minimum of correlation in eDNA concentration and density of the target species. We aimed to explore whether eDNA concentrations in the water correlate with observed relative density of noble crayfish. However, even at the highest noble crayfish densities we had very few samples where the eDNA copy number exceeded the LOQ both for qPCR data and for ddPCR data. If the highly uncertain DNA copy numbers obtained below the LOQ are plotted against CPUE, a weak positive correlation is found. Also in other studies where a week positive correlation between eDNA concentrations and crayfish density estimates is observed (Dougherty et al., 2016; Cai et al., 2017; Larson et al., 2017), the eDNA data is rarely above what would be the recommended LOQ (Klymus et al., 2020). Such data must be interpreted with great caution, and we argue that our data is not suited for statistical correlations between eDNA concentrations and relative density of noble crayfish. With our rather intensive sample effort in terms of sample replicates and water volumes, our study is a strong documentation on the missing correspondence between noble crayfish density and noble crayfish eDNA concentrations in the water, at least for the time-window where we did the sampling.

Several factors contribute to why crayfish eDNA copy number is a poor predictor of crayfish density. Abiotic and environmental factors (Stewart, 2019) combined with persistence and degradation of eDNA in the lake (Dejean et al., 2011; Barnes et al., 2014) are universal challenges in eDNA studies. High water flow, heavy rain, clay particles and water turbidity reduce the eDNA detection likelihood (Roussel et al., 2015; Dougherty et al., 2016), which probably explains our failure to detect noble crayfish eDNA after heavy rainfall in one of the lakes. Sampling techniques of crayfish eDNA, including choices of season, depths, volume and filters, and downstream choices of molecular techniques, and ultimately LOD/LOQ stringency, also impact on the results (Hinlo et al., 2017; Hunter et al., 2017, 2019; Strand et al., 2019; Klymus et al., 2020; Troth et al., 2020). Our results might also be prone to downstream analytical issues, e.g., a recent report found that using the same filter type, but a different DNA extraction procedure than we used increases DNA yield and detectability of crayfish and fish eDNA (Fossøy et al., 2020).

Biotic factors, such as seasonality, life cycle and behavioral traits of crayfish (Dunn et al., 2017; Stewart, 2019) are particularly important in the context of why crayfish eDNA concentrations seemingly correlate poorly with crayfish densities. Many biological features might impede eDNA detectability of crayfish compared to for example fish, where correlations between population density and eDNA concentrations are seen (e.g., Doi et al., 2017; Salter et al., 2019; Stewart, 2019). A recent study report from Norway found significantly more DNA from fish compared to crayfish in the same eDNA samples (Fossøy et al., 2020). Crayfish commonly stay buried or hide under rocks during daytime, and their hard carapace emits little if any superfluous mucus or epidermal cells, suggesting reduced emission of DNA to the ambient water compared to many other aquatic organisms (Dougherty et al., 2016; Forsström and Vasemägi, 2016; Mauvisseau et al., 2019b). Spawning and reproduction events are known to increase eDNA signals markedly from aquatic species groups such as fish and amphibians (Stewart, 2019 and references therin). For crayfish, molting periods and spawning periods with elevated aggressive behavior (Moore, 2007), have been found to elevate DNA copy numbers in the water column (Dunn et al., 2017; Laurendz, 2017; Harper et al., 2018). In tank experiments, Dunn et al. (2017) found a correlation between eDNA concentration and abundance of egg-bearing females. Thus, the ovigerous period in the late autumn could be a good period for eDNA correlations to crayfish density. However, in cooler climate such as the Nordic countries, the proportion of egg-bearing females may vary considerably annually, both between and within populations (Taugbøl et al., 1988), which may bias the results. Furthermore, if trying to compare eDNA concentrations and CPUE data during the ovigerous period, possible correlations will probably be biased, as both low temperatures and trap avoidance from berried females will affect the catches (Abrahamsson, 1983). Nevertheless, compared to relatively short-term molting and reproduction events, the relatively long-lasting ovigerous period may at least be a recommendable period for eDNA sampling for confirming presence/absence of freshwater crayfish. In our study, we monitored the crayfish after the molting season and prior to the reproduction- and ovigerous season. In this respect, we might have selected a time window that was recommendable for achieving CPUE data that takes the crayfish biological needs into account, but where crayfish eDNA shedding from the crayfish population was very low.

The Use of eDNA in Monitoring and Conservation of Freshwater Crayfish

Environmental DNA methods are moving toward the stage of being ready for, and sometimes also employed for, biodiversity

inventories and monitoring of native or invasive species (Leese et al., 2016; Stewart, 2019; Sepulveda et al., 2020). In Norway, comparative data obtained with eDNA monitoring and traditional methods (cages and trapping) of native noble crayfish, introduced signal crayfish and the crayfish plague pathogen *A. astaci,* convinced the authorities to include eDNA as a monitoring method (Strand et al., 2019). In 2016, eDNA was officially integrated into the national crayfish plague monitoring program commissioned by the National Food Safety Authorities (Vrålstad et al., 2017), and replaced a controversial cage-surveillance strategy with live, naïve noble crayfish for the monitoring of crayfish plague from 2017 and onward. From 2018, the national surveillance program on noble crayfish also included eDNA monitoring methods, both for noble crayfish and the invasive signal crayfish. With an integrated synergistic approach, including joint fieldwork and sharing of results, the two monitoring programs now focus on the pathogen (*A. astaci*), and its hosts and carriers [noble crayfish and signal crayfish, see Johnsen et al. (2019)], resulting in a more holistic monitoring approach for noble crayfish and its threats.

Monitoring programs are usually put out to tender, which to varying degrees request and emphasize competence and quality versus costs. If cost-effectiveness is regarded as important, compromises with the number of samples, water volumes and number of sites must often be made to deliver a competitive offer. Consequently, the monitoring programs might not be able to afford a sufficient sampling effort that ensures high probability of detection at very low densities of crayfish, or any other rare or elusive target organism. eDNA monitoring is often promoted as a cost-saving method. These savings might be at the expense of precision and the ability to provide reliable presence-absence data. In cases where the sampling effort is deficient, it must be expected that the absence data covers a large portion of false negatives, and that the prospects of uncovering rare threatened species or the early invasion phase of invasive species will diminish. It is therefore of great importance to develop smarter sampling methods, identify time windows for sampling with elevated eDNA detection probabilities, identify the molecular methods recovering the highest eDNA concentrations from various environmental samples, and at the same time build up acceptance and understanding among the stakeholders and authorities that quality and reliability come at a cost. Saving Europe's crustaceans, cannot be achieved by saving money on insufficient and cost-effective eDNA sampling strategies.

CONCLUSION

The use of eDNA in ecological research, monitoring and conservation has grown tremendously in recent years. However, there are several scientists requesting a more balanced appreciation of this method's strengths and limitations (Thomsen and Willerslev, 2015; Cristescu and Hebert, 2018; Beng and Corlett, 2020). Among the several mentioned aspects requiring more examination is the use of eDNA concentrations to predict relative density or biomass of species (Beng and Corlett, 2020). We found no evidence for a correlation between eDNA concentrations and relative crayfish density with the chosen methods for field sampling, DNA extraction protocol and period of sampling. Combined with previous studies, it seems increasingly clear that eDNA concentrations of a target species cannot replace CPUE data or serve as a proxy for relative crayfish density estimates. Conventional methods are still needed to monitor changes in population size and densities over time, and for providing additional information on length and weight distribution, sex ratio, fecundity, and maturation. However, as eDNA monitoring has proven to detect crayfish reliably even at very low densities, this method is a powerful supplement to monitor the presence or absence of crayfish in a larger number of localities than feasible with the traditional trapping methods. If the eDNA samples are exploited for the monitoring of multiple target species, for example alien crayfish species and diseases that threaten the native crayfish species (Strand et al., 2019; Rusch et al., 2020) it is also possible to implement a more holistic and cost-efficient monitoring approach.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in the **Supplementary Material** as dataset 1–3.

AUTHOR CONTRIBUTIONS

DS, SJ, and TV: conception and design of the study. DS, SJ, and JR: carried out the fieldwork. DS and JR: molecular methods development, analyses, and results. DS and SJ: statistics and drafting of the manuscript. All authors contributed to content, editing of the manuscript, and approved the final version.

FUNDING

This work was funded by the Norwegian Research Council through the project "Targeted strategies for safeguarding the noble crayfish against alien and emerging threats" (TARGET; NFR-243907), the Norwegian Veterinary Institute, the Norwegian Institute for Nature Research, and the Norwegian Environmental Agency.

ACKNOWLEDGMENTS

We thank Bjørn Spilsberg for help with ddPCR troubleshooting, and Frode Fossøy and Brett K. Sandercock for help with occupancy modeling.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2020.612253/full#supplementary-material

REFERENCES

Abrahamsson, S. (1983). Trappabillity, locomotion, and diel pattern of activity of the crayfish *Astacus astacus* and *Pacifastacus leniusculus* Dana. *Freshw. Crayfish* 5, 239–253.

Ackefors, H. (1998). The culture and capture crayfish fisheries in Europe. *World Aquac. Magaz.* 29, 18–67.

Agersnap, S., Larsen, W. B., Knudsen, S. W., Strand, D., Thomsen, P. F., Hesselsøe, M., et al. (2017). Monitoring of noble, signal and narrow-clawed crayfish using environmental DNA from freshwater samples. *PLoS One* 12:e0179261. doi: 10.1371/journal.pone.0179261

Barnes, M. A., Turner, C. R., Jerde, C. L., Renshaw, M. A., Chadderton, W. L., and Lodge, D. M. (2014). Environmental conditions influence eDNA persistence in aquatic systems. *Environ. Sci. Technol.* 48, 1819–1827. doi: 10.1021/es404734p

Beng, K. C., and Corlett, R. T. (2020). Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. *Biodivers. Conserv.* 29, 2089–2121. doi: 10.1007/s10531-020-01980-1980

Berdal, K., Bøydler, C., Tengs, T., and Holst-Jensen, A. (2008). A statistical approach for evaluation of PCR results to improve the practical limit of quantification (LOQ) of GMO analyses (SIMQUANT). *Eur. Food Res. Technol.* 227, 1149–1157. doi: 10.1007/s00217-008-0830-831

Bohmann, K., Evans, A., Gilbert, M. T. P., Carvalho, G. R., Creer, S., Knapp, M., et al. (2014). Environmental DNA for wildlife biology and biodiversity monitoring. *Trends Ecol. Evol.* 29, 358–367. doi: 10.1016/j.tree.2014.04.003

Brys, R., Halfmaerten, D., Neyrinck, S., Mauvisseau, Q., Auwerx, J., Sweet, M., et al. (2020). Reliable eDNA detection and quantification of the European weather loach (*Misgurnus fossilis*). *J. Fish Biol.* 1–16. doi: 10.1111/jfb.14315

Bylemans, J., Gleeson, D. M., Duncan, R. P., Hardy, C. M., and Furlan, E. M. (2019). A performance evaluation of targeted eDNA and eDNA metabarcoding analyses for freshwater fishes. *Environ. DNA* 1, 402–414. doi: 10.1002/edn3.41

Cai, W., Ma, Z., Yang, C., Wang, L., Wang, W., Zhao, G., et al. (2017). Using eDNA to detect the distribution and density of invasive crayfish in the Honghe-Hani rice terrace World Heritage site. *PLoS One* 12:e0177724. doi: 10.1371/journal.pone.0177724

Capo, E., Spong, G., Norman, S., Königsson, H., Bartels, P., and Byström, P. (2019). Droplet digital PCR assays for the quantification of brown trout (*Salmo trutta*) and Arctic char (*Salvelinus alpinus*) from environmental DNA collected in the water of mountain lakes. *PLoS One* 14:226638. doi: 10.1371/journal.pone.0226638

Creed, R. P. (1994). Direct and indirect effects of crayfish grazing in a stream community. *Ecology* 75, 2091–2103. doi: 10.2307/1941613

Cristescu, M. E., and Hebert, P. D. N. (2018). Uses and misuses of environmental DNA in biodiversity science and conservation. *Ann. Rev. Ecol. Evol. Syst.* 49, 209–230. doi: 10.1146/annurev-ecolsys-110617-162306

Deiner, K., Bik, H. M., Machler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., et al. (2017). Environmental DNA metabarcoding: transforming how we survey animal and plant communities. *Mol. Ecol.* 26, 5872–5895. doi: 10.1111/mec.14350

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P., et al. (2011). Persistence of environmental DNA in freshwater ecosystems. *PLoS One* 6:e023398. doi: 10.1371/journal.pone.0023398

Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E., and Miaud, C. (2012). Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog *Lithobates catesbeianus*. *J. Appl. Ecol.* 49, 953–959. doi: 10.1111/j.1365-2664.2012.02171.x

Demeke, T., and Dobnik, D. (2018). Critical assessment of digital PCR for the detection and quantification of genetically modified organisms. *Analyt. Bioanalyt. Chem.* 410, 4039–4050. doi: 10.1007/s00216-018-1010-1011

Dingle, T. C., Sedlak, R. H., Cook, L., and Jerome, K. R. (2013). Tolerance of droplet-digital PCR vs real-time quantitative PCR to inhibitory substances. *Clin. Chem.* 59, 1670–1672. doi: 10.1373/clinchem.2013.211045

Dobnik, D., Spilsberg, B., Kosir, A. B., Holst-Jensen, A., and Zel, J. (2015). Multiplex quantification of 12 European union authorized genetically modified maize lines with droplet digital polymerase chain reaction. *Analyt. Chem.* 87, 8218–8226. doi: 10.1021/acs.analchem.5b01208

Doi, H., Inui, R., Akamatsu, Y., Kanno, K., Yamanaka, H., Takahara, T., et al. (2017). Environmental DNA analysis for estimating the abundance and biomass of stream fish. *Freshw. Biol.* 62, 30–39. doi: 10.1111/fwb.12846

Doi, H., Takahara, T., Minamoto, T., Matsuhashi, S., Uchii, K., and Yamanaka, H. (2015a). Droplet digital polymerase chain reaction (PCR) outperforms real-time PCR in the Detection of environmental DNA from an invasive fish species. *Environ. Sci. Technol.* 49, 5601–5608. doi: 10.1021/acs.est.5b00253

Doi, H., Uchii, K., Takahara, T., Matsuhashi, S., Yamanaka, H., and Minamoto, T. (2015b). Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. *PLoS One* 10:e0122763. doi: 10.1371/journal.pone.0122763

Dorazio, R. M., and Erickson, R. A. (2018). Ednaoccupancy: an r package for multiscale occupancy modelling of environmental DNA data. *Mol. Ecol. Resour.* 18, 368–380. doi: 10.1111/1755-0998.12735

Dougherty, M. M., Larson, E. R., Renshaw, M. A., Gantz, C. A., Egan, S. P., Erickson, D. M., et al. (2016). Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes rusticus* at low abundances. *J. Appl. Ecol.* 53, 722–732. doi: 10.1111/1365-2664.12621

Dunn, N., Priestley, V., Herraiz, A., Arnold, R., and Savolainen, V. (2017). Behavior and season affect crayfish detection and density inference using environmental DNA. *Ecol. Evol.* 7, 7777–7785. doi: 10.1002/ece3.3316

Edsman, L., Füreder, L., Gherardi, F., and Souty-Grosset, C. (2010). Astacus astacus. *IUCN Red List Threat. Spec.* 2010:e.T2191A9338388.

Forsström, T., and Vasemägi, A. (2016). Can environmental DNA (eDNA) be used for detection and monitoring of introduced crab species in the Baltic Sea? *Mar. Pollut. Bull.* 109, 350–355. doi: 10.1016/j.marpolbul.2016.05.054

Fossøy, F., Strand, D. A., Sandercock, B. K., and Johnsen, S. I. (2020). *Environmental DNA: Comparison of Sampling Methodology and Laboratory Analysis for Detection of Crayfish and Fish in Freshwater*. NINA Report 1778, Norway: Norwegian Institute for Nature Research.

Goldberg, C. S., Turner, C. R., Deiner, K., Klymus, K. E., Thomsen, P. F., Murphy, M. A., et al. (2016). Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods Ecol. Evol.* 7, 1299–1307. doi: 10.1111/2041-210x.12595

Gomes, G. B., Hutson, K. S., Domingos, J. A., Chung, C., Hayward, S., Miller, T. L., et al. (2017). Use of environmental DNA (eDNA) and water quality data to predict protozoan parasites outbreaks in fish farms. *Aquaculture* 479, 467–473. doi: 10.1016/j.aquaculture.2017.06.021

Goutte, A., Molbert, N., Guérin, S., Richoux, R., and Rocher, V. (2020). Monitoring freshwater fish communities in large rivers using environmental DNA metabarcoding and a long-term electrofishing survey. *J. Fish Biol.* 97, 444–452. doi: 10.1111/jfb.14383

Hamaguchi, M., Shimabukuro, H., Hori, M., Yoshida, G., Terada, T., and Miyajima, T. (2018). Quantitative real-time polymerase chain reaction (PCR) and droplet digital PCR duplex assays for detecting *Zostera marina* DNA in coastal sediments. *Limnol. Oceanogr. Methods* 16, 253–264. doi: 10.1002/lom3.10242

Hänfling, B., Lawson Handley, L., Read, D. S., Hahn, C., Li, J., Nichols, P., et al. (2016). Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. *Mol. Ecol.* 25, 3101–3119. doi: 10.1111/mec.13660

Harper, K. J., Anucha, N. P., Turnbull, J. F., Bean, C. W., and Leaver, M. J. (2018). Searching for a signal: environmental DNA (eDNA) for the detection of invasive signal crayfish, *Pacifastacus leniusculus* (Dana, 1852). *Manag. Biol. Invas.* 9, 137–148. doi: 10.3391/mbi.2018.9.2.07

Hempel, C. A., Peinert, B., Beermann, A. J., Elbrecht, V., Macher, J.-N., Macher, T.-H., et al. (2020). Using environmental DNA to monitor the reintroduction success of the *Rhine sculpin* (*Cottus rhenanus*) in a restored stream. *Front. Ecol. Evol.* 8:81. doi: 10.3389/fevo.2020.00081

Henriksen, S., and Hilmo, O. (2015). *Norsk Rødliste for Arter 2015. Artsdatabanken, Norge*. Available online at: https://artsdatabanken.no/Rodliste2015/rodliste2015/Norge/16528 (accessed January 9, 2020).

Hinlo, R., Gleeson, D., Lintermans, M., and Furlan, E. (2017). Methods to maximise recovery of environmental DNA from water samples. *PLoS One* 12:e0179251. doi: 10.1371/journal.pone.0179251

Holdich, D. M., Reynolds, J. D., Souty-Grosset, C., and Sibley, P. J. (2009). A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. *Knowl. Manag. Aquat. Ecosyst.* 394–395:11. doi: 10.1051/kmae/2009025

Hunter, M. E., Dorazio, R. M., Butterfield, J. S. S., Meigs-Friend, G., Nico, L. G., and Ferrante, J. A. (2017). Detection limits of quantitative and digital PCR assays

and their influence in presence-absence surveys of environmental DNA. *Mol. Ecol. Resour.* 17, 221–229. doi: 10.1111/1755-0998.12619

Hunter, M. E., Ferrante, J. A., Meigs-Friend, G., and Ulmer, A. (2019). Improving eDNA yield and inhibitor reduction through increased water volumes and multi-filter isolation techniques. *Sci. Rep.* 9:5259. doi: 10.1038/s41598-019-40977-w

Jerde, C. L., Mahon, A. R., Chadderton, W. L., and Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conserv. Lett.* 4, 150–157. doi: 10.1111/j.1755-263X.2010.00158.x

Johnsen, S. I., Skurdal, J., Taugbøl, T., and Garnås, E. (2014). Effect of mesh size on baited trap catch composition for noble crayfish (*Astacus Astacus*). *Knowl. Manag. Aquat. Ecosyst.* 413, 1–7. doi: 10.1051/kmae/2014013

Johnsen, S. I., Strand, D. A., Rusch, J., and Vrålstad, T. (2019). *National Surveillance of Noble Crayfish and the Spread of Signal Crayfish – Presentation of Surveilance Data and Population Status*. NINA Report 1590, Norway: Norwegian Institute for Nature Research.

Johnsen, S. I., and Vrålstad, T. (2017). *Noble Crayfish (Astacus astacus) – Scientific Investigation and Proposal for Coordination of the Monitoring Programs for Crayfish and Crayfish Plague*. NINA Report 1339, Norway: Norwegian Institute for Nature Research.

Kelly, R. P., Port, J. A., Yamahara, K. M., Martone, R. G., Lowell, N., Thomsen, P. F., et al. (2014). Harnessing DNA to improve environmental management. *Science* 344, 1455–1456. doi: 10.1126/science.1251156

Klymus, K. E., Merkes, C. M., Allison, M. J., Goldberg, C. S., Helbing, C. C., Hunter, M. E., et al. (2020). Reporting the limits of detection and quantification for environmental DNA assays. *Environ. DNA* 2, 271–282. doi: 10.1002/edn3.29

Kouba, A., Petrusek, A., and Kozák, P. (2014). Continental-wide distribution of crayfish species in Europe: update and maps. *Knowl. Manag. Aquat. Ecosyst.* 413:5. doi: 10.1051/kmae/2014007

Lacoursière-Roussel, A., Côté, G., Leclerc, V., and Bernatchez, L. (2016). Quantifying relative fish abundance with eDNA: a promising tool for fisheries management. *J. Appl. Ecol.* 53, 1148–1157. doi: 10.1111/1365-2664.12598

Laramie, M. B., Pilliod, D. S., and Goldberg, C. S. (2015). Characterizing the distribution of an endangered salmonid using environmental DNA analysis. *Biol. Conserv.* 183, 29–37. doi: 10.1016/j.biocon.2014.11.025

Larson, E. R., Renshaw, M. A., Gantz, C. A., Umek, J., Chandra, S., Lodge, D. M., et al. (2017). Environmental DNA (eDNA) detects the invasive crayfishes *Orconectes rusticus* and *Pacifastacus leniusculus* in large lakes of North America. *Hydrobiologia* 800, 173–185. doi: 10.1007/s10750-017-3210-3217

Laurendz, C. (2017). *Impact of Temperature, Food Availability and Lifehistory Stages on the eDNA Emission from Pacifastacus leniusculus and its obligate parasite Aphanomyces astaci*. Master thesis, University of Oslo, Oslo.

Leese, F., Altermatt, F., Bouchez, A., Ekrem, T., Hering, D., Meissner, K., et al. (2016). DNAqua-Net: developing new genetic tools for bioassessment and monitoring of aquatic ecosystems in Europe. *Res. Ideas Outcom.* 2:e11321. doi: 10.3897/rio.2.e11321

Lodge, D. M., Turner, C. R., Jerde, C. L., Barnes, M. A., Chadderton, L., Egan, S. P., et al. (2012). Conservation in a cup of water: estimating biodiversity and population abundance from environmental DNA. *Mol. Ecol.* 21, 2555–2558. doi: 10.1111/j.1365-294X.2012.05600.x

Mason, D. H., Dysthe, J. C., Franklin, T. W., Williams, C. L., Young, M. K., McKelvey, K. S., et al. (2020). Certain detection of uncertain taxa: eDNA detection of a cryptic mountain sucker (*Pantosteus jordani*) in the Upper Missouri River, USA. *Environ. DNA* 1–9. doi: 10.1002/edn3.133

Mauvisseau, Q., Coignet, A., Delaunay, C., Pinet, F., Bouchon, D., and Souty-Grosset, C. (2018). Environmental DNA as an efficient tool for detecting invasive crayfishes in freshwater ponds. *Hydrobiologia* 805, 163–175. doi: 10.1007/s10750-017-3288-y

Mauvisseau, Q., Davy-Bowker, J., Bulling, M., Brys, R., Neyrinck, S., Troth, C., et al. (2019a). Combining ddPCR and environmental DNA to improve detection capabilities of a critically endangered freshwater invertebrate. *Sci. Rep.* 9, 1–9. doi: 10.1038/s41598-019-50571-50579

Mauvisseau, Q., Tönges, S., Andriantsoa, R., Lyko, F., and Sweets, M. (2019b). Early detection of an emerging invasive species: eDNA monitoring of a parthenogenetic crayfish in freshwater systems. *Manag. Biol. Invas.* 10, 461–472. doi: 10.3391/mbi.2019.10.3.04

McElroy, M. E., Dressler, T. L., Titcomb, G. C., Wilson, E. A., Deiner, K., Dudley, T. L., et al. (2020). Calibrating environmental DNA Metabarcoding to conventional surveys for measuring fish species richness. *Front. Ecol. Evol.* 8:276. doi: 10.3389/fevo.2020.00276

McKee, A. M., Spear, S. F., and Pierson, T. W. (2015). The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. *Biol. Conserv.* 183, 70–76. doi: 10.1016/j.biocon.2014.11.031

Momot, W. T. (1995). Redefining the role of crayfish in aquatic ecosystems. *Rev. Fish. Sci.* 3, 33–63. doi: 10.1080/10641269509388566

Moore, P. A. (2007). "Agonistic behavior in freshwater crayfish," in *Evolutionary Ecology of Social and Sexual Systems: Crustaceans as Model Organisms*, eds J. E. Duffy and M. Thiel (Oxford: Oxford University Press).

Pecoraro, S., Berben, G., Burns, M., Corbisier, P., De Giacomo, M., De Loose, M., et al. (2019). *Overview and Recommendations for the Application of Digital PCR*. Luxembourg: Publications Office of the European Union. EUR 29673 EN. doi: 10.2760/192883

Quan, P. L., Sauzade, M., and Brouzes, E. (2018). dPCR: a technology review. *Sensors* 18:1271. doi: 10.3390/s18041271

Rački, N., Morisset, D., Gutierrez-Aguirre, I., and Ravnikar, M. (2014). One-step RT-droplet digital PCR: a breakthrough in the quantification of waterborne RNA viruses. *Analyt. Bioanalyt. Chem.* 406, 661–667. doi: 10.1007/s00216-013-7476-y

Reynolds, J., Souty-Grosset, C., and Richardson, A. (2013). Ecological roles of crayfish in freshwater and terrestrial habitats. *Freshw. Crayfish* 19, 197–218.

Rice, C. J., Larson, E. R., and Taylor, C. A. (2018). Environmental DNA detects a rare large river crayfish but with little relation to local abundance. *Freshw. Biol.* 63, 443–455. doi: 10.1111/fwb.13081

Robinson, C. V., Webster, T. M. U., Cable, J., James, J., and Consuegra, S. (2018). Simultaneous detection of invasive signal crayfish, endangered white-clawed crayfish and the crayfish plague pathogen using environmental DNA. *Biol. Conserv.* 222, 241–252. doi: 10.1016/j.biocon.2018.04.009

Roussel, J. M., Paillisson, J. M., Tréguier, A., and Petit, E. (2015). The downside of eDNA as a survey tool in water bodies. *J. Appl. Ecol.* 52, 823–826. doi: 10.1111/1365-2664.12428

Rusch, J. C., Hansen, H., Strand, D. A., Markussen, T., Hytterød, S., and Vrålstad, T. (2018). Catching the fish with the worm: a case study on eDNA detection of the monogenean parasite *Gyrodactylus salaris* and two of its hosts, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). *Parasit. Vect.* 11:333. doi: 10.1186/s13071-018-2916-2913

Rusch, J. C., Mojžišová, M., Strand, D. A., Svobodová, J., Vralståd, T., and Petrusek, A. (2020). Simultaneous detection of native and invasive crayfish and *Aphanomyces astaci* from environmental DNA samples in a wide range of habitats in Central Europe. *Neobiota* 58, 1–32. doi: 10.3897/neobiota.58.49358

Salter, I., Joensen, M., Kristiansen, R., Steingrund, P., and Vestergaard, P. (2019). Environmental DNA concentrations are correlated with regional biomass of Atlantic cod in oceanic waters. *Commun. Biol.* 2:461. doi: 10.1038/s42003-019-06968

Sepulveda, A. J., Nelson, N. M., Jerde, C. L., and Luikart, G. (2020). Are environmental DNA methods ready for aquatic invasive species management? *Trends Ecol. Evol.* 35, 668–678. doi: 10.1016/j.tree.2020.03.011

Souty-Grosset, C., Holdich, D. M., Noël, P. Y., Reynolds, J. D., and Haffner, P. (2006). *Atlas of Crayfish in Europe*. Paris: Muséum national d'Histoire naturelle.

Spens, J., Evans, A. R., Halfmaerten, D., Knudsen, S. W., Sengupta, M. E., Mak, S. S. T., et al. (2016). Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. *Methods Ecol. Evol.* 8, 635–645. doi: 10.1111/2041-210X.12683

Stewart, K. A. (2019). Understanding the effects of biotic and abiotic factors on sources of aquatic environmental DNA. *Biodivers. Conserv.* 28, 983–1001. doi: 10.1007/s10531-019-01709-1708

Strand, D. A., Holst-Jensen, A., Viljugrein, H., Edvardsen, B., Klaveness, D., Jussila, J., et al. (2011). Detection and quantification of the crayfish plague agent in natural waters: direct monitoring approach for aquatic environments. *Dis. Aquat. Organ.* 95, 9–17. doi: 10.3354/Dao02334

Strand, D. A., Johnsen, S. I., Rusch, J. C., Agersnap, S., Larsen, W. B., Knudsen, S. W., et al. (2019). Monitoring a Norwegian freshwater crayfish tragedy – eDNA snapshots of invasion, infection and extinction. *J. Appl. Ecol.* 56, 1661–1679. doi: 10.1111/1365-2664.13404

Strand, D. A., Jussila, J., Johnsen, S. I., Viljamaa-Dirks, S., Edsman, L., Wiik-Nielsen, J., et al. (2014). Detection of crayfish plague spores in large freshwater systems. *J. Appl. Ecol.* 51, 544–553. doi: 10.1111/1365-2664.12218

Strand, D. A., Rusch, J., Johnsen, S. I., Tarpai, A., and Vrålstad, T. (2020). *The Surveillance Programme for Aphanomyces astaci in Norway 2019*. Annual report, Oslo: Norwegian Veterinary Institute.

Sylvestre, S., Sekela, M., Tuominen, T., and Moyle, G. (2002). Water quality assessment of agricultural and residential run-off using the crayfish *Pacifastacus leniusculus* in British Columbia, Canada. *Freshw. Crayfish* 13, 383–395.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., and Kawabata, Z. (2012). Estimation of fish biomass using environmental DNA. *PLoS One* 7:e035868. doi: 10.1371/journal.pone.0035868

Taugbøl, T., Skurdal, J., and Fjeld, E. (1988). Maturity and fecundity of *Astacus astacus* females in Norway. *Freshw. Crayfish* 7, 107–114.

Thomsen, P. F., Kielgast, J., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., et al. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Mol. Ecol.* 21, 2565–2573. doi: 10.1111/j.1365-294X.2011.05418.x

Thomsen, P. F., and Willerslev, E. (2015). Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. *Biol. Conserv.* 183, 4–18. doi: 10.1016/j.biocon.2014.11.019

Tréguier, A., Paillisson, J.-M., Dejean, T., Valentini, A., Schlaepfer, M. A., and Roussel, J.-M. (2014). Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. *J. Appl. Ecol.* 51, 871–879. doi: 10.1111/1365-2664.12262

Troth, C. R., Burian, A., Mauvisseau, Q., Bulling, M., Nightingale, J., Mauvisseau, C., et al. (2020). Development and application of eDNA-based tools for the conservation of white-clawed crayfish. *Sci. Total Environ.* 748, 141394. doi: 10.1016/j.scitotenv.2020.141394

Uchii, K., Doi, H., Okahashi, T., Katano, I., Yamanaka, H., Sakata, M. K., et al. (2019). Comparison of inhibition resistance among PCR reagents for detection and quantification of environmental DNA. *Environ. DNA* 1, 359–367. doi: 10.1002/edn3.37

Usio, N., and Townsend, C. R. (2001). The significance of the crayfish *Paranephrops zealandicus* as shredders in a New Zealand headwater stream. *J. Crustacean Biol.* 21, 354–359. doi: 10.1163/20021975-99990135

Villacorta-Rath, C., Adekunle, A. I, Edmunds, R. C., Strugnell, J. M., Schwarzkopf, L., and Burrows, D. (2020). Can environmental DNA be used to detect first arrivals of the cane toad, *Rhinella marina*, into novel locations? *Environ. DNA* 2, 635–646. doi: 10.1002/edn3.114

Vrålstad, T., Knutsen, A. K., Tengs, T., and Holst-Jensen, A. (2009). A quantitative TaqMan (R) MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. *Vet. Microbiol.* 137, 146–155. doi: 10.1016/j.vetmic.2008.12.022

Vrålstad, T., Strand, D. A., Rusch, J. C., Toverud, Ø, Johnsen, S. I., Tarpai, A., et al. (2017). *The Surveillance Programme for Aphanomyces astaci in Norway 2016*. Annual report, Oslo: Norwegian Veterinary Institute.

Westman, K., and Pursiainen, M. (1982). Size and structure of crayfish (*Astacus astacus*) populations on different habitats in Finland. *Hydrobiologia* 86, 67–72. doi: 10.1007/BF00005789

Wood, S. A., Pochon, X., Laroche, O., von Ammon, U., Adamson, J., and Zaiko, A. (2019). A comparison of droplet digital polymerase chain reaction (PCR), quantitative PCR and metabarcoding for species-specific detection in environmental DNA. *Mol. Ecol. Resour.* 19, 1407–1419. doi: 10.1111/1755-0998.13055

Zhao, Y., Xia, Q. Y., Yin, Y. P., and Wang, Z. K. (2016). Comparison of droplet digital PCR and quantitative PCR assays for quantitative detection of *Xanthomonas citri* Subsp *citri*. *PLoS One* 11:e0159004. doi: 10.1371/journal.pone.0159004

Zimmerman, J. K. M., and Palo, R. T. (2011). Reliability of catch per unit effort (CPUE) for evaluation of reintroduction programs – A comparison of the mark-recapture method with standardized trapping. *Knowl. Manag. Aquat. Ecosyst.* 401:07. doi: 10.1051/kmae/2011016

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Environmental Science

ORIGINAL RESEARCH
published: 09 December 2020
doi: 10.3389/fenvs.2020.609129

Measures to Control Invasive Crayfish Species in Switzerland: A Success Story?

*Raphael Krieg, Alex King and Armin Zenker**

School of Life Sciences, Institute for Ecopreneurship, University of Applied Sciences and Arts Northwestern Switzerland, Muttenz, Switzerland

Invasive crayfish species were first documented in Switzerland in the 1970s. Today, North American crayfish species dominate in most major lakes and streams in Switzerland. In combination with the crayfish plague, they pose a substantial threat to our native crayfish. Over the past 20 years, various techniques have been applied to reduce negative impacts of these invasive crayfish in Switzerland: eradication (temporary drainage or destruction of a water system, biocides), suppression (intensive trapping, electricity introduction of predatory fish) and containment (construction of crayfish barriers). Temporary drainage or filling-in of isolated ponds, in combination with calcium hydroxide application has been successful in eradicating populations of invasive crayfish. However, trapping and introduction of predatory fish led to a reduction in population density but neither method has ever caused the extinction of a population. Invasive crayfish have not yet reached crayfish barriers, therefore, long-term functionality of these barriers still needs to be proven. Nevertheless, functional controls with native crayfish have shown that barriers prevent their upstream movement. Implementation of crayfish barriers is the most promising method to protect native crayfish from displacement by invasive crayfish species. Many measures are expensive, time consuming, and show little or no success in controlling invasive crayfish. Therefore, we recommend to focus on implementing drastic measures, such as filling-in or draining of isolated waters or a combination of various methods to maximise the reduction of population size.

Keywords: biocides, trapping, barriers, infilling, electric-fishing, drainage, function control, migration

OPEN ACCESS

Edited by:
Ivana Maguire,
University of Zagreb, Croatia

Reviewed by:
Elena Tricarico,
University of Florence, Italy
Christoph Chucholl,
Fischereiforschungsstelle Baden-Württemberg, Germany

***Correspondence:**
Armin Zenker
armin.zenker@fhnw.ch

Specialty section:
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Environmental Science

Received: *22 September 2020*
Accepted: *17 November 2020*
Published: *09 December 2020*

Citation:
Krieg R, King A and Zenker A (2020) Measures to Control Invasive Crayfish Species in Switzerland: A Success Story? Front. Environ. Sci. 8:609129. doi: 10.3389/fenvs.2020.609129

INTRODUCTION

Preventing the widespread disappearance of indigenous crayfish species (ICS) in Europe is an on-going challenge. In addition to habitat destruction by river engineering and water pollution, invasive non-indigenous crayfish species (NICS) from North America are becoming increasingly widespread in Europe and pose a major threat to ICS (Kouba et al., 2014). Their dominance over native species is reflected in their rapid reproduction (Burič et al., 2011), physical superiority and aggressiveness (Gherardi, 2006) as well as their tolerance to poor water quality (Nyström, 2002). Displacement of ICS with NICS is predicted whenever they occupy the same habitat niche (Westman et al., 2002); this is if the crayfish plague *(Aphanomyces astaci)* has not already initiated extinction of the native species. This fungus like pathogen belonging to the Oomycetes (Saprolegniales) was responsible for the first mass mortalities of native crayfish in Europe in the

late ninetieth century and it continues to cause huge problems in waterways today (Holdich et al., 2009).

Various methods have already been applied and tested across the world to reduce or eradicate the negative impacts of the unwanted intruders as well as to prevent them from spreading further. These methods include: intensive trapping (Bills and Marking, 1988; Hein et al., 2007), male sterilisation (Piazza et al., 2015), the use of biocides (Cecchinelli et al., 2012), habitat destruction, the release of predators (Musseau et al., 2015), the construction of barriers (Cowart et al., 2018) or a multi-method approach combining different combinations of these methods (Hein et al., 2006; Freeman et al., 2010; Stebbing et al., 2014). The success of these measures varies and so far only attempts which fill-in isolated still waters or the use poison (Ballantyne et al., 2019) have been successful in completely eradicating populations of NICS.

In Switzerland, the three native species *Astacus astacus* (Linnaeus, 1758), *Austropotamobius pallipes* (Lereboullet, 1858) and *Austropotamobius torrentium* (von Paula Schrank, 1803) are threatened by the presence of three North American crayfish species. *Faxonius limosus* (Rafinesque, 1817) was detected for the first time in Switzerland in 1976. It was followed by *Pacifastacus leniusculus* (Dana, 1852) at the end of the 1980s and *Procambarus clarkii* (Girard, 1852) in the 1990s. *F. limosus* and *P. leniusculus* have spread rapidly across Switzerland and are, through transmission of the crayfish plague, the main reason for population extinction of ICS (Stucki and Zaugg, 2011).

The three native species in Switzerland are defined as internationally protected in Appendix III of the Convention on the Conservation of European Wildlife and Natural Habitats which was decided upon in the Bern Convention 1979. In order to protect these species, Switzerland is tackling not only reintroduction, conservation and habitat management but also containment, suppression and eradication of NICS (Hefti and Stucki, 2006).

This paper summarises and assesses the success of various measures taken so far to combat and prevent the spread of NICS in Switzerland. The results serve as a basis for the future strategy to manage invasive crayfish species in Switzerland and help other countries to implement similar measures.

MATERIALS AND METHODS

Cantonal fisheries departments in Switzerland provided information regarding which measures they had already taken and provided existing data about implementation and success of the methods. The data received was examined to assess whether it could be used to indicate the success of a control measure. When catch per unit effort (CPUE) values were recorded over several years or when the functionality of a crayfish barrier was tested in the field (function control) with ICS, data was assessed as "valid." Personal assessments, measures which have started within the last 3 years and catch figures without indication of the sampling effort were rated as "insufficient" to produce valid data and are classified and referred to as "unclear" success in this paper. "Success" was defined by the data which showed a reduction in CPUE by at least 75%; when no invasive crayfish were detected above a crayfish barrier as well as when there was no further evidence of a crayfish appearance after an eradication campaign over a 5 year period. If CPUE decreased only by $\leq$ 25% after the eradication measurements were enforced this was judged as "no success."

RESULTS

Overall, nine cantons provided data regarding 40 control measures carried out at a total of 27 sites, 9 lotic and 18 lentic waterbodies (**Table 1**). Data on catch effort in large watersystems, such as Lake Zurich, Geneva and Neuchâtel as well as in the river Rhine, were insufficient and not included. When excluding sites which were classified as "unclear", ∼83% of eradication, 20% of suppression, and 100% of containment methods were successful (**Figure 1**).

Eradication of NICS

Temporary drainage or destruction of habitat and the use of biocides are methods used to eradicate unwanted crayfish species in Switzerland. Attempts to eradicate populations of crayfish were successful at five out of seven sites including one isolated lotic waterbody. In one case success could not be evaluated because of insufficient data and the other attempt was unsuccessful.

Temporary Drainage or Destruction of Habitat

A 300-m section of the small brook, *Stadtbach, Baden*, which is isolated by culverts, contained a population of *P. leniusculus*. The stream section was completely drained in January and February 2004 and 2005. Crayfish were removed by hand following drainage but even after this crayfish could still be found. For the entire summer of 2013 this section of stream was left drained again and since then no crayfish have been detected (Stucki, 2018).

In 2013, complete drainage and hand removal of crayfish at two small quarry ponds, (*Steinbruch Mellikon*) led to the successful eradication of a population of *P. clarkii*. The project was carried out in close cooperation with the amphibian managers at this site (Stucki, 2018).

There was an attempt to eradicate *P. clarkii* inhabiting an artificial pond in a public park *(l'étang de Vidy)* by draining away all of the water and treating the soil with calcium hydroxide. After treatment, the soil at the bottom of the pond was removed. One year after the procedure, crayfish were still caught in traps in the pond. The process was repeated again, but this time refuges in the banks were also concreted over (Girardet et al., 2012). No crayfish have been detected since completing the second procedure.

Similar success was also achieved with another pond *(Kunzareal, Rheinfelden)* inhabited by *P. clarkii*. In 2008, the pond was successfully restocked with *A. astacus* after it had been drained from winter 2006 until summer 2007 and following subsequent calcium hydroxide treatment (Stucki, 2018).

A pond populated by *P. leniusculus* was filled with excavated material to restore a disused gravel pit (*Kiesgrube Rohr)*. This measure was successful in eradicating the isolated crayfish population (Stucki, 2018).

TABLE 1 | Measures to control populations of NICS which were applied in Swiss waters.

Site name	Water type	Species	Aim	Method	Duration	Change of CPUE	Success
Aubach	Lotic	*P. leniusculus*	Suppression	Trapping	Continous since 2009	Increase by 100%	No**
Birsig	Lotic	*P. leniusculus*	Suppression	Trapping	Continous since 1997	–	Unclear*
Brook Near Origlio	Lotic	*P. clarkii*	Suppression	Trapping	Since 2013	Increase by 30%	No**
Brook Near Origlio	Lotic	*P. clarkii*	Suppression	Electricity	Since 2013	Increase by 30%	No**
Brook Near Origlio	Lotic	*P. clarkii*	Suppression	Hand catch	Since 2013	Increase by 30%	No**
Dättwiler Weiher	Lentic	*P. leniusculus*	Suppression	Trapping	Continous since 1997	Decrease by 75%	Yes**
Dättwiler Weiher	Lentic	*P. leniusculus*	Containment	Overflow pipe	Since 2002	–	Yes**
Dättwiler Weiher	Lentic	*P. leniusculus*	Suppression	Predatory fish	Campagin in the 90s	Decrease by 75%	Yes**
Depotsee Bern	Lentic	*O. limosus*	Suppression	Hand catch	1999–2004	–	Unclear*
Egelsee	Lentic	*F. limosus*	Containment	Overflow pipe	Since 2013	–	Unclear*
Eisweiher	Lentic	*P. leniusculus*	Suppression	Trapping	Since 2013	Increase by 20%	No**
Etzgerbach	Lotic	*P. leniusculus*	Containment	Crayfish barrier	Since 2015	100%	Yes**
Fischzucht Heuwies	Lentic	*P. leniusculus*	Eradication	Drainage	2012–2014 and 2019	–	Unclear*
Greifensee	Lentic	*O. limosus*	Suppression	Predatory fish	Campaign 2019	–	Unclear*
Katzensee	Lentic	*P. clarkii*	Suppression	Trapping	Since 2015	–	Unclear*
Katzensee	Lentic	*P. clarkii*	Suppression	Predatory fish	2019	–	Unclear*
Kiesgrube Rohr	Lentic	*P. clarkii*	Eradication	Filled-in	Campagin in the 90s	–	Yes**
Kunzareal Rheinfelden	Lentic	*P. clarkii*	Eradication	Drainage	2006/2007	–	Yes**
L'étang de Vidy	Lentic	*P. clarkii*	Eradication	Drainage	2008 et 2010	–	Yes**
Lützel	Lotic	*P. leniusculus*	Containment	Crayfish barrier	Since 2016	–	Unclear*
Mellinger Lanklagerweiher	Lentic	*P. clarkii*	Suppression	Trapping	Since 1997	–	No**
Mellinger Tanklagerweiher	Lentic	*P. clarkii*	Suppression	Predatory fish	Since 1997	–	No**
Mellinger Tanklagerweiher	Lentic	*P. clarkii*	Eradication	Biocide	Campagin in 2007	–	No**
Mellinger Tanklagerweiher	Lentic	*P. clarkii*	Containment	Catch basket	Since 2000–2016	–	Unclear*
Mellinger Tanklagerweiher	Lentic	*P. clarkii*	Containment	infiltration system	since 2016	–	Unclear*
Pisciculture de Saint-Victor	Lentic	*P. leniusculus*	Suppression	Predatory fish	Campaign in 2020	–	Unclear*
Pisciculture de Saint-Victor	Lentic	*P. leniusculus*	Suppression	Trapping	Since 2020	–	Unclear*
Pond Near Passeiry	Lentic	*P. leniusculus*	Suppression	Predatory fish	Campaign in 2020	Decrease by 100%	Yes**
Pond Near Passeiry	Lentic	*P. leniusculus*	Suppression	Trapping	Since 2020	Decrease by 100%	Yes**
Pfaffnern	Lotic	*P. leniusculus*	Containment	Crayfish barrier	Since 2017	–	Yes**
Riehenteich	Lentic	*P. leniusculus*	Suppression	Trapping	Since 2009	Increase by 70%	No**
Roulave	Lotic	*P. leniusculus*	Containment	Crayfish barrier	Planned for 2021	–	Unclear*
Rumensee	Lentic	*P. clarkii*	Suppression	Trapping	2007 and 2018	–	Unclear*
Schübelweiher	Lentic	*P. clarkii*	Suppression	Trapping	Start 2002	Decrease by 75%	Yes**
Schübelweiher	Lentic	*P. clarkii*	Suppression	Predatory fish	Start 2002	Decrease by 75%	Yes**
Stadtbach, Baden	Lotic	*P. leniusculus*	Suppression	Hand catch	Since 1997	–	No**
Stadtbach, Baden	Lotic	*P. leniusculus*	Suppression	Electricity	Campagne in the 90s	–	No**
Stadtbach, Baden	Lotic	*P. leniusculus*	Eradication	Drainage	2004 and 2005	–	Yes**
Steinbruch Mellikon	Lentic	*P. clarkii*	Eradication	Drainage	Campaign 2013	–	Yes**
Wenkenweiher	Lentic	*A. leptodactylus*	Suppression	Trapping	Since 2009	Increase by 30%	No**

*Le, Lentic water; L, lotic water; Data rating: * = insufficient; ** = valid.*

In *Fischzucht Heuwies*, an attempt to completely remove a population of *P. leniusculus* by draining the fish pond from December 2012 to April 2014 was almost successful. No crayfish was found during monitoring from 2015 to 2018. In 2019, the pond was drained again to carry out construction works to reconnect the pond to the brook. In the drained pond,

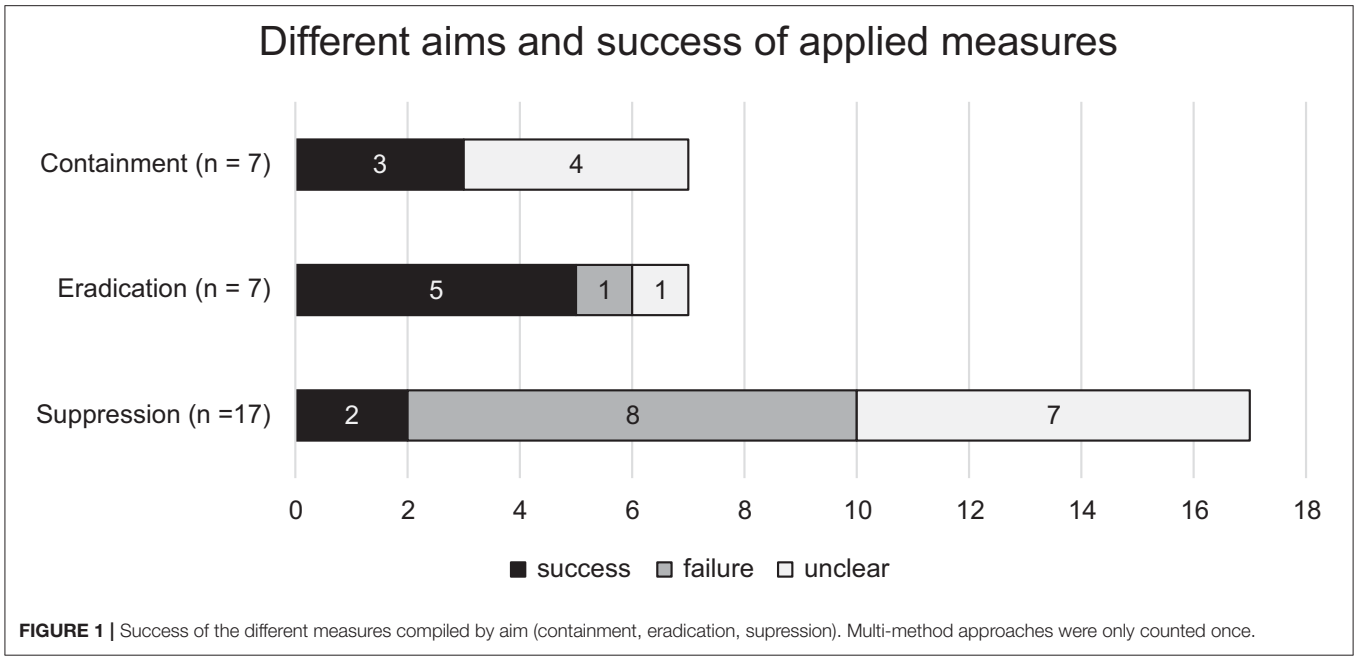

FIGURE 1 | Success of the different measures compiled by aim (containment, eradication, supression). Multi-method approaches were only counted once.

12 *P. leniusculus* were found in multiple hand catches. During construction work, the drained pond was mechanically treated and the majority of the soil at the bottom of the pond was removed (∼10,000 m^{-3}). Since reconstruction, no invasive crayfish have been detected with traps or using eDNA. The success of the eradication measure in *Fischzucht Heuwies* is still open as it was conducted only one year before publication of this article.

Biocides

Liquid manure was used in an attempt to eradicate a population of *P. clarkii* living in an artificial outflow culvert of a pond *(Mellinger Tanklagerweiher)*. The culvert was blocked at one end and filled with liquid manure from cows and hens and left for 48 h before being removed. Complete eradication of crayfish in the treated area was not possible using this method (Stucki, 2018).

In the 1990s, the fishing authorities planned to treat two ponds *(Schübelweiher, Rumensee)* inhabited by invasive *P. clarkii* with fenthion, an insecticide used to kill mosquitos, fleas and ticks as well as any unwanted fish and crayfish in fish breading stations. However, the project was stopped due to protests from nature conservation organisations including the WWF and local activists about the predicted negative impacts which fenthion would have had on other aquatic life. An alternative action plan was carried out avoiding the use of harmful chemicals; the combined use of predatory fish and trapping were sufficient to regulate the invasive crayfish population and reduce their negative impacts on ecosystem (Borner et al., 1997, 1998; Frutiger and Müller, 2002).

Suppression of NICS

To suppress NICS mechanical removal by traps, hand-catch, electricity and introduction of predatory fish were applied. In 2 of 12 lentic waters (∼17%) trapping of *P. leniusculus* in combination with the introduction of predatory fish led to a 75% reduction in CPUE. In six of the lentic waters, data was classified as "unclear" and in four cases there was no success despite valid data. In lotic waters, three of the control methods applied were so far unsuccessful and one success was unclear.

Mechanical Removal

Tapping of crayfish was carried out by cantonal fishery authorities (pond near *Passeiry* and *Pisciculture de Saint-Victor)*, community service workers *(Aubach, Birsig, Eisweiher, Riehenteich,* and *Wenkenweiher)*, water tenants *(Dättwiler Weiher, Katzensee, Mellinger Tanklagerweiher, Rumensee,* and *Schübelweiher)* and by a private environmental company (brook near *Origlio*). A corresponding permit from the responsible department for fisheries is needed to be allowed to catch invasive crayfish in Switzerland. Water tenants were obliged to remove crayfish of all age groups and of both sexes and were forbidden to put any back after they had been caught. Crayfish from *Mellinger Tanklagerweiher*, *Schübelweiher*, und *Katzensee* were sold to restaurants to be eaten.

At sites where trapping was combined with introduction of predatory fish a reduction in CPUE of 75% was observed in *Dättwiler Weiher, Schübelweiher* and 100% in *Pisciculture de Saint-Victor*. In *Mellinger Tanklagerweiher*, no change in CPUE was observed over 19 years despite the introduction of pike. In the pond near *Passeiry*, the CPUE decreased from 10.9 to 0 from mid-July to beginning of September. At this site, artificial refuge traps (ARTs) were used in addition to "pirate" traps (Bock-Ås Ltd., Finland). However, only 0.4% of the 2,480 individuals

caught at *Passeiry* were trapped in ARTs; these were mainly small indiviudals (<8 cm total length, sex ratio 5:4).

Electricity

Three methods of electricity exposure were tested to try to eradicate *P. leniusculus* in a 300 m long stretch of a culvert-isolated stream *(Stadtbach, Baden);* direct current (electric fishing gear, 500 V, 5 A, 30 s exposure), impulse current (electric fishing gear, 1,000 V, 1 A, 100 Hz, 5 s exposure) and alternating current (fish killing gear, 40 V, 0.35 A, 50 Hz, 60 s exposure). Crayfish exhibited strong behavioural reactions to the impulse current when alternating and direct current were applied but there were no mortalities of crayfish (Stucki, 2018).

However, electrofishing devices were successful in catching *P. clarkii* in a small brook near *Origlio;* on average four electrofishing-campaigns were implemented each year. At this site, electrofishing, trapping and hand catch have been used in combination for 5 years. In this brook more crayfish could be caught using electrofishing compared to trapping and hand-catch during night-time inspections. Nevertheless, there has not yet been significant decrease in the number of crayfish caught so far.

Introduction of Predatory Fish

Pike, *Esox lucius* (Linnaeus, 1758), perch, *Perca fluviatilis* (Linnaeus, 1758), zander, *Sander lucioperca* (Linnaeus, 1758), and *A. anguilla* are all predatory species which were introduced individually or combination into seven lentic waters to reduce the occurrence of three invasive crayfish; *P. lenisculus (Dättwiler Weiher, Pisciculture de Saint-Victor,* and pond near *Passeiry)*, *P. clarkii (Mellinger Tanklagerweiher, Katzensee, Schübelweiher)*, and *F. limosus (Greifensee)*.

The introduction of eel into *Pisciculture de Saint-Victor* and the pond near *Passeiry* was conducted in summer 2019 and results are from autumn of the same year. In two of the lentic waters, the release of predatory fish in combination with the use of trapping, led to a reduction in CPUE of 75%; after three (*Dättwilerweiher*) and six years (*Schübelweiher*). In four cases, success cannot be assessed due to the insufficient data gathered so far *(Greifensee, Katzensee, Pisciculture de Saint-Victor,* and pond *near Passeiry)*. A reduced tendency of *P. clarkii* to spread over land was reported by the local fishery department after implementation of control measures in *Schübelweiher, Katzensee*, and *Mellinger Tanklagerweiher*.

Containment of NICS

To prevent further spread of NICS several barriers were constructed in Switzerland. Three out of eight barriers have so far proven their success. For the other five, an assessment of success is not yet possible and have so far been classified as "unclear".

Crayfish Barriers in Lentic Waters

It was attempted to stop the spread of *F. limosus* from a two hectare lake *(Egelsee)* into an adjoining river by covering the outlet drain with a metal mesh cage which was filled with stones. This construction must be regularly cleaned because the banks will be flooded if it becomes clogged. It was later discovered that the protected river had been populated by *F. limosus* from another river section and therefore success of this construction was deemed "unclear".

In a pond *(Dättwiler Weiher),* an overflow pipe was installed several meters away from the bank in order to stop the migration of *P. leniusculus* from the pond into the adjoining brook. So far there has been no evidence of invasive crayfish present in the outflow brook.

A catch basket was installed at the end of the culvert in order to stop *P. clarkii* spreading from the two-hectare lake *Tanklagerweiher Mellingen* into the outflowing brook. The catch basket had to be emptied and cleaned when clogged; the frequency of cleaning was dependant on season. An infiltration system with gravel was installed directly at the outflow of the lake in order to reduce maintenance efforts at this site. The success could not be confirmed so far, as some of the invasive crayfish have already established downstream of the construction. These individuals must first be eliminated before the success of the catch basket as a control method can be assessed.

Crayfish Barriers in Lotic Waters

In the *Etzgerbach*, a crayfish barrier was built to prevent *P. leniusculus* from migrating into the headwaters where native *A. torrentium* are found. The barrier consisted of a 30 cm free fall with a steel overhanging lip. A function control with native *A. astacus* could confirm that the barrier functioned in preventing crayfish movement upstream while also allowing migration of trout (*Salmo trutta fario* Linnaeus 1758) with a body length ranging from 11.5 to 49 cm.

Another barrier with the same design as in *Etzgerbach* was built in the river *Lützel* in order to protect the habitat of *A. pallipes* from *P. leniusculus* invasion. There has been no detection of *P. leniusculus* in traps upstream of the barrier, even 4 years after its construction.

A 40 cm high crayfish barrier made out of stainless steel with a fish passable mid-section was built with connection to the river bed in the *Pfaffnern*. The stainless steel created smooth surfaces that in combination with high water velocities, ≥0.65 m/s, aimed to stop *P. leniusculus* from spreading further upstream (Frings et al., 2013). PIT-tagged *A. astacus* were released downstream of the barrier to verify if crayfish could overcome the barrier. No released *A. astacus* was detected upstream of the barrier during the 8 month study.

DISCUSSION

Eradication of NICS

Temporary Drainage or Destruction of a Habitat

Complete destruction of an isolated waterbody has led to a successful eradication of the invasive crayfish population in *Kiesgrube Rohr* and is the most promising method for future eradication attempts. Although drainage of waterbodies has been successful *(Steinbruch Mellikon* and *l'étange de Vidy)*, if the water is only drained for a few months, there is always the risk that some crayfish will survive in burrows or humid places and can rebuild

a stock (*Fischzucht Heuwies)*. Treatment with calcium hydroxide can in this case increase success possibility *(l'étange de Vidy* and *Kunzareal Rheinfelden)*.

It is difficult to definitively state that there are no crayfish inhabiting a particular waterbody after an eradication effort. Eradication is only considered successful after 2–5 years of monitoring without a crayfish detection (Peay et al., 2006). Trapping, night inspections and eDNA testing should be used in combination to effectively evaluate the presence of crayfish.

Biocides

Although the use of biocides is considered the cheapest and most efficient method to eradicate unwanted crayfish populations (Manfrin et al., 2019), they have never been used in Switzerland, with the exception of liquid manure. Examples from Scotland (Peay et al., 2019) and Sweden (Ljunggren and Sundin, 2010) demonstrate that the use of biocides can be successful in eradicating NICS in isolated waters systems and that non-target invertebrates start recolonisation within a month after treatment.

Difficulties in Eradicating NICS

The extinction vortex and minimum viable population density is species-specific and depends on predation, reproduction strategy and environmental factors-food source, habitat size, and disease (Fagan and Holmes, 2006). In theory, only a single male and female are needed to successfully rebuild a population. One study showed that recolonisation could theoretically begin with as little as eleven individuals in an isolated water of 1 km^2, this figure was calculated from walking distances of male *P. leniusculus* during breeding season (Peay, 2001). With such a small original population there can be problems with inbreeding which can result in low genetic diversity, meaning an entire population could more easily be wiped out by disease or by a natural disaster. Establishment of all populations of *F. limosus* in Europe can be traced back to the introduction of only 90 individuals (Filipová et al., 2011), underlining the fact that even if only a few individuals remain after a control measure this can be sufficient enough to build up the population again (Henttonen and Huner, 1997). For marbled crayfish (*Procambarus virginalis* Lyko, 2017), a species which reproduce by parthenogenesis, it is theoretical even possible to rebuild a population with only one individual (Ercoli, 2019). In this case, the probability of extinction is again much higher due to environmental factors, predation and failure to breed.

Suppression of NICS

Mechanical Removal

At sites where mainly adult crayfish were caught in traps, no reduction in population size was observed *(Aubach, Wenkenweiher Riehen, Eisweiher, Riehenteich)*. According to our data and in agreement with previous studies, the use of various techniques to reduce the population size clearly achieves better results than a single control approach (Manfrin et al., 2019). Species with high fecundity and early maturity react undesirably to harvest control measures because the niche of the removed adult crayfish is quickly taken over by younger individuals (Zipkin et al., 2009). This fact can explain the increase in CPUE at *Aubach, Eiweiher, Riehenteich*, *Wenkenweiher,* and in the brook near *Origlio*. Evaluating whether NICS eradication measures have been successful can take several years (Peay, 2001). Therefore, in order to prevent the population from rebuilding, the use of traps and other control methods must continue even when no more animals are caught.

In the *Passeiry,* the CPUE decreased to zero after one year of intense trapping with "pirate" traps and ARTs as well as after the introduction of eels. The treatment was only conducted for one year so success cannot yet be guaranteed. Trapping was stopped when no more crayfish were caught with traps. Traps have been shown to only catch a minority of the population (Chadwick et al., 2020), therefore, it can be assumed that there are still some crayfish occurring in the *Passeiry* and so trapping should be continued to avoid population numbers bouncing back. There was limited success to reduce invasive crayfish populations using ARTs in the *Passeiry*. In a typical upland river in the south-west of England more juvenile and female *P. leniusculus* were caught in ARTs than in conventional traps indicating the advantage of applying this method (Green et al., 2018). The poor catch rate in ARTs in the *Passeiry* could be because there are many naturally existing refuges already present in the banks, which makes the traps a less attractive refuge for the crayfish. Another reason for the lack of success of ARTs in Switzerland could be the fact that the number of crayfish was quickly lowered by the eels introduced at the same time as the traps.

For *Birsig, Katzensee, Greifensee*, and *Rumensee* no data is available regarding effort and catch numbers: therefore, it is not possible to evaluate success in suppressing these populations of NICS. For this reason it is important to use CPUE values or the capture-mark-recapture (CMR) technique to estimate the population size and assess the success of the method (Zimmerman and Palo, 2011).

Invasive crayfish fishing and sale has never led to eradication of a NICS population in Switzerland. The creation of a culinary market for invasive crayfish promotes illegal stocking and means that the population of invaders is maintained instead of eradicated (Nuñez et al., 2012). This issue is also highlighted in the IAA Gotland Resolution, which was formulated at the "IAA Gotland 2019 Crayfish conference" (Edsman, 2019).

Electricity

In Switzerland, the use of electricity in an isolated section of the *Stadtbach, Baden* was not effective in eradicating *P. leniusculus*. However, the use of repeated high intensity (69 W, direct current 1,600 V, 57.8 A, at 7 Hz) shocks resulted in high mortality (86–97%) of *P. leniusculus* inhabiting a stony headwater stream in England (Peay et al., 2014). In the treatment in England, they used 1.6 V and amps eleven times higher than in *Stadtbach, Baden.* This could be the reason for the failure to eradicate the invasive population at this site.

In a small stream (brook near *Origlio)*, even the use of electrofishing combined with night inspections and the use of traps, could not reduce the catch number of *P. clarkii* over 5 years. One reason for this could be due to the specific life-history traits

of *P. clarkii* including its high fecundity and early maturity which allows fast population recovery (Chucholl, 2011).

Introduction of Predatory Fish

In Switzerland, introduction of predatory fish in combination with the use of traps has been successful in reducing recorded overland movements of *P. clarkii.* However, it is assumed that the population size will grow again if trapping is stopped and the number of predatory fish decrease (Paragamian, 2010). The use of native predatory fish is regarded as a good way to reduce the number of juvenile crayfish and complements trapping which removes reproducing adults (Elvira et al., 1996; Aquiloni et al., 2010; Musseau et al., 2015).

Non-native predators can also reduce crayfish densities (Miyake and Miyashita, 2011). Thus, the removal of invasive predatory fish, namely, pike, perch or catfish, from conservation ponds, can lead to an increase in the population size of invasive crayfish; consequently, causing negative effects on other animal and plant species. The release of native predatory fish in previously uninhabited waters can also have a negative impact on the other species present, including amphibians (Braña et al., 1996; Hecnar and M'Closkey, 1997). Since invasive crayfish also have a negative influence on native plants and animals, the pros and cons of each control method must be considered to determine which will be most beneficial for each site.

Catching of predatory fish in waterways with NICS should be prohibited or regulated. If recreational fishing is allowed in certain ponds, new groups of predatory fish should be regularly introduced to the water system to ensure that their numbers are kept high; therefore, maximising the effect of predatory fish on crayfish.

Containment of NICS

Preventing the further spread of NICS through artificial or natural barriers is an important tool for invasive species management. The use of artificial barriers or modification to existing structures prevents the further spread of invasive crayfish and is a more cost-effective method compared to carrying out never-ending stock control when crayfish have invaded new sections of a lotic water. In Switzerland, the erection of barriers is part of the national strategy to prevent NICS from interfering with further waters (Stucki and Zaugg, 2006).

The crayfish barriers in the *Etzgerbach*, Lützel, and *Pfaffnern* which are mentioned in this paper were so far able to prevent upstream movement of crayfish. Barriers are the only known way to stop the natural spread of NICS within a water system. On the other hand, crayfish barriers can impede the migration of poor swimming fish; therefore, each new case a balance of interests has to be made as to whether fish migration or the prevention of the spread of NICS is more important (Krieg and Zenker, 2020). Most crayfish barriers will be constructed to protect side waters which are mainly inhabited by trout. Functional controls carried out at the barrier in the *Etzgerbach* highlighted that marked trout were able to overcome the crayfish barrier here; indicating that trout are not negatively affected by artificial crayfish barriers.

Despite the lack of function control in *Mellinger Tanklagerweiher*, the use of an infiltration system is a promising method to prevent the spread of NICS from a pond into an adjoining brook as it is not physically possible for crayfish to dig through this infiltration system.

Combination of Different Methods

According to the results of this study, success in suppression of an NICS population can be better achieved by using a combination of several control methods (electricity, ARTs, hand-catch, traps, and predatory fish) rather than applying just one method on its own. The reduction in size of populations of NICS was achieved exclusively in lentic waters, when combining the use of traps with the stocking of predatory fish. Predatory fish have a larger impact on preventing population growth rate than traps because they target the offspring which are responsible for future population growth. Traps on the other hand have a larger impact on breeding adults and they often miss juveniles which are small and can escape through gaps in the traps (Hein et al., 2006). The selectiveness of each method means that they should be used in combination to be most effective. If the majority of adult crayfish are caught in traps, there can be an increase in reproduction in the population whereby females produce more eggs at a younger age and there is a higher rate of survival of juveniles because of lower intraspecific predation and increased food availability (Momot, 1998).

Data Collection

In this study, data showed that no conclusions could be drawn about the success of the control measures in ∼43% of the sites ($n = 12$). However, in 57% of treated waters ($n = 16$), the data could be used to assess the success of the method and whether its continuation is appropriate.

The meticulous collection of catch data, in particular the determination of the CPUE, is mandatory in order to assess the growth of a population over time, thus the success of a control measure (Schwarz and Seber, 1999). By catching and measuring all age groups, it is also possible to make an assessment of the number of reproducing individuals and highlight any visible population growth trends (Rabeni et al., 1997; Paillisson et al., 2011). Another technique to evaluate population size is CMR which is a promising option in smaller ponds (Coignet et al., 2012).

Conclusion

It is more effective to completely eradicate an invasive species with drastic measures as soon as it is found inhabiting a water rather than continuously reducing population densitiy by suppression or isolating a population by containment (Simberloff 2014). Populations of NICS should be removed as soon as possible to avoid further damage and destruction of the habitat for native species. If the use of biocides is not possible, it is recommended to drain or destroy water bodies whenever possible to eradicate populations of NICS. Suppression and containment methods can be used to minimise the negative effects which NICS have on a habitat.

A combination of trapping and introduction of predatory fish has proven successful as a suppression multi-method approach. Crayfish barriers are the only way to contain an established NICS population to ensure that they are isolated from invading further upstream. Global warming may pose further problems as invasive species are advantaged by the increasing water temperatures, meaning they will be able to spread even faster and colonise previously inappropriate habitats (Rahel and Olden, 2008).

It is important that measures to control invasive species are carried out and agreed nationwide and across countries to prevent spread from places where no measures are taken. This is always a difficult issue to overcome when trying to control an invasive species of animal or plant, as land or municipal boundaries set by humans do not apply to them (Fernandes et al., 2019; Beaury et al., 2020). The development of a common strategy based on the experience gained to date can significantly increase success of invasive species eradication as well as saving money, the environment and the species inhabiting it.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

RK, AK, and AZ conceived the ideas and designed the methodology. RK collected and analysed the data. AK and AZ led to the writing of the manuscript and contributed critically to the drafts and all authors gave final approval for publication.

ACKNOWLEDGMENTS

The Authors would like to thank the following people for providing the data for this paper: Cédric Henry, Daniel Zopfi, Daniel Bernet, Danilo Foresti, Gabriel Sutter, Gottlieb Dandliker, Hans-Peter Jermann, Lukas Bammatter, Michael Kugler, Mirko Zanini, Rainer Kühnis, and Thomas Stucki.

REFERENCES

Aquiloni, L., Brusconi, S., Cecchinelli, E., Tricarico, E., Mazza, G., Paglianti, A., et al. (2010). Biological control of invasive populations of crayfish: the European eel *(Anguilla anguilla)* as a predator of *Procambarus clarkii*. *Biol. Invasions* 12, 3817–3824. doi: 10.1007/s10530-010-9774-z

Ballantyne, L., Baum, D., Bean, C. W., Long, and, J., and Whitaker, S. (2019). "Successful eradication of signal crayfish (*Pacifastacus leniusculus*) using a non-specific biocide in a small isolated water body in Scotland," in *2019 Isl. Invasives Scaling up to Meet Challenge. Occas. Pap. SSC no. 62,* eds C. R. Veitch, M. N. Clout, A. R. Martin, J. C. Russell C. J. West (Dundee: IUCN), 443–446.

Beaury, E. M., Fusco, E. J., Jackson, M. R., Laginhas, B. B., Lyn, T., Jenica, M., et al. (2020). Incorporating climate change into invasive species management: insights from managers. *Biol. Invasions* 22, 233–252. doi: 10.1007/s10530-019-02087-6

Bills, T. D., and Marking, L. L. (1988). Control of nuisance populations of crayfish with traps and toxicants. *Progres Fish Cultu.* 50, 103–106. doi: 10.1577/1548-8640(1988)050<0103:CONPOC>2.3.CO;2

Borner, S., Büsser, T., Eggen, R., Fent, K., and Frutiger, A. (1997). Der Rote Sumpfkrebs - Situationsanalyse und Vorschläge für Bekämpfungsmassnahmen. *Eawag Swiss Fed. Inst. Aquat. Sci. Technol. Dübendorf.* 27.

Borner, S., Büsser, T., Eggen, R., Frutiger, A., Müller, R., Müller, S., et al. (1998). Der Rote Sumpfkrebs im Schübelweiher - Auswertung der Massnahmen 1997. *Eawag Swiss Fed. Inst. Aquat. Sci. Technol. Dübendorf.* 23.

Braña, F., Frechilla, L., and Orizaola, G. (1996). Effect of introduced fish on amphibian assemblages in mountain lakes of northern Spain. *Herpetol. J.* 6, 145–148.

Burič, M., Hulák, M., Kouba, A., Petrusek, A., and Kozák, P. (2011). A successful crayfish invader is capable of facultative parthenogenesis: a novel reproductive mode in decapod crustaceans. *PLoS ONE* 6:e0020281. doi: 10.1371/journal.pone.0020281

Cecchinelli, E., Aquiloni, L., Maltagliati, G., Orioli, G., Tricarico, E., and Gherardi, F. (2012). Use of natural pyrethrum to control the red swamp crayfish *Procambarus clarkii* in a rural district of Italy. *Pest Manag. Sci.* 68, 839–844. doi: 10.1002/ps.2335

Chadwick, D. D. A., Pritchard, E. G., Bradley, P., Sayer, C. D., Chadwick, M. A., Eagle, L. J. B., et al. (2020). A novel 'triple drawdown' method highlights deficiencies in invasive alien crayfish survey and control techniques. *J. Appl. Ecol.* 29. doi: 10.1111/1365-2664.13758

Chucholl, C. (2011). Population ecology of an alien "warm water" crayfish (*Procambarus clarkii*) in a new cold habitat. *Knowl. Manag. Aquat. Ecosyst.* 401, 1–21. doi: 10.1051/kmae/2011053

Coignet, A., Pinet, F., and Souty-Grosset, C. (2012). Estimating population size of the red swamp crayfish (*Procambarus clarkii*) in fish-ponds (Brenne, Central France). *Knowl. Manag. Aquat. Ecosyst.* 406:11 doi: 10.1051/kmae/2012019

Cowart, D. A., Breedveld, K. G. H., Ellis, M. J., Hull, J. M., and Larson, E. R. (2018). Environmental DNA (eDNA) applications for the conservation of imperiled crayfish (*Decapoda*: *Astacidea*) through monitoring of invasive species barriers and relocated populations. *J. Crustac. Biol.* 38, 257–266. doi: 10.1093/jcbiol/ruy007

Edsman, L. (2019). Gotland Resolution. *Crayfish News* 41, 10–13.

Elvira, B., Gnicola, G., and Almodovar, A. (1996). Pike and red swamp crayfish: a new case on predator-prey relationship between aliens in central Spain. *J. Fish Biol.* 48, 437–446. doi: 10.1006/jfbi.1996.0043

Ercoli, F. (2019). First record of an established marbled crayfish *Procambarus virginalis* (Lyko, 2017) population in Estonia. *BioInvasions Rec.* 8, 675–683. doi: 10.3391/bir.2019.8.3.25

Fagan, W. F., and Holmes, E. E. (2006). Quantifying the extinction vortex. *Ecol. Lett.* 9, 51–60. doi: 10.1111/j.1461-0248.2005.00845.x

Fernandes, R. F., Honrado, J. P., Guisan, A., Roxo, A., Alves, P., Martins, J., et al. (2019). Species distribution models support the need of international cooperation towards successful management of plant invasions. *J. Nat. Conserv.* 49, 85–94. doi: 10.1016/j.jnc.2019.04.001

Filipová, L., Lieb, D. A., Grandjean, F., and Petrusek, A. (2011). Haplotype variation in the spiny-cheek crayfish *Orconectes limosus:* colonization of Europe and genetic diversity of native stocks. *J. North Am. Benthol. Soc.* 30, 871–881. doi: 10.1899/10-130.1

Freeman, M. A., Turnbull, J. F., Yeomans, W. E., Bean, C. W., Lumpur, K., Foundation, C. R., et al. (2010). Prospects for management strategies of invasive crayfish populations with an emphasis on biological control. *Aquat. Conserv. Mar. Freshw. Ecosyst.* 20, 211–223. doi: 10.1002/aqc.1065

Frings, R. M., Vaeßen, S. C. K., Gro,ß, H., Roger, S., Schüttrumpf, H., and Hollert, H. (2013). A fish-passable barrier to stop the invasion of non-indigenous crayfish. *Biol. Conserv.* 159, 521–529. doi: 10.1016/j.biocon.2012.12.014

Frutiger, A., and Müller, R. (2002). Der Rote Sumpfkrebs im Schübelweiher: Auswertung der Massnahmen 1998 – 2001 und Erkenntnisse. *Eawag Swiss Fed. Inst. Aquat. Sci. Technol. Dübendorf.* 26.

Gherardi, F. (2006). Crayfish invading Europe: the case study of *Procambarus clarkii*. *Mar. Freshw. Behav. Physiol.* 39, 175–191. doi: 10.1080/10236240600869702

Girardet, M., Cherix, D., Hofmann, F., and Rubin, J. (2012). Eradication d'une population d'écrevisses de Louisiane, *Procambarus clarkii,* à l'étang de Vidy et situation des populations d'écrevisses à Lausanne, Suisse. *Bull. Soc. Vaud. Sc. Nat.* 93:11. Available online at: https://serval.unil.ch/en/notice/serval:BIB_67962B5CA8CB

Green, N., Bentley, M., Stebbing, P., Andreou, D., and Britton, R. (2018). Trapping for invasive crayfish: comparisons of efficacy and selectivity of baited traps versus novel artificial refuge traps. *Knowl. Manag. Aquat. Ecosyst.* 419:9. doi: 10.1051/kmae/2018007

Hecnar, S. J., and M'Closkey, R. T. (1997). The effects of predatory fish on amphibian species richness and distribution. *Biol. Conserv.* 79, 123–131. doi: 10.1016/S0006-3207(96)00113-9

Hefti, D., and Stucki, P. (2006). Crayfish management for Swiss waters. *Bull. Français la Pêche la Piscic.* 937–949. doi: 10.1051/kmae:2006033

Hein, C. L., Roth, B. M., Ives, A. R., and vander Zanden, M. J. (2006). Fish predation and trapping for rusty crayfish *(Orconectes rusticus)* control: a whole-lake experiment. *Can. J. Fish. Aquat. Sci.* 63, 383–393. doi: 10.1139/f05-229

Hein, C. L., Vander Zanden, M. J., and Magnuson, J. J. (2007). Intensive trapping and increased fish predation cause massive population decline of an invasive crayfish. *Freshw. Biol.* 52, 1134–1146. doi: 10.1111/j.1365-2427.2007.01741.x

Henttonen, P., and Huner, J. V. (1997). "The introduction of alien species of crayfish in Europe: A historical introduction," in *Crayfish in Europe as Alien Species: How to Make the Best of the Bad Situation?* eds *F.* Gherardi, and D. M. Holdich (Rotterdam: A. A. Balkema), 13–22. doi: 10.1201/9781315140469-2

Holdich, D. M., Reynolds, J. D., Souty-Grosset, C., and Sibley, P. J. (2009), A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. *Knowl. Manag. Aquat. Ecosyst.* 11, 394–395. doi: 10.1051/kmae/2009025

Kouba, A., Petrusek, A., and Kozák, P. (2014). Continental-wide distribution of crayfish species in Europe: update and maps. *Knowl. Manag. Aquat. Ecosyst.* 413, 31. doi: 10.1051/kmae/2014007

Krieg, R., and Zenker, A. (2020). A review of the use of physical barriers to stop the spread of non-indigenous crayfish species. *Rev. Fish Biol. Fish* 30, 423–435. doi: 10.1007/s11160-020-09606-y

Ljunggren, N., and Sundin, J. (2010). *Eliminering av Signalkräfta på Gotland - En Redovisning av Utförda Åtgärder Inom Åtgärdsprogrammet för Bevarande av Flodkräfta Under 2007–2009*, Technical Report. Visby, 1–51.

Manfrin, C., Souty-grosset, C., Anast, P. M., Reynolds, J., and Giulianini, P. G. (2019). Detection and control of invasive freshwater crayfish: from traditional to innovative methods. *Diversity* 11, 1–16. doi: 10.3390/d11010005

Miyake, M., and Miyashita, T. (2011). Identification of alien predators that should not be removed for controlling invasive crayfish threatening endangered odonates. *Aquat. Conserv. Mar. Freshw. Ecosyst.* 21, 292–298. doi: 10.1002/aqc.1178

Momot, W. T. (1998). "An example of how exploitation can increase production and yield in a northern crayfish *(Orconectes virilis)* population," in *Proceedings of the North Pacific Symposium on Invertebrate Stock Assessment and Management*, eds G. S. Jamieson, and A. Campbell (Ottawa, ON: Natinoal research council of Canada), 225–234.

Musseau, C., Boulenger, C., Crivelli, A. J., Lebel, I., Pascal, M., Boulêtreau, S., et al. (2015). Native European eels as a potential biological control for invasive crayfish. *Freshw. Biol.* 60, 636–645. doi: 10.1111/fwb.12510

Nuñez, M. A., Kuebbing, S., Dimarco, R. D., and Simberloff, D. (2012). Invasive Species: To eat or not to eat, that is the question. *Conserv. Lett.* 5, 334–341. doi: 10.1111/j.1755-263X.2012.00250.x

Nyström, P. (2002). "Ecology," in *Biology of Freshwater Crayfish,* ed D. M. *Holdich (Oxford, UK: Blackwell Science)*, 192–235.

Paillisson, J.-M., Soudieux, A., and Damien, J.-P. (2011). Capture efficiency and size selectivity of sampling gears targeting red-swamp crayfish in several freshwater habitats. *Knowl. Manag. Aquat. Ecosyst.* 401:12. doi: 10.1051/kmae/2011015

Paragamian, V. L. (2010). Increase in abundance of signal crayfish may be due to decline in predators. *J. Freshw. Ecol.* 25, 155–157. doi: 10.1080/02705060.2010.9664369

Peay, S. (2001). *Eradication of alien crayfish populations. R&D Technical Report W1-037/TR1. Environment Agency*. Bristol: Almondsbury.

Peay, S., Dunn, A. M., Kunin, W. E., McKimm, R., and Harrod, C. (2014). A method test of the use of electric shock treatment to control invasive signal crayfish in streams. *Aquat. Conserv. Mar. Freshw. Ecosyst.* 25, 874–880. doi: 10.1002/aqc.2541

Peay, S., Hiley, P. D., Collen, P., and Martin, I. (2006). Biocide treatment of ponds in Scotland to eradicate signal crayfish. *Bull. Français la Pêche la Piscic.* 380–381, 1363–1379. doi: 10.1051/kmae:2006041

Peay, S., Johnsen, S., Bean, C., Dunn, A., Sandodden, R., and Edsman, L. (2019). Biocide treatment of invasive signal crayfish: successes, failures and lessons learned. *Diversity* 11:29. doi: 10.3390/d11030029

Piazza, F., Aquiloni, L., Peruzza, L., Manfrin, C., Simi, S., Marson, L., et al. (2015). Managing of *Procambarus clarkii* by X-ray sterilisation of males: cytological damage to gonads. *Micron* 77, 32–40. doi: 10.1016/j.micron.2015.05.016

Rabeni, C. F., Collier, K. J., Parkyn, S. M., and Hicks, B. J. (1997). Evaluating techniques for sampling stream crayfish *(Paranephrops planifrons). New Zeal. J. Mar. Freshw. Res.* 31, 693–700. doi: 10.1080/00288330.1997.9516799

Rahel, F. J., and Olden, J. D. (2008). Assessing the effects of climate change on aquatic invasive species. *Consercation Biol.* 22, 521–533. doi: 10.1111/j.1523-1739.2008.00950.x

Schwarz, C. J., and Seber, G. A. F. (1999). Estimating animal abundance: review III. *Stat. Sci.* 14, 427–456. doi: 10.1214/ss/1009212521

Stebbing, P., Longshaw, M., and Scott, A. (2014). Review of methods for the management of non-indigenous crayfish, with particular reference to Great Britain. *Ethol. Ecol. Evol.* 26, 204–231. doi: 10.1080/03949370.2014.908326

Stucki, T. (2018). *Bekämpfung Amerikanischer Krebsarten im Kanton Aargau. Bot. Gesellschaft Liechtenstein-Sargans-Werdenberg*, 35, 33–40.

Stucki, P., and Zaugg, B. (2006). *Nationaler Aktionsplan Flusskrebse.* Bern: Bundesamt für Umwelt.

Stucki, P., and Zaugg, B. (2011). *Aktionsplan Flusskrebse Schweiz. Artenförderung von Edelkrebs, Dohlenkrebs und Steinkrebs.* Bern: Bundesamt füür Umwelt. 61.

Westman, K., Savolainen, R., and Julkunen, M. (2002). Replacement of the native crayfish *Astacus astacus* by the introduced species *Pacifastacus leniusculus* in a small, enclosed Finnish lake: a 30-year study. *Ecography* 25, 53–73. doi: 10.1034/j.1600-0587.2002.250107.x

Zimmerman, J. K. M., and Palo, R. T. (2011). Reliability of catch per unit effort (CPUE) for evaluation of reintroduction programs – a comparison of the mark-recapture method with standardized trapping. *Knowl. Manag. Aquat. Ecosyst.* 401:7. doi: 10.1051/kmae/2011016

Zipkin, E. F., Kraft, C. E., Cooch, E. G., and Sullivan, P. J. (2009). When can efforts to control nuisance and invasive species backfire? *Ecol. Appl.* 19, 1585–1595. doi: 10.1890/08-1467.1

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

BRIEF RESEARCH REPORT
published: 11 January 2021
doi: 10.3389/fevo.2020.622434

Coexistence of Two Invasive Species, *Procambarus clarkii* and *Aphanomyces astaci*, in Brackish Waters of a Mediterranean Coastal Lagoon

Laura Martín-Torrijos[1], Angel Jose Correa-Villalona[1], Antonio Pradillo[2] and Javier Diéguez-Uribeondo[1]*

[1] Department of Mycology, Real Jardín Botánico-CSIC (RJB-CSIC), Madrid, Spain, [2] Conselleria d'Agricultura, Desenvolupament Rural, Emergència Climàtica i Transició Ecològica, Generalitat Valenciana, Valencia, Spain

OPEN ACCESS

Edited by:
Hideyuki Doi,
University of Hyogo, Japan

Reviewed by:
Adam Petrusek,
Charles University, Czechia
Jiří Patoka,
Czech University of Life Sciences Prague, Czechia

***Correspondence:**
Laura Martín-Torrijos
lmtorrijos@rjb.csic.es

Specialty section:
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *28 October 2020*
Accepted: *14 December 2020*
Published: *11 January 2021*

Citation:
Martín-Torrijos L, Correa-Villalona AJ, Pradillo A and Diéguez-Uribeondo J (2021) Coexistence of Two Invasive Species, Procambarus clarkii and Aphanomyces astaci, in Brackish Waters of a Mediterranean Coastal Lagoon. Front. Ecol. Evol. 8:622434. doi: 10.3389/fevo.2020.622434

Procambarus clarkii is a worldwide freshwater invasive crustacean from North America and was first introduced into Europe the 1970s. Along with *P. clarkii*, another invasive alien species was also spreading: *Aphanomyces astaci*. This pathogen is listed among the 100 World's worst invasive species and involved in the European native crayfish decline. Although both species live in freshwater ecosystems, *P. clarkii* can withstand brackish waters and inhabit estuarine habitats. However, the presence of *A. astaci* associated to North American crayfishes has never been described in brackish waters. In this study, we have investigated the presence of *A. astaci* in a *P. clarkii* population of a Mediterranean coastal lagoon in the Albufera Natural Park, Valencia, Spain introduced in 1976. Our study confirmed the presence of this pathogen, and suggests that *A. astaci* has been spreading for more than four decades in the mentioned estuarine environment. Mitochondrial ribosomal rnnS and rnnL indicated that the isolated pathogen belongs to d1-haplotype (i.e., D-haplogroup) typically hosted by *P. clarkii*. The presence of this pathogen in a brackish environment may suggest a better adaptation than other *A. astaci* strains to adverse conditions, such as high salinity levels. This is a matter of concern for the conservation of European native freshwater crayfish and highlights once more the risk of introducing invasive alien crustaceans.

Keywords: salinity, oomycetes, pathogen, crayfish plague, estuarine habitat, biological invasions, crayfish

INTRODUCTION

Biological invasions represent one of the main threats to biodiversity. Some of the alien species causing these invasions are now common throughout the world and are driving the existing biodiversity toward homogenization (Piscart et al., 2011). Among the diverse ecosystems affected by invasive alien species, freshwater ecosystems are particularly rapidly altered and according to the European Union the impact of invaders accounts for billions of euros yearly (Tollington et al., 2015).

The success of freshwater invasive species seems to be due to a combination of several traits, such as aggressive behavior, higher metabolic, and growth rates, greater fecundity, omnivory, and great tolerance to different pH and salinities (Firkins, 1993; Tollington et al., 2015). In particular, freshwater crustaceans represent some of the most successful aquatic alien invaders (Hänfling et al., 2011). In European freshwater ecosystems, these represent about 53% of all macroinvertebrate invasive species, and almost half of the freshwater decapods in European waters, ca 46%, are invasive species (Karatayev et al., 2009). Five species of freshwater decapods of North American origin, i.e., the freshwater crayfish *Faxonius limosus, F. virilis, Pacifastacus leniusculus, Procambarus clarkii,* and *P. virginalis*, are included in a list of invasive alien species of the European Union concern pursuant to Regulation (EUR-lex, 2016) because of the alarming increasing impact to the flora and fauna of European ecosystems (Simberloff and Rejmánek, 2011).

Specifically, the red swamp crayfish, *P. clarkii*, was introduced into Europe in 1973 from Louisiana (see review by Alonso et al., 2000) and was identified as a high-risk species (Gherardi et al., 2011; Souty-Grosset et al., 2016; Oficialdegui et al., 2019). The high ecological plasticity of this species allows it to colonize most types of water bodies (Fidalgo et al., 2001; Scalici et al., 2010). Besides its omnivore condition, this invasive species is a major predator of aquatic vertebrates (i.e., amphibians and fishes) and invertebrates, and is responsible for the decline and local extinctions of many native species (Cruz and Rebelo, 2005; Geiger et al., 2005; Rodríguez et al., 2005; Casellato and Masiero, 2011). Since its introduction, *P. clarkii* has negatively affected freshwater ecosystems (Gherardi et al., 2011; Arce and Diéguez-Uribeondo, 2015), and native crayfish species (Diéguez-Uribeondo et al., 1997; Rezinciuc et al., 2015; Souty-Grosset et al., 2016; Martín-Torrijos et al., 2019). The latter was due to the fact that *P. clarkii* is a chronic carrier of the crayfish plague pathogen *Aphanomyces astaci* (Diéguez-Uribeondo and Söderhäll, 1993; Diéguez-Uribeondo et al., 1995; Aquiloni et al., 2010; Rezinciuc et al., 2014; Martín-Torrijos et al., 2019). This pathogenic oomycete is listed among the 100 World's worst invasive species (Lowe et al., 2000) and has provoked a rapid decline of native European freshwater crayfishes due to a disease named crayfish plague (see review by Rezinciuc et al., 2015).

This *A. astaci* carrier has colonized several coastal and saline environments in Europe (Fidalgo et al., 2001; Scalici et al., 2010; Sousa et al., 2013; Meineri et al., 2014) due to its resilience. Scalici et al. (2010) demonstrated that a *P. clarkii* population was capable of live and reproduce in a brackish wetland in Italy, with salinity varying between 16,200 and 29,600 ppm. *Procambarus clarkii*'s ability consists in regulating its metabolism (Casellato and Masiero, 2011) and adapting it to saline environments by osmoregulating the ions and compatible solutes in their hemolymph. This adaptation has enabled this invasive species to live and reproduce in diverse water salinities (Fidalgo et al., 2001; Scalici et al., 2010; Casellato and Masiero, 2011; Sousa et al., 2013; Meineri et al., 2014; Bissattini et al., 2015; Vodovsky et al., 2017; Dörr et al., 2020). However, there are scarce studies focusing on the tolerance, survival and dispersion in relation to salinity conditions of the crayfish plague pathogen, that is chronically carried in the cuticle of this invasive North American crayfish species.

First studies on *A. astaci* physiological response and zoospore production under different salt concentrations showed that high mineral salt concentrations inhibited the growth and sporulation (Unestam, 1969a,b). Moreover, Persson and Söderhäll (1986) and Rantamäki et al. (1992) suggested that *A. astaci* would barely survive for long or spread under brackish water conditions. However, investigations carried out in other *Aphanomyces* spp. such as *A. invadans* (Kiryu et al., 2005), and other closely related genera such as *Phytophthora* or *Saprolegnia* (Harrison and Jones, 1975; Padgett, 1984; Hearth and Padgett, 1990; Ali, 2009; Preuett et al., 2016) suggested that some oomycetes might survive under high salinity conditions and sporulate when salinity decreases.

So far, no studies regarding presence of *A. astaci* in their native carriers, i.e., North American freshwater species, living under adverse conditions of high salinity, have been performed in nature. The naturalized population of *P. clarkii* from the shallow Mediterranean coastal lagoon of the Albufera Natural Park of Valencia, in the east coast of the Iberian Peninsula, represents an ideal opportunity to investigate the survival of *A. astaci* in its original host under saline conditions. This Natural Park is listed in the Ramsar List of Wetlands of International Importance (RAMSAR, 2020) and Natura 2000 site (EU, 2020), and during the past decades have suffered from several anthropogenic impacts, shaping the lake to a seriously deteriorated ecosystem (Martín et al., 2020). The invasive *P. clarkii* was introduced there in 1976 and since then, the decline of the native white clawed crayfish populations, *Austropotamobius pallipes*, in Valencia has been associated to the co-introduction of both *P. clarkii* and *A. astaci* (Galindo et al., 2000; Martín-Torrijos et al., 2019).

The detection of crayfish plague pathogen and its genetic diversity can currently accurately be performed in clinical samples (Oidtmann et al., 2004, 2006; Vrålstad et al., 2009; Makkonen et al., 2018). Therefore, this work aims to detect whether the introduced *P. clarkii* still carry this pathogen in a Mediterranean brackish lagoon after four decades of its introduction. This information is necessary to better understand crayfish plague epidemiology and its survival in its chronically infected carriers in different ecosystems.

MATERIALS AND METHODS

Crayfish Sampling and Environmental Conditions

The lagoon of the Albufera Natural Park (Valencia) has the surface area of 2,433 ha, and comprises brackish water (salinity 1,280–1,920 ppm). We used one baited funnel trap during 24 h to collect a total of 40 individuals of *P. clarkii* in December 2018 from this Natural Park. The individuals collected were transferred to aquaria in the Real Jardín Botánico (RJB-CSIC) facilities in Madrid. To test the prevalence of the pathogen *A. astaci* in the sampled *P. clarkii*, we maintained the aquaria at 17°C until the crayfish molted.

Molecular Analyses: Genomic Isolation, PCR Amplification, and Sequencing

Molts were first kept in sterile distilled water for 3 days and observed for the presence of melanization spots and growing hyphae (**Figure 1**). If any growing hyphae were detected, we preserved part of the sample in 96% ethanol for further molecular analyses. Genomic DNA was isolated with the E.Z.N.A.® Insect DNA Kit (Omega Bio-Tek, Norcross, Georgia, United States). We performed a single round of PCR for the extracted DNA with the *A. astaci* diagnostic primers 42 (Oidtmann et al., 2006) and 640 (Oidtmann et al., 2004) (which amplify the ITS1 and ITS2 surrounding the 5.8S rDNA), according to the assay described by Oidtmann et al. (2006). All samples which PCR products were sequenced and matched with *A. astaci* (i.e., specific primers 42 and 640) were further analyzed in order to describe their genetic diversity (i.e., haplogroup and haplotype pathogen characterization). In order to do it, we amplified mitochondrial ribosomal small (rnnS) and large (rnnL) subunits as described in Makkonen et al. (2018). For all PCR reactions, we used a positive control (i.e., genomic DNA from a *A. astaci* SAP-Málaga5 pure culture; Makkonen et al., 2016) and a negative control (distilled Milli-Q water). Amplified products were visualized by electrophoresis in 1% agarose TAE gels stained with 0.5 μM SBYR1 Safe (Thermo Fisher Scientific, Waltham, MA, United States). Both strands of amplified PCR products (ITS, rnnS, and rnnL) were sequenced using an automated sequencer (Applied Biosystems 3730xl DNA, Macrogen, The Netherlands). Consensus sequences were assembled and edited with Geneious Prime 2019.2.1.

RESULTS

Pathogen Characterization and Molecular Analyses

Procambarus clarkii from Albufera Natural Park exhibited characteristic melanized areas in the soft abdominal cuticle and pereiopods. Microscopically, in these melanized areas we observed hyphal growth and also sporangia of *A. astaci* (**Figure 1**).

For a total of 40 analyzed crayfish, we obtained five positive samples for *A. astaci* based on amplification of the nuclear ribosomal ITS region (amplified by diagnostic primers 42 and 640 for *A. astaci*). These five sequences (GenBank accession numbers MW332633–MW332637) were identical and showed a 99.82% similarity to other *A. astaci* deposited in GenBank (e.g., sequence FM999249 of the isolate SAP302). Moreover, the mitochondrial rnnS and rnnL subunits belonged to the D-haplogroup, allowing us to concatenate both regions to obtain the d1-haplotype

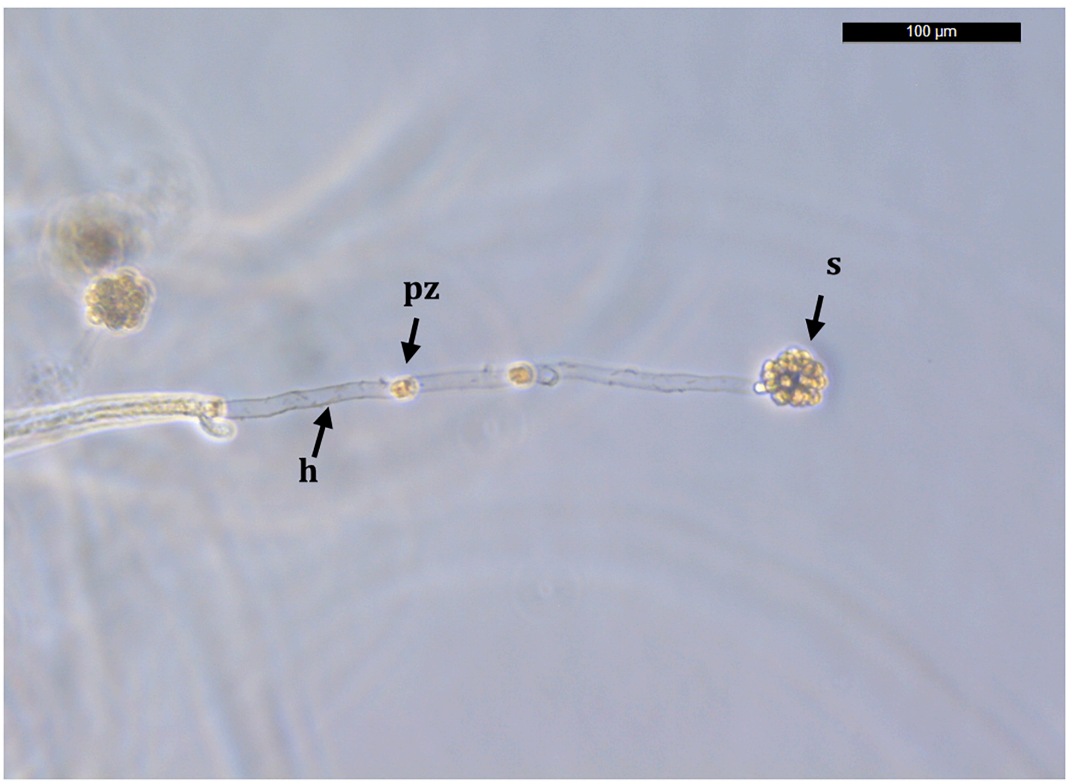

FIGURE 1 | An *Aphanomyces astaci* growing hypha and sporangium. The sporangium is formed from a hypha (h) growing from the cuticle of a *Procambarus clarkii* specimen originated from Albufera Natural Park, Valencia. Primary zoospores (pz) move toward the hyphal tip to form a characteristic spore ball sporangium (s) that will eventually produce secondary zoopores, i.e., infective unit. Bar = 100 μm.

(GenBank accession number for rnnS MW174856–MW174860 and for rnnL MW174851–MW174855).

DISCUSSION

The ability of *P. clarkii* to survive and reproduce in saline environments have been widely reported (Fidalgo et al., 2001; Scalici et al., 2010; Casellato and Masiero, 2011; Sousa et al., 2013; Meineri et al., 2014; Bissattini et al., 2015; Vodovsky et al., 2017; Dörr et al., 2020). However, the tolerance of *A. astaci* to grow in saline environments in its natural carriers, i.e., North American crayfish, have never been investigated. Panteleit et al. (2018) did not find *A. astaci* when tested its prevalence in nine marine decapods from the Black Sea. However, in this study, we report and describe for the first time the presence of the crayfish plague pathogen in a *P. clarkii* population that lives in a saline environment, a Mediterranean coastal lagoon of the Albufera Natural Park. The presence of the crayfish plague within a similar scenario, had only been reported in the Danube Delta and in the narrow-clawed crayfish (*Astacus leptodactylus*) (Schrimpf et al., 2012). Although these crayfish were in the vicinity of the river mouth, they might represent a real threat if their *A. astaci* strain could survive salinity concentrations, such as those of the Albufera Natural Park.

Procambarus clarkii was introduced in the Albufera Natural Park in 1976 (Galindo et al., 2000) and this population has survived in this lagoon until today. Our results show the presence of the pathogen *A. astaci* in this population, which corresponds to the same genetic group, i.e., D-haplogroup, identified in founder population of *P. clarkii* introduced in Spain in 1973 (Diéguez-Uribeondo et al., 1995; Makkonen et al., 2018; Martín-Torrijos et al., 2019). This lagoon is a changing environment with high salinity, accumulations of sediments, hypertrophic status, and intense daily oscillations in pH and dissolved oxygen (Martín et al., 2020). Previous studies on physiological adaptations of *A. astaci* isolates of the D-haplogroup showed that they can grow and sporulate at warmer temperatures than other genetic groups (Diéguez-Uribeondo et al., 1995). Physiological characteristics of this D-haplogroup may indicate a better ability to adapt to adverse conditions (e.g., higher temperatures, high salinity levels or low dissolved oxygen concentrations). Although brackish conditions are known to prevent *A. astaci* transmission (Rantamäki et al., 1992), the salinity of the Albufera Natural Park decreases during the months of May and September due to the rainfall and rice field irrigations. This fact appears to allow the dispersion of the pathogen by formation of the infection units of the pathogen, i.e., the swimming zoospores that infect other crayfish (Rantamäki et al., 1992). A similar effect was described by Kiryu et al. (2005) for the survival and spread of *A. invadans* in estuarine environments.

The resilience of both *P. clarkii* and *A. astaci* in saline environments constitutes an additional difficulty to the management of threatened populations of native European freshwater crayfish, which are susceptible to the crayfish plague pathogen, and, especially, to the native species of the Iberian Peninsula, *A. pallipes*. Currently, only 25 populations of this endangered native crayfish species remain in the Valencia province in small isolated highland streams (Generalitat Valenciana, 2019). Specimens of *P. clarkii* are continuously translocated and can transport this disease to surrounding areas, and potentially transmit it to certain decapods that might become vectors for this pathogen (e.g., the Chinese mitten crab, *Eriocheir sinensis*, or the semi-terrestrial *Potamon potamios* appear to be capable of transmitting *A. astaci* to the European native crayfish; Schrimpf et al., 2014; Svoboda et al., 2014). In the Albufera Natural Park, *P. clarkii* coexists with the blue crab, *Callinectes sapidus* that migrates into freshwater habitats during its live cycle (Hines et al., 1987) and since we found that *P. clarkii* can carry and transmit the crayfish plague pathogen, this dispersion could be favored by this invasive crab.

Therefore, this work alerts to the authorities and decision makers to rapidly develop and implement action plans to avoid the translocation from the Albufera Natural Park of *P. clarkii* and other potential carriers of *A. astaci* such as *C. sapidus*. Thus, future studies on salinity tolerance should be designed in order to determine the physiological adaptations of the different *A. astaci* genetic groups, highlighting brackish environments as favorable habitats for the maintenance of *A. astaci*. Moreover, transmission experiments in saline environments should be performed in order to get future insights about the biological characteristics of this pathogen and its possible transmission to other crustaceans. Furthermore, considering the current distribution of *P. clarkii* introductions and their negative impacts in non-European freshwater ecosystems (specifically for non-North American crayfish), these findings should be of worldwide aware and concern.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

AUTHOR CONTRIBUTIONS

LM-T and JD-U contributed to the design, supervision, and writing of this manuscript. AC-V conducted the sample field collection and carried out the molecular analyses (DNA extraction and haplotyping). AP conducted the sample field collection. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We acknowledge support of the publication fee to the Frontiers Fee Support program and the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI). We want to thank the assistance CCEDCV-EL PALMAR (Conselleria d'Agricultura, Desenvolupament Rural, Emergència Climàtica

i Transició Ecològica, Generatlitat Valenciana) for allowing the sampling and the performance of this research. We also want to thank the Master in Biodiversidad en Áreas Tropicales y su Conservación UIMP-CSIC, and María Martinez-Ríos and Gloria Casabella-Herrero from the RJB–CSIC for their technical assistance.

REFERENCES

Ali, E. H. (2009). Antifungal activity of sodium chloride on *Saprolegnia diclina* and *Aphanomyces* sp. *Acta Mycol.* 44, 125–138. doi: 10.5586/am.2009.011

Alonso, F., Temiño, C., and Diéguez-Uribeondo, J. (2000). Status of the white clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858) in Spain: distribution and legislation. *B. Fr. Peche Piscic.* 356, 31–54. doi: 10.1051/kmae:2000003

Aquiloni, L., Martín, M. P., Gherardi, F., and Diéguez-Uribeondo, J. (2010). The North American crayfish *Procambarus clarkii* is the carrier of the oomycete *Aphanomyces astaci* in Italy. *Biol. Invasions* 13, 359–367. doi: 10.1007/s10530-010-9828-2

Arce, J., and Diéguez-Uribeondo, J. (2015). Structural damage caused by the invasive crayfish *Procambarus clarkii* (Girard, 1852) in rice fields of the Iberian Peninsula: a study case. *J. Fundam. Appl. Limnol.* 186, 259–269. doi: 10.1127/fal/2015/0715

Bissattini, A. M., Traversetti, L., Bellavia, G., and Scalici, M. (2015). Tolerance of increasing water salinity in the red swamp crayfish *Procambarus clarkii* (Girard, 1852). *J. Crustac. Biol.* 35, 682–685. doi: 10.1163/1937240X-00002366

Casellato, S., and Masiero, L. (2011). Does *Procambarus clarkii* (Girard, 1852) represent a threat for estuarine brackish ecosystems of Northeastern Adriatic coast (Italy)? *J. Life Sci.* 5, 549–554.

Cruz, M. J., and Rebelo, R. (2005). Vulnerability of Southwest Iberian amphibians to an introduced crayfish. *Procambarus clarkii*. *Amphib-reptil.* 26, 293–303. doi: 10.1163/156853805774408577

Diéguez-Uribeondo, J., and Söderhäll, K. (1993). *Procambarus clarkii* as a vector for the crayfish plague fungus *Aphanomyces astaci* Schikora. *Aquacult. Fish. Manag.* 24, 761–765. doi: 10.1111/j.1365-2109.1993.tb00655.x

Diéguez-Uribeondo, J., Huang, T.-S., Cerenius, L., and Söderhäll, K. (1995). Physiological adaptation of an *Aphanomyces astaci* strain isolated from the freshwater crayfish *Procambarus clarkii*. *Mycol. Res.* 99, 574–578. doi: 10.1016/s0953-7562(09)80716-8

Diéguez-Uribeondo, J., Temiño, C., and Muzquiz, J. L. (1997). The crayfish plague fungus. Aphanomyces astaci in Spain. *B. Fr. Peche Piscic*. 347, 753–763.

Dörr, A. J. M., Scalici, M., Caldaroni, B., Magara, G., Scoparo, M., Goretti, E., et al. (2020). Salinity tolerance of the invasive red swamp crayfish *Procambarus clarkii* (Girard, 1852). *Hydrobiologia* 847, 2065–2081. doi: 10.1007/s10750-020-04231-z

EU, (2020). *Thirteenth update of the list of sites of community importance for the Mediterranean Biogeographical Region*. Belgium: *OJEU*.

EUR-lex, (2016). *Commission Implementing Regulation (EU) 2016/1141 of 13 July 2016 adopting a list of invasive alien species of union concern pursuant to Regulation (EU) No 1143/2014 of the European Parliament*. Belgium: Official Journal of the European Union.

Fidalgo, M. L., Carvalho, A. P., and Paulo, S. (2001). Population dynamics of the red swamp crayfish, *Procambarus clarkii* (Girard, 1852) from the Aveiro region, Portugal (Decapoda, Cambaridae). *Crustaceana* 74, 369–375. doi: 10.1163/156854001300104453

Firkins, I. (1993). Environmental tolerances of three species of freshwater crayfish. [PhD thesis]. [Nottingham (United Kingdom)]: University of Nottingham.

Galindo, J., Monzó, J., and Sancho, V. (2000). Estatus del cangrejo de río (*Austropotamobius pallipes*) en Valencia. *Dugastella* 1, 9–11.

Geiger, W., Alcorlo, P., Baltanás, A., and Montes, C. (2005). Impact of an introduced crustacean on the trophic webs of Mediterranean wetlands. *Biol. Invasions* 7, 49–73. doi: 10.1007/1-4020-3870-4_6

Generalitat Valenciana, (2019). *Actuaciones de conservación del cangrejo de río en la Comunitat Valenciana. Campaña 2019*. Valencia: Generalitat Valenciana.

Gherardi, F., Aquiloni, L., Diéguez-Uribeondo, J., and Tricarico, E. (2011). Managing invasive crayfish: Is there a hope? *Aquat. Sci.* 73, 185–200. doi: 10.1007/s00027-011-0181-z

Hänfling, B., Edwards, F., and Gherardi, F. (2011). Invasive alien crustacea: dispersal, establishment, impact and control. *BioControl* 56, 573–595. doi: 10.1007/s10526-011-9380-8

Harrison, J. L., and Gareth Jones, E. B. (1975). The effect of salinity on sexual and asexual sporulation of members of the Saprolegniaceae. *Trans. Brit. Mycol. Soc.* 65, 389–394. doi: 10.1016/s0007-1536(75)80035-0

Hearth, J. H., and Padgett, D. E. (1990). Salinity tolerance of an *Aphanomyces isolate* (Oomycetes) and its possible relationship to ulcerative mycosis (UM) of Atlantic menhaden. *Mycologia* 82, 364–369. doi: 10.1080/00275514.1990.12025892

Hines, A. H., Lipcius, R. N., and Haddon, A. M. (1987). Population dynamics and habitat partitioning by size, sex, and molt stage of blue crabs *Callinectes sapidus* in a subestuary of central Chesapeake Bay. *Mar. Ecol. Prog. Ser.* 36, 55–64. doi: 10.3354/meps036055

Karatayev, A. Y., Burlakova, L. E., Padilla, D. K., Mastitsky, S. E., and Olenin, S. (2009). Invaders are not a random selection of species. *Biol. Invasions* 11, 2009–2019. doi: 10.1007/s10530-009-9498-0

Kiryu, Y., Blazer, V. S., Vogelbein, W. K., Kator, H., and Shields, J. D. (2005). Factors influencing the sporulation and cystformation of *Aphanomyces invadans*, etiological agent of ulcerative mycosis in Atlantic menhaden. *Brevoortia tyrannus*. *Mycologia* 97, 569–575. doi: 10.1080/15572536.2006.1183278

Lowe, S., Browne, M., Boudjelas, S., and De Poorter, M. (2000). *100 of the world's worst invasive alien species. A selection from the Global Invasive Species Database*. Switzerland: The Invasive Species Specialist Group (ISSG) a specialist group of the Species Survival Commission (SSC) of the World Conservation Union (IUCN).

Makkonen, J., Jussila, J., Panteleit, J., Keller, N. S., Schrimpf, A., Theissinger, K., et al. (2018). MtDNA allow the sensitive detection and haplotyping of the crayfish plague disease agent *Aphanomyces astaci* showing clues about its origin and migration. *Parasitology* 145, 1210–1218. doi: 10.1017/S0031182018000227

Makkonen, J., Vesterbacka, A., Martin, F., Jussila, J., Dieguez-Uribeondo, J., Kortet, R., et al. (2016). Mitochondrial genomes and comparative genomics of *Aphanomyces astaci* and *Aphanomyces invadans*. *Sci. Rep.* 6:36089. doi: 10.1038/srep36089

Martín, M., Hernández-Crespo, C., Andrés-Doménech, I., and Benedito-Durá, V. (2020). Fifty years of eutrophication in the Albufera Lake (Valencia, Spain): causes, evolution and remediation strategies. *Ecol. Eng.* 155:105932. doi: 10.1016/j.ecoleng.2020.105932

Martín-Torrijos, L., Kokko, H., Makkonen, J., Jussila, J., and Diéguez-Uribeondo, J. (2019). Mapping 15 years of crayfish plague in the Iberian Peninsula: The impact of two invasive species on the endangered native crayfish. *PloS One* 14:e0219223. doi: 10.1371/journal.pone.0219223

Meineri, E., Rodriguez-Perez, H., Hilaire, S., and Mesleard, F. (2014). Distribution and reproduction of *Procambarus clarkii* in relation to water management, salinity and habitat type in the Camargue. *Aquat. Conserv.* 24, 312–323. doi: 10.1002/aqc.2410

Oficialdegui, F. J., Clavero, M., Sánchez, M. I., Green, A. J., Boyero, L., Michot, T. C., et al. (2019). Unravelling the global invasion routes of a worldwide invader, the red swamp crayfish (*Procambarus clarkii*). *Freshw. Biol.* 64, 1382–1400. doi: 10.1111/fwb.13312

Oidtmann, B., Geiger, S., Steinbauer, P., Culas, A., and Hoffmann, R. W. (2006). Detection of *Aphanomyces astaci* in North American crayfish by polymerase chain reaction. *Dis. Aquat. Organ.* 72, 53–64. doi: 10.3354/dao072053

Oidtmann, B., Schaefers, N., Cerenius, L., Söderhäll, K., and Hoffmann, R. W. (2004). Detection of genomic DNA of the crayfish plague fungus *Aphanomyces astaci* (Oomycete) in clinical samples by PCR. *Vet. Microbiol.* 100, 269–282. doi: 10.1016/j.vetmic.2004.01.019

Padgett, D. E. (1984). Evidence for extreme salinity tolerance in saprolegniaceous fungi (Oomycetes). *Mycologia* 76, 372–375. doi: 10.2307/3793118

Panteleit, J., Keller, N. S., Diéguez-Uribeondo, J., Makkonen, J., Martín-Torrijos, L., Patrulea, V., et al. (2018). Hidden sites in the distribution of the crayfish plague pathogen *Aphanomyces astaci* in Eastern Europe: Relicts of genetic groups from

older outbreaks? *J. Invertebr. Pathol.* 157, 117–124. doi: 10.1016/j.jip.2018.05.006

Persson, M., and Söderhäll, K. (1986). CaCl2-Induced germination and peptidase secretion in *Aphanomyces astaci*. *Exp. Mycol.* 10, 205–213. doi: 10.1016/0147-5975(86)90005-8

Piscart, C., Kefford, B. J., and Beisel, J. N. (2011). Are salinity tolerances of non-native macroinvertebrates in France an indicator of potential for their translocation in a new area? *Limnologica* 41, 107–112. doi: 10.1016/j.limno.2010.09.002

Preuett, J. A., Collins, D. J., Luster, D. G., and Widmer, T. L. (2016). The effect of salinity on the survival, growth, sporulation and infection of *Phytophthora ramorum*. *Fungal Ecol.* 23, 123–130. doi: 10.1016/j.funeco.2016.08.002

RAMSAR (2020). *The List of Wetlands of International Importance*. RAMSAR: RAMSAR.

Rantamäki, J., Cerenius, L., and Söderhäll, K. (1992). Prevention of transmission of the crayfish plague fungus (*Aphanomyces astaci*) to the freshwater crayfish *Astacus astacus* by treatment with MgCl2. *Aquaculture* 104, 11–18. doi: 10.1016/0044-8486(92)90133-6

Rezinciuc, S., Gallindo, J., Montserrat, J., and Diéguez-Uribeondo, J. (2014). AFLP-PCR and RAPD-PCR evidences of the transmission of the pathogen *Aphanomyces astaci* (Oomycetes) to wild populations of European crayfish from the invasive crayfish species. *Procambarus clarkii*. *Fungal Biol.* 118, 612–620. doi: 10.1016/j.funbio.2013.10.007

Rezinciuc, S., Sandoval-Sierra, J. V., Oidtmann, B., and Diéguez-Uribeondo, J. (2015). "The biology of crayfish plague pathogen *Aphanomyces astaci*. Current answers to most frequent questions," in *Freshwater Crayfish: A Global Overview*, eds T. Kawai, Z. Faulkes, and G. Scholtz, (Boca Raton, FL: CRC Press), 182–204. doi: 10.1201/b18723-12

Rodríguez, C. F., Bécares, E., Fernández-Aláez, M., and Fernández-Aláez, C. (2005). Loss of diversity and degradation of wetlands as a result of introducing exotic crayfish. *Biolog. Invasions* 7, 75–85. doi: 10.1007/1-4020-3870-4_7

Scalici, M., Chiesa, S., Scuderi, S., Celauro, D., and Gibertini, G. (2010). Population structure and dynamics of *Procambarus clarkii* (Girard, 1852) in a Mediterranean brackish wetland (Central Italy). *Biol. Invasions* 12, 1415–1425. doi: 10.1007/s10530-009-9557-6

Schrimpf, A., Pârvulescu, L., Copilaş-Ciocianu, D., Petrusek, A., and Schulz, R. (2012). Crayfish plague pathogen detected in the Danube Delta-A potential threat to freshwater biodiversity in Southeastern Europe. *Aquat. Invasions* 7, 503–510. doi: 10.3391/ai.2012.7.4.007

Schrimpf, A., Schmidt, T., and Schulz, R. (2014). Invasive Chinese mitten crab (*Eriocheir sinensis)* transmits crayfish plague pathogen (*Aphanomyces astaci*). *Aquat. Invasions* 9, 203–209. doi: 10.3391/ai.2014.9.2.09

Simberloff, D., and Rejmánek, M. (2011). *Encyclopedia of Biological Invasions*. Berkeley,CA: University of California Press.

Sousa, R., Freitas, F. E. P., Mota, M., Nogueira, A. J. A., and Antunes, C. (2013). Invasive dynamics of the crayfish *Procambarus clarkii* (Girard, 1852) in the international section of the river Minho (NW of the Iberian Peninsula). *Aquat. Conserv.* 23, 656–666. doi: 10.1002/aqc.2323

Souty-Grosset, C., Anastácio, P. M., Aquiloni, L., Banha, F., Choquer, J., Chucholl, C., et al. (2016). The red swamp crayfish *Procambarus clarkii* in Europe: Impacts on aquatic ecosystems and human well-being. *Limnologica* 58, 78–93. doi: 10.1016/j.limno.2016.03.003

Svoboda, J., Strand, D. A., Vrålstad, T., Grandjean, F., Edsman, L., Kozák, P., et al. (2014). The crayfish plague pathogen can infect freshwater-inhabiting crabs. *Freshw. Biol.* 59, 918–929. doi: 10.1111/fwb.12315

Tollington, S., Turbé, A., Rabitsch, W., Groombridge, J. J., Scalera, R., Essl, F., et al. (2015). Making the EU legislation on invasive species a conservation success. *Conserv. Lett.* 10, 112–120. doi: 10.1111/conl.12214

Unestam, T. (1969a). On the adaptation of *Aphanomyces astaci* as a parasite. *Physiol. Plant.* 22, 221–235. doi: 10.1111/j.1399-3054.1969.tb07371.x

Unestam, T. (1969b). On the physiology of zoospore production in *Aphanomyces astaci*. *Physiol. Plant.* 22, 236–246. doi: 10.1111/j.1399-3054.1969.tb07372.x

Vodovsky, N., Patoka, J., and Kouba, A. (2017). Ecosystem of Caspian Sea threatened by pet-traded non-indigenous crayfish. *Biol. Invasions* 19, 2207–2217. doi: 10.1007/s10530-017-1433-1

Vrålstad, T., Knutsen, A. K., Tengs, T., and Holst-Jensen, A. (2009). A quantitative TaqMan® MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. *Vet. Microbiol.* 137, 146–155. doi: 10.1016/j.vetmic.2008.12.022

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

REVIEW
published: 14 January 2021
doi: 10.3389/fevo.2020.621723

Review of Dam Effects on Native and Invasive Crayfishes Illustrates Complex Choices for Conservation Planning

Zanethia C. Barnett and Susan B. Adams*

Aquatic Conservation and Ecology Team, Center for Bottomland Hardwoods Research, Southern Research Station, United States Department of Agriculture (USDA) Forest Service, Oxford, MS, United States

Dams are among the most prevalent and extreme alterations humans have perpetrated on fluvial systems. The dramatic physical and biological changes caused by dams have been synthesized for many aquatic faunal groups, but not for crayfishes. In addition, invasive crayfish species are an increasing threat to global biodiversity, and dams have both costs and benefits with respect to crayfish invasions. North American crayfishes have imperiled native crayfishes in Europe, largely by hosting and spreading the crayfish plague pathogen *Aphanomyces astaci* that is lethal to European crayfishes. The differential effects of *A. astaci* on North American vs. European crayfishes contribute to differences between the continents in the costs and benefits of dams. We reviewed literature on both the detrimental and beneficial effects of dams on crayfishes, with emphasis on conservation of European crayfishes. We also suggested additional potential dam effects that warrant investigation. Our review illustrates the challenges and opportunities dams create for crayfish conservation. Dams create detrimental effects to native crayfishes, including reducing suitable habitats necessary for native habitat-specialist species and creating habitats suitable for non-native habitat-generalist species; fragmenting crayfish populations; and reducing species' ability to recolonize upstream habitats. Conversely, dams can have beneficial effects by creating barriers that slow or halt upstream invasions by non-native crayfishes and spread of the crayfish plague. The complexity of the issues and the limited ecological information available highlights the need for future studies on the effects of dams on crayfishes. Crayfishes are one of the most imperiled groups of aquatic fauna globally; therefore, understanding the beneficial and detrimental effects of dams is essential for effective conservation of many crayfish species.

Keywords: impoundment, dams, European crayfish, crayfish plague, fragmentation, dispersal, migration, non-native species

OPEN ACCESS

Edited by:
Javier Dieguez-Uribeondo, Real Jardín Botánico (RJB), Spain

Reviewed by:
Pavel Kozak, University of South Bohemia in Ceské Budějovice, Czechia
Leopold Füreder, University of Innsbruck, Austria

****Correspondence:***
Zanethia C. Barnett
Zanethia.C.Barnett@usda.gov

Specialty section:
This article was submitted to Evolutionary and Population Genetics, a section of the journal Frontiers in Ecology and Evolution

Received: *26 October 2020*
Accepted: *14 December 2020*
Published: *14 January 2021*

Citation:
Barnett ZC and Adams SB (2021) Review of Dam Effects on Native and Invasive Crayfishes Illustrates Complex Choices for Conservation Planning. Front. Ecol. Evol. 8:621723. doi: 10.3389/fevo.2020.621723

INTRODUCTION/SCOPE

Flowing water is the defining characteristic of fluvial systems, with flow influencing hydrologic structure, geomorphology, temperature regime, nutrient cycling, and the distribution and evolution of biota (e.g., Poff et al., 1997). Dams can control river flows, dramatically changing streams and creating new ecosystems (Baxter, 1977) and are among the most prevalent and

extreme alterations humans have perpetrated on fluvial systems (The Heinz Center, 2002; Liermann et al., 2012; Grill et al., 2015). With dams on 59% of rivers globally and planned on another 16% (Grill et al., 2015), they have drastically altered rivers on every continent except Antarctica. Impacts range from physical alterations of stream channels and water quality to effects on most biological kingdoms.

The largest biological effects of dams often result from river fragmentation and flow regulation (Stanford and Ward, 2001; Grill et al., 2015). As of 2010, an estimated 35% of the world's river volume was heavily to severely impacted by fragmentation; 35% impacted by flow regulation; and <10% unaffected by either (Grill et al., 2015). Dams nearly always create discontinuities in the river continuum and alter aquatic communities, reducing "natural" biodiversity and facilitating invasions by non-native organisms (Stanford and Ward, 2001). They also dramatically alter stream physiochemical properties, including flow and temperature regimes, channel geomorphology, and water chemistry (Baxter, 1977) that all influence riverine communities (Stanford and Ward, 2001; Grill et al., 2015; Hanks and Hartman, 2019).

Dams are built for various purposes (e.g., water storage, flood control, navigation, hydropower, recreation) that dictate their modes of operation, with irrigation the primary purpose of an estimated 50% of the world's dams (Mulligan et al., 2020). A dam's purpose and operations, as well as its geomorphic, climatic, and biotic settings, all powerfully influence how it affects faunal communities (Stanford and Ward, 2001; The Heinz Center, 2002). For example, water storage dams in mountainous settings typically impound deep, high-volume reservoirs with long water residence times. Dams in such systems usually create insurmountable upstream migration barriers to lotic fauna, and the large reservoirs they impound may further inhibit both up- and downstream migrations. Conversely, reservoirs can also facilitate upstream invasion by flooding natural barriers (Júnior et al., 2009). Unlike water storage dams, run-of-the-river hydropower or navigation dams may impound little or no water and have negligible effects on water residence times, but instead may facilitate upstream passage and expansion of some species (Taugbøl et al., 1993; Kim and Mandrak, 2016). This is especially true of dams with locks.

As of 2019, European rivers had more than 21,000 hydropower dams, with another 8,507 planned and 278 under construction (Schwarz, 2019). Of the existing hydropower dams, 19,344 were "small," producing 0.1–<10.0 megawatts (MW) of power, and 686 were "large" (≥50 MW). These totals excluded the considerable number of non-hydropower dams. In the Iberian Peninsula, for example, most dams are managed for irrigation or water abstraction (Schwarz, 2019). The US National Inventory of Dams (NID) included 91,457 dams, including 6,346 dams >15 m high (https://nid.sec.usace.army.mil/ords/f?p=105:113:15689164071610::NO:::, accessed 25 June 2020). The primary purposes of inventoried dams included recreation (32%), flood control (19%), irrigation (9%), and hydropower (2%). Small dams are much more abundant than large dams but are poorly quantified (Liermann et al., 2012; Grill et al., 2015; Schwarz, 2019), even though "the cumulative effects of many small dams can differ from and even exceed those of a single large dam" (Liermann et al., 2012).

The dramatic physical and biological changes caused by dams have been synthesized for many aquatic faunal groups, but not for crayfishes. Crayfishes are one of the most imperiled groups of aquatic fauna globally (Richman et al., 2015), so understanding how dams affect them is important for conservation. Dams and water management threaten an estimated 25% of at-risk crayfishes in Australia, 35% in the US, and 70% in Mexico (Richman et al., 2015). In Europe, the major threat to native crayfish persistence is the crayfish plague, caused by the oomycete *Aphanomyces astaci*, native to North America (Diéguez-Uribeondo, 2010; Füreder, 2016). North American crayfishes generally carry the pathogen but are relatively resistant to the crayfish plague. Their introduction to other continents also introduced the plague, which is often more devastating to the native crayfish fauna than the introduced crayfishes themselves (Reynolds, 1988; Taugbøl et al., 1993; Kozubíková et al., 2008). Due to the overwhelming impact of the crayfish plague, habitat modifications are of only intermediate concern to crayfish conservation in much of Europe (Füreder, 2016). This creates a scenario in which the costs and benefits of dams to crayfish populations may differ between the continents.

We synthesized literature on the effects of dams on crayfishes. Because the literature exploring this topic is limited, we first briefly summarized general effects of dams on riverine ecosystems and on other aquatic faunal groups that are more thoroughly studied. We then summarized effects of dams on crayfishes in non-European continents, followed by effects in Europe, including some benefits of dams for conservation of European crayfishes (**Table 1**). Finally, we addressed several additional effects that dams are likely to have on crayfishes, based on what is known about other faunal groups. Finally, we suggested additional dam-related research that may facilitate crayfish conservation.

GENERAL ECOSYSTEM EFFECTS OF DAMS

Dam effects on rivers and biota differ fundamentally in three longitudinal zones: the reservoir (area converted from lotic to lentic habitat) impounded by the dam, the river segment(s) upstream of the reservoir, and the river segment(s) downstream of the dam. The spatial extent and degree of impacts in each zone vary according to geomorphology, dam height, and dam operation and often vary temporally as well. Because dam effects on riverine physiochemical properties and biological communities are synthesized elsewhere (e.g., Poff and Zimmerman, 2010; Ellis and Jones, 2013), we present only a brief synopsis of the main physiological and biotic impacts of dams.

Impacts in Reservoirs

Within a reservoir, a river's natural current velocity is greatly reduced, changing a lotic environment to a lentic one and often inundating areas that were previously floodplain or terrestrial habitats (Baxter, 1977). Thus, species adapted to lentic conditions

TABLE 1 | Summary of documented effects of dams on crayfishes in Europe, North America, and other continents.

Impact mechanisms	EU	NA	Other	References
Adverse effects				
Genetic fragmentation		•		Hartfield, 2010 Barnett et al., 2020
Changes in native crayfish assemblage	•	•	•	Roell and Orth, 1992 Joy and Death, 2001 Westhoff et al., 2006 *Meyer et al., 2007 *Bubb et al., 2008 DiStefano et al., 2009b Hartfield, 2010 Lieb et al., 2011 Adams, 2013 *Füreder, 2016 Barnett, 2019
Crayfish introductions	•	•	•	*Hobbs et al., 1989 *Rogers and Holdich, 1995 Lodge et al., 2000 Puth and Allen, 2004 Johnson et al., 2008 *Simić et al., 2008 DiStefano et al., 2009a Kilian et al., 2012 Nunes et al., 2017 Madzivanzira et al., 2020
Crayfish plague introductions	•		•	*Reynolds, 1988 *Taugbøl et al., 1993 *Gerrard et al., 2003 *Longshaw, 2011 *Kozubíková-Balcarová et al., 2014 Mrugala et al., 2017 *Kokko et al., 2018
Stream segments favor invasive species		•	•	Light, 2003 Bobeldyk and Lamberti, 2008 Nunes et al., 2017
Increased fish predation		•		Barnett, 2019
Beneficial effects				
Barriers to invasive species	•	•	•	Light, 2003 *Kozák et al., 2004 Kerby et al., 2005 *Krieg and Zenker, 2020 Nunes et al., 2017 *Dana et al., 2011 *Gherardi et al., 2011 Lieb et al., 2011 *Rahel, 2013 *Rosewarne et al., 2013
Reduced spread of crayfish plague	•			*Taugbøl et al., 1993 *Kozubíková-Balcarová et al., 2014 *Krieg and Zenker, 2020
[a]Expanded habitat for native species	•	•		Sheldon, 1989 Light, 2003 Parkyn et al., 2011 *Zaikov et al., 2011 *Yuksel et al., 2013 Barnett, 2019

[a]*From these studies, we inferred that the habitat of native species would expand. No study compared abundances of crayfishes in riverine vs. reservoir habitats.*

*EU, Europe; NA, North America; Other, other continents; •effects documented on continent; *European studies.*

dominate reservoirs, resulting in fewer native, habitat-specialist species and more non-native, habitat-generalist species (Johnson et al., 2008; Kanno and Vokoun, 2010; Santos et al., 2017). However, even native species tolerant of lentic conditions may have to adapt (e.g., morphologically) to new conditions in order to survive (Baxter, 1977; Haas et al., 2010). Decreased velocities in reservoirs also cause fine sediment accumulations, especially where undammed tributaries are present (Ward, 1976; Maneux et al., 2001; Schleiss et al., 2016). Fine sediments can eliminate gravel-associated species, such as some Unionid mussels and gravel-spawning salmonid fishes that require unembedded substrates for reproduction (Bates, 1962; Magilligan et al., 2016). Fine sediments can also cover and suffocate mussels or reduce their filter-feeding efficiencies (Vaughn and Taylor, 1999; McAllister et al., 2001).

The amount of water impounded differs greatly among dams (Baxter, 1977), impacting the physiochemical characteristics of the water impounded. For a run-of-the-river dam, where flow is minimally regulated, the entire water column of the reservoir may remain well-mixed thermally due to water flows throughout the reservoir and relatively shallow depths, leading to isothermal and orthograde temperature and oxygen profiles, as well as nutrient concentrations uniformly distributed throughout the water column (Worth, 1995). In storage reservoirs, where water residence times may be months or years (Maavara et al., 2014), inflows may be turbulent, and well-mixed, whereas water is thermally stratified throughout much of the reservoir. In summer, oxygen and temperature levels are highest in the epilimnion (upper layer) and lowest in the hypolimnion (bottom layer) (Cassidy, 1989). Furthermore, water storage and retention times influence reservoir temperature regimes, water quality, and other physiochemical and ecological processes (Winton et al., 2019). Changes to these processes often degrade habitats for native, riverine species and lead to communities more tolerant of anthropogenic perturbation, decreasing or eliminating native species intolerant of degraded conditions (Havel et al., 2005).

When rivers are dammed, recreational access and water-based activities often increase, which increases species introductions (Roell and Orth, 1992; Johnson et al., 2008; Adams et al., 2015). Non-native species often abound in reservoirs, due to intentional (legal or illegal) and unintentional introductions and to "the young age, increased niche availability, and high disturbance regime characteristic of most impoundments" (Johnson et al., 2008). Unintentional species introductions may occur by many means, including inadvertent transport on boats, fishing gear, or other recreational equipment (Rothlisberger et al., 2010; Cole et al., 2019) or with shipments of intentionally stocked fishes (Davies et al., 2013).

Impacts Upstream of Reservoirs

The primary physical changes to river segments upstream of reservoirs are the reduced water velocity and increased sedimentation (Graf, 2005; Hu et al., 2009). As water velocities slow, sediment deposition occurs in the river bed, river margins, and interstitial spaces, increasing water levels, flooding events, and channel migration (Baxter, 1977; Wood and Petts, 1994; Graf, 2005; Hu et al., 2009; Schleiss et al., 2016). Decreased

interstitial spaces reduces shelter available to lotic species, which subsequently decreases growth and survival (Finstad et al., 2007; Magilligan et al., 2016). As in reservoirs, increased sediment deposition upstream may eliminate gravel-associated species (Bates, 1962; Magilligan et al., 2016). Sedimentation also increases the amount of invertebrates entering the drift, reducing the standing stock of benthic invertebrates and changing the abundance and composition of the remaining invertebrate community upstream of reservoirs (Jones et al., 2012). Thus, increased sedimentation and the loss of interstitial spaces can greatly reduce the abundance, reproduction, growth, and survival of numerous riverine taxa directly upstream of reservoirs. River segments upstream of reservoirs are also impacted by reservoir taxa (e.g., stocked or introduced fishes) that move into upstream river segments (Swink and Jacobs, 1983; Herbert and Gelwick, 2003; Hladík and Kubečka, 2003). These effects diminish with distance upstream of reservoirs (Ellis and Jones, 2013).

Dams and reservoirs create physical and behavioral barriers to animal movements through river networks, interrupting longitudinal connectivity and isolating upstream populations (Falke and Gido, 2006; Branco et al., 2012; Crook et al., 2015). Such river fragmentation alters patterns of ecological connectivity, potentially impacting life history strategies and habitat colonization (Pringle, 2000; Crook et al., 2015), leading to local extinction of migratory organisms (Gehrke et al., 2002; Hall et al., 2011; Liermann et al., 2012), and reducing abundances of non-migratory, upstream populations (Winston et al., 1991; Morita and Yamamoto, 2002). Inhibiting movements through river systems can also reduce gene flow among populations, decreasing population genetic diversity and population fitness (Lande and Barrowclough, 1987; Fullerton et al., 2010; Fluker et al., 2014). Because dams have fragmented riverine systems only recently relative to evolutionary timescales, the full extent of their effects on species may ultimately be much greater than currently estimated, due to time lags in population responses (Richmond et al., 2009; Abernathy et al., 2013).

Impacts Downstream of Dams

Dams and associated reservoirs can profoundly alter natural flow regimes downstream, causing subsequent changes in fauna composition (Baxter, 1977; Watters, 1996; Cumming, 2004; Graf, 2006). Some dams (e.g., hydroelectric dams) create a rapidly fluctuating downstream hydrologic regime, matching discharges to hourly water or power demands (Richter and Thomas, 2007). Such dam operation often results in a low abundance and low diversity of species downstream (Ward, 1976; Armitage, 1978; Baumgartner et al., 2020). Conversely, some dams only release high volumes of water during certain seasons, with minimum water discharged during other seasons (Graf, 2006). These dam operations lead to a high abundance, but low diversity of species (Ward, 1976; Armitage, 1978; Baumgartner et al., 2020). To meet management needs, dam managers may also stop the release of water from reservoirs or divert water from the river, completely dewatering long river segments downstream (Pringle, 2000; Perkin et al., 2015). Dry segments downstream of dams often eliminate most lotic species, especially those that are not adapted to harsh, unstable physical conditions (Anderson et al., 2006). Additionally, dams prevent movement and migration of fauna through riverine systems, which can lead to the reduced abundance or local extinction of native species downstream (Gehrke et al., 2002; Liermann et al., 2012).

Dams typically alter downstream temperature regimes, water quality, and physiochemical and ecological processes, and dams with long water-retention times often cause the greatest changes (Baxter, 1977; Watters, 1996; Maneux et al., 2001; Cumming, 2004; Maavara et al., 2014). In thermally stratified reservoirs, the seasonal timing and depth (hypolimnetic vs. epilimnetic) of water releases influence downstream water quality (Hanks and Hartman, 2019). Water released downstream from reservoirs can cool water in the summer and warm it in the winter, as well as alter oxygen and nutrient loads relative to natural levels, causing subsequent changes in downstream fauna (Baxter, 1977; Lessard and Hayes, 2003; Graf, 2005; Kunz et al., 2011; Mejia et al., 2020). Nonetheless, with distance downstream from dams and input from downstream tributaries, water conditions (e.g., thermal regime, nutrient content) often gradually returns to a more natural state (Mejia et al., 2020). Species community structure also reverts toward its pre-impoundment state with distance downstream from dams (Hanks and Hartman, 2019). Recovery distance varies by taxa and species (Voelz and Ward, 1991; Camargo and Voelz, 1998; McGregor and Garner, 2003; Phillips and Johnston, 2004b; Hanks and Hartman, 2019), with hundreds of kilometers needed for the recovery of some rare species (Vaughn and Taylor, 1999).

The reduced sediment loads carried by water released from storage reservoirs alter channel morphology downstream of dams. Reduced sedimentation creates coarser riverbeds and greater channel erosion, increasing channel incision (i.e., lowering of bed level) and width (Baxter, 1977; Chien, 1985; Wood and Petts, 1994; Gordon et al., 2004; Graf, 2005; Simon and Rinaldi, 2006). Channel incision can lower local water tables, affecting river flows and riparian conditions (Scott et al., 2000; Schilling et al., 2004). The loss of fine sediments creates riverbeds armored with large rocks and boulders (Graf, 2005) and reduces the geomorphic complexity of downstream segments, resulting in fewer sand/gravel bars and shallow-water habitats compared to unregulated rivers (Poff et al., 1997; Graf, 2006). These geomorphic changes can propagate hundreds of kilometers downstream of dams (Graf, 2006). Native species that are poorly adapted to the altered habitat conditions are decreased or eliminated, often with non-native species filling the new niches (Caiola et al., 2014).

ADVERSE EFFECTS OF DAMS ON CRAYFISHES

Genetic Fragmentation of Crayfish Populations

Dams and reservoirs can restrict crayfish dispersal and gene flow, genetically fragmenting populations, as demonstrated in the southern Appalachian Mountains, USA (Hartfield, 2010;

Barnett et al., 2020). An older, low-head dam (>160 years, <8 m high) and three newer, large dams (36–104 years, >15 m high) reduced passage of crayfishes (*Cambarus* sp. and *Faxonius* spp.), genetically fragmenting populations (Hartfield, 2010; Barnett et al., 2020).

In impounded Alabama, USA, streams, crayfish populations shared few mitochondrial DNA (mtDNA) cytochrome oxidase subunit I gene haplotypes between segments up- and downstream of dams (Hartfield, 2010; Barnett et al., 2020), but in unimpounded streams most haplotypes were shared between up- and downstream segments (Barnett et al., 2020). Also, crayfish gene flow in most impounded streams, was one-way, downstream past dams, whereas in unimpounded streams, it was bidirectional (Barnett et al., 2020). Barnett et al. (2020) inferred that time since dam closure, reservoir size, as well as dispersal abilities and ecological tolerances of the species studied impacted the magnitude of genetic differences between impounded and unimpounded streams. For example, no gene flow occurred between up- and downstream populations in the stream with the oldest dam; only downstream gene flow occurred between populations in the largest, young dam; and both up- and downstream gene flow occurred for one species in the smallest, young dam (Barnett et al., 2020). Additionally, upstream gene flow in impounded streams was detected only for *Faxonius validus* (powerful crayfish), a species preferring small to medium streams; conversely, *F. erichsonianus* (reticulate crayfish), a species preferring medium to large streams, did not display upstream gene flow in impounded streams (Barnett et al., 2020).

One-way downstream migration isolates and reduces gene flow to upstream populations (Fagan, 2002; Fuller et al., 2015; Barnett et al., 2020). Nevertheless, the genetic diversity and effective population size of crayfish populations upstream of impoundments remained high in both impounded and unimpounded streams (Hartfield, 2010; Barnett et al., 2020), suggesting that overall loss of genetic diversity in impounded streams may be a slow process.

Genetic signatures of crayfish population fragmentation by dams in place for only a few decades suggest that substantial genetic changes are ongoing (Liermann et al., 2012; Barnett et al., 2020). Because dam disruption of gene flow in crayfishes was observed using a mtDNA marker that does not mutate at exceptionally fast rates (Hartfield, 2010; Fluker et al., 2014; Barnett et al., 2020), dams likely have had a more profound impact on crayfish population genetics than has been demonstrated to date (Lacy, 1987; Dixo et al., 2009; Hartfield, 2010; Barnett et al., 2020). The effects of genetic fragmentation caused by dams may be exacerbated in smaller crayfish populations and may heighten extinction risk of species with small natural ranges (Lodge et al., 2000; Richman et al., 2015). Although extirpation or extinction caused by dams has not been documented for crayfishes, to our knowledge, the relatively young ages of many dams makes the presence of an extinction debt likely (Tilman et al., 1994; Liermann et al., 2012). Moreover, the historic data necessary to document such extirpations is extremely limited in North America.

Changes in Native Crayfish Assemblages

Changes in resources and habitat availability can influence crayfish presence and abundance (Ellis and Jones, 2013). Shifts in relative abundances of crayfishes have been documented in streams with both small (< 20 ha) and large (> 400 ha) reservoirs (Joy and Death, 2001; Westhoff et al., 2006; DiStefano et al., 2009b; Hartfield, 2010; Adams, 2013; Barnett, 2019).

Small dams altered crayfish species assemblages up- and downstream of reservoirs relative to unimpounded streams (Joy and Death, 2001; Adams, 2013). In the New Zealand Taranaki Ring Plain, the abundance of *Paranephrops planifrons* (northern koura), the only crayfish species collected in the region, was higher upstream of reservoirs than in unimpounded streams (Joy and Death, 2001). In Mississippi, the catch-per-unit-effort (CPUE) of *F. etnieri* spp. complex [*Orconectes chickasawae* in Adams (2013)] was lower downstream of small dams than in unimpounded streams, whereas CPUE of *Procambarus* spp. were either higher downstream of dams or similar between impounded and unimpounded streams (Adams, 2013). Presumably as a result of differing assemblage compositions, seasonal changes in crayfish CPUE also trended in opposite directions downstream of dams vs. in unimpounded streams (Adams, 2013). Impounded streams had higher abundances of species that were better adapted to the newly formed habitats and flow regimes (Adams, 2013).

In Alabama, crayfish density was higher in unimpounded streams than up- and downstream of large impoundments (Barnett, 2019). In unimpounded relative to impounded streams, juvenile density tended to be higher upstream and adult density higher downstream (Barnett, 2019). Higher juvenile and adult crayfish densities in unimpounded than impounded streams were correlated with more aquatic vegetation, lower top predator fish biomass, higher turbidities, higher discharge, and lower minimum temperatures (Barnett, 2019).

Impoundments reset the natural river continuum for physical and biotic variables (Ward and Stanford, 1983), interrupting longitudinal changes in assemblage structure. In Alabama, sites directly below impoundments shared similar physical and biotic characteristics with sites in headwaters, modifying habitat conditions that historically occurred downstream (Barnett, 2019). The gradual up- to downstream shifts in species composition and dominant species in unimpounded streams were not observed in impounded streams (Barnett, 2019). Instead, one or two crayfish species dominated all sites in impounded streams, with low densities of other species (Barnett, 2019). Some species [e.g., *Cambarus striatus* (ambiguous crayfish), *F. compressus* (slender crayfish)] that were common in unimpounded streams were rarely encountered in impounded streams (Adams et al., 2015; Barnett, 2019). Crayfish density, richness, and evenness increased with distance downstream of dams, indicating assemblage recovery (Barnett, 2019). Similarly, in a West Virginia river, crayfish density 15 km downstream from a dam was higher compared to just downstream of it, although no statistical comparison was made (Roell and Orth, 1992). Thus, it appears that crayfish communities, like those of fishes, mussels, and insects recover to some degree with distance downstream of

dams, as tributaries enter and river conditions return to a more natural state (Voelz and Ward, 1991; Kinsolving and Bain, 1993; Camargo and Voelz, 1998; McGregor and Garner, 2003; Phillips and Johnston, 2004b). However, regardless of impoundment status, crayfish richness tends to increase with distance upstream unlike fishes (Hicks, 2003).

Impoundments have greater impacts on crayfish populations in and downstream of reservoirs than upstream of reservoirs. In Alabama streams, total crayfish CPUE was higher upstream of small reservoirs than directly below them (Hartfield, 2010). Similarly, in Pennsylvania, USA, most populations of an extremely rare crayfish (*Cambarus* sp.) occurred upstream of reservoirs, with the invasive *F. rusticus* (rusty crayfish) often abundant in and downstream of reservoirs (Lieb et al., 2011). In Missouri, USA, repeated summer trapping upstream of a reservoir produced the common crayfish *F. neglectus* (ringed crayfish) and two imperiled species, *F. williamsi* (Williams' crayfish) and *F. meeki meeki* (Meek's crayfish), whereas trapping within the reservoir produced the common *F. neglectus* and *F. longidigitus* (long-pincered crayfish) and only one individual of an imperiled species (DiStefano et al., 2009b). Reservoirs reduced the amount of suitable stream habitat for *F. williamsi,* reducing population sizes and isolating upstream populations, although populations may have been isolated even before impoundment (Westhoff et al., 2006).

Recurring themes in papers documenting changes to crayfish abundances in impounded streams were changes to stream habitats caused by dams (Joy and Death, 2001; DiStefano et al., 2009b; Adams, 2013; Barnett, 2019). These changes to stream habitats may decrease the availability of habitat types necessary for survival of some crayfish species, causing a subsequent decrease in the abundance of these crayfishes in impounded stream systems.

Crayfish Introductions to Reservoirs

Crayfishes are one of the most commonly introduced freshwater organisms because the wide niches of some crayfish species allow them to survive in diverse ecosystems (Hobbs et al., 1989; Lodge et al., 1998; Zeng et al., 2015). Unlike many aquatic species, some crayfishes can survive out of water for relatively long periods of time (Banha and Anastácio, 2014), making them more likely than many other aquatic taxa to survive unintentional translocations and overland dispersal and facilitating extensive secondary spread after initial introductions (Krieg and Zenker, 2020).

Human-mediated introductions of non-native crayfishes—and their associated diseases—tend to increase after damming, due to greater human access to and use of water bodies (Muirhead and Macisaac, 2005). For example, in a region rich with natural lakes, five aquatic invasive species, including the *F. rusticus* were 2.5 to 300 times more likely to occur in reservoirs than in natural lakes (Johnson et al., 2008). Non-native crayfishes are commonly introduced to reservoirs through intentional (legal or illegal; often to create new crayfisheries) or unintentional pathways (Lodge et al., 2000; Krieg and Zenker, 2020; Madzivanzira et al., 2020). Introductions of non-native crayfishes potentially also add crayfish commensals and pathogens (Longshaw, 2011; Mrugala et al., 2017; Madzivanzira et al., 2020), most notably the crayfish plague pathogen, to ecosystems (Souty-Grosset et al., 2006). Reservoir fisheries create at least three possible vectors for crayfish introductions: introduction with stocked fishes (Simić et al., 2008), introduction for fish forage (Madzivanzira et al., 2020), and release or escape of bait (DiStefano et al., 2009a). Several countries and US states have banned the transport and possession of live crayfishes as bait, but crayfish introduction through use as bait is still a concern in many places (Puth and Allen, 2004; DiStefano et al., 2009a; Kilian et al., 2012; Banha and Anastácio, 2015). Crayfishes are also introduced to reservoirs through the release or escape of aquarium, classroom, or research crayfishes (Rogers and Holdich, 1995; Lodge et al., 2000; Madzivanzira et al., 2020). Many characteristics of crayfishes sold in the aquarium trade include characteristics that promote invasion success, such as the ability to reproduce under warm aquarium conditions and a preference for lentic habitats (Chucholl and Wendler, 2017).

River Segments Hydrologically Altered by Dams Favor Invasive Species

Flow regimes are critical in structuring fluvial biotic communities (Poff and Ward, 1989; Power et al., 2008; Matthews, 2012). Native crayfishes have evolved traits and life history strategies favoring persistence under natural flow regimes that, in some systems, include extreme hydrological events (Flinders and Magoulick, 2003; Lynch et al., 2019). The numerous changes that dams cause in flow, often create environments that native, habitat-specialist species are not adapted to—or that favor native generalist or non-native species—thereby decreasing the abundance and diversity of native species and increasing biological homogenization (Bunn and Arthington, 2002; Rahel, 2002; Light, 2003; Bobeldyk and Lamberti, 2008; Nunes et al., 2017). Many invasive crayfishes have very wide physiological tolerances, flexible behaviors, high dispersal abilities, and high phenotypic plasticity allowing them to establish in the new habitats created by dams (Perry et al., 2013; Crook et al., 2015; Zeng et al., 2015). Under natural flow regimes, extreme flows can prevent, or even reverse, the establishment of invasive crayfishes (Light, 2003); however, impounded streams with more stable flow regimes (e.g., decreases in annual flow variation), sometimes allow invasive species to thrive, displacing native species through competition and predation (Rahel, 2002).

Crayfish mating, spawning, foraging, and growth are linked to flow regimes (Lowery, 1988; Mead, 2008; Barnett, 2017). Thus, changes in flow regimes can facilitate non-native crayfish invasions. In the Ontonagon River, Michigan, USA, invasive *F. rusticus* were abundant in stream segments closer to impoundments, where the flow regime was more stable, compared to segments further downstream (Bobeldyk and Lamberti, 2008). Similarly, in South Africa, invasive Australian crayfish *Cherax quadricarinatus* (Australian redclaw crayfish) were more abundant in river systems with irrigation dams than in less regulated rivers (Nunes et al., 2017). Nunes et al. (2017) inferred that less suitable habitats and higher flow velocities decreased the abundance of *C. quadricarinatus* in less regulated

rivers. In the Lake Tahoe Basin, California, USA, invasive *Pacifastacus leniusculus* (signal crayfish) was present only in impounded streams (Light, 2003). *Pacifastacus leniusculus* was positively associated with proximity to reservoirs (both up and downstream), declining significantly in abundance upstream of reservoirs following the resumption of normal or high wet-season flows (Light, 2003). In addition, *P. leniusculus* moved long distances (up to 120 m/day) and occupied unimpounded streams only sporadically, leading Light (2003) to infer that they migrated into unimpounded tributaries during low-flow seasons to exploit highly productive habitats or escape fish predation but returned to the hydrologically stable reservoir before high flow seasons began. Thus, dams in the basin protected downstream reaches from high flows and allowed repeated recolonization of upstream reaches—from source populations in the reservoirs—after high-flow events (Light, 2003). Furthermore, reservoirs appear to function as invasion hubs for invasive crayfishes, by harboring source populations of invaders and increasing pathways for range expansion (e.g., irrigation canals connected to dams) that facilitate the subsequent spread and establishment of invaders into natural waterbodies (Light, 2003; Muirhead and Macisaac, 2005; Johnson et al., 2008; Nunes et al., 2017).

Increased Fish Predation on Crayfishes

Predatory fishes are often more abundant in impounded streams due to more favorable habitat conditions and fish stocking (Taylor et al., 2001; Phillips and Johnston, 2004a). Pringle (1997) noted that "until the last few decades, dams and reservoirs in the western US were often viewed as opportunities to introduce game fishes..." Because >40% of the diets of many game fish species (e.g., basses and catfishes) consists of crayfishes (Dorn and Mittelbach, 1999), increases in game fishes potentially increases predation pressure on crayfishes in reservoirs and the rivers that connect to them (Westhoff et al., 2006). Fishes introduced to reservoirs can impact entire stream systems, with the ability to freely leave the reservoirs and move to river segments upstream (Ruhr, 1956; Winston et al., 1991; Pringle, 1997), as well as potentially dispersing downstream of dams. For example, top crayfish predators (e.g., *Micropterus* spp.) were more abundant in impounded than unimpounded Alabama streams, and in impounded streams, top predator fish density was inversely correlated with crayfish density (Barnett, 2019).

Smaller crayfishes are more susceptible to predation than larger crayfishes. Larger crayfishes secure and retain shelter better than smaller individuals (Rabeni, 1985; Nakata and Goshima, 2003) and are less susceptible to predation by gape-limited predators, such as fishes (Stein and Magnuson, 1976; Rahel and Stein, 1988). Additionally, habitats such as tree roots, used especially by small crayfishes for protection from predators (Bohl, 1987; Smith et al., 1996; Parkyn and Collier, 2004), may become inhospitable or inaccessible due to rapidly fluctuating stream flows downstream of dams. In Alabama, the mean size of adult crayfishes was smaller in unimpounded than impounded streams due to higher densities of small-bodied species in the unimpounded streams (Barnett, 2019). The small species *F. compressus,* with an adult length 62% shorter than that of other species collected, occupied unimpounded, but not nearby impounded, streams. Adult crayfishes averaged 25% larger in impounded than unimpounded streams. Additionally, the density of top predator fishes was negatively correlated with juvenile crayfish density and positively correlated with adult crayfish size (Barnett, 2019).

BENEFICIAL EFFECTS OF DAMS ON CRAYFISHES

Barriers to Invasive Species

Dams often block access to upstream river segments, thereby fragmenting populations and generally increasing conservation risks. However, in some circumstances, such movement barriers can facilitate conservation efforts by halting—or slowing—invasions by non-native species, including fishes, crayfishes, pathogens, and parasites (Ruhr, 1956; Liermann et al., 2012; Rahel, 2013; Füreder, 2016). Often, barriers may be the only means to prevent further invasion (Krieg and Zenker, 2020). In California, upstream movements by the invasive crayfish *P. clarkii* were stopped or greatly reduced by artificial and natural barriers, including dams, ranging from 1 to 3 m in height (Kerby et al., 2005). Interestingly, some barriers also deterred downstream movements. The presence of natural and artificial vertical barriers also appeared to stop or slow the spread of non-native *P. leniusculus* in Lake Tahoe Basin, California (Light, 2003). In South Africa, the upstream invasion by *C. quadricarinatus* in the Lomati River was apparently stopped by the Driekoppies Dam; however, an alternative explanation was that increased elevation or lower water temperatures hindered the invasion (Nunes et al., 2017). Dams can be so effective at blocking upstream animal movements that, in some cases, small dams or other artificial obstructions have been installed with the intent of blocking the upstream spread of non-native fishes, crayfishes, or diseases (Pringle, 1997; Gherardi et al., 2011; Rahel, 2013; Manfrin et al., 2019; Krieg and Zenker, 2020).

Barriers are often seen as the best method to stop the spread of invasive species (Krieg and Zenker, 2020); however, they are not wholly reliable. For example, in California, barriers were ineffective at stopping upstream invasion of *P. leniusculus*, but the barriers may have been breached during construction (Cowart et al., 2018). Factors influencing the effectiveness of barriers to crayfish movements include height, water velocity, angle, surface smoothness, bank characteristics (Kerby et al., 2005; Dana et al., 2011; Frings et al., 2013), and presumably, crayfish species is also important.

The tension between the conservation costs and benefits of dams can set the stage for competing conservation interests related to barrier construction or dam removals: on one hand, dams reduce population connectivity and access to habitat for native species, but on the other hand, dams may halt aquatic invasions (although as noted above, reservoirs may also increase chances of introductions). Benefits may accrue to one faunal group while costs are borne by a different group

(Frings et al., 2013; Krieg and Zenker, 2020). Lieb et al. (2011) noted that neglecting to consider the role of dams in halting crayfish invasions may lead to further endangerment of rare crayfish species upstream of dams proposed for removal. They recommended (1) assessing invasion risks prior to dam removals, and (2) not removing dams located downstream of imperiled crayfishes in invasion-prone areas, even in the absence of non-natives. At the least, native crayfishes—as well as invasive and potentially invasive aquatic species—should be considered in assessments prior to dam removals. Maintaining dams to protect upstream populations from invasions may not always be an all-or-nothing choice. In some instances, dams may be retrofitted by lowering or other means so that their impacts on habitat and hydrology are reduced, while they still block invasions of certain species from downstream. Finally, barriers are likely to be less effective in low gradient habitats unless water velocities exceed the critical swimming speeds of the crayfishes (Frings et al., 2013). Most examples of dams as barriers to upstream crayfish movements have come from mountainous environments (e.g., Light, 2003; Kerby et al., 2005; Dana et al., 2011).

Expanded Habitat for Some Crayfish Species

Reservoirs can create new or expanded suitable habitat for crayfish species that are well-adapted to the reservoir's biophysical conditions. Certain crayfish species may thrive in such habitats. In Table Rock Reservoir, Missouri the native crayfish *F. longidigitus,* among the largest crayfishes in North America, supported a popular sport fishery (Parkyn et al., 2011). Likewise in Montana, USA, three reservoirs along the Clark Fork River supported commercial fisheries for *P. leniusculus* for several years, beginning in the late 1980's (Sheldon, 1989). The studies did not compare capture rates in rivers vs. reservoirs, but the bulk of harvest effort was concentrated in reservoirs. In Alabama, the dominant crayfish species, *F. erichsonianus* and *F. validus*, in lotic segments of impounded streams were also the most dominant species collected from guts of fishes in the reservoirs (Barnett, 2019). Louisiana, USA, is well-known in crayfish aquaculture circles for its high production rates of *P. clarkii* (red swamp crayfish), *P. acutus* (white river crayfish), and *P. zonangulus* (southern white river crayfish); in this slightly different context, crayfishes are produced in essentially very shallow reservoirs—with water pumped in from surface or groundwater sources—that are managed solely for the production of crayfishes, or of crayfishes and rice (Gillespie et al., 2012). Dams sometimes increase the habitat and resources available to crayfish species with broad niches that are adapted to lentic, or to both lentic and lotic, conditions (Light, 2003; Barnett, 2019).

EFFECTS OF DAMS ON CRAYFISHES IN EUROPE

As elsewhere, dams appear to have both negative and positive effects on native crayfishes in Europe. Most European studies of dam effects on crayfishes examined dams as barriers to upstream dispersal of non-natives (**Table 1**), but we also apply the lessons from other continents to the European situation, being ever mindful that the differential effects of crayfish plague in Europe versus North America shift the balance of the costs and benefits of dams between the continents.

Negative Effects

All of the native crayfish species in Europe are adapted to both lotic and lentic waters (Reynolds and Souty-Grosset, 2011), and in general, the European crayfish fauna is better adapted to large lake ecosystems (Skurdal and Taugbøl, 2002) than are many crayfishes native to the southeastern US stream ecosystems, where several studies of dam effects on North American crayfishes were conducted (Hartfield, 2010; Adams, 2013; Barnett, 2019; Barnett et al., 2020). Reservoirs may not present the major loss of habitat to lacustrine species that they do to fluvial species, although the characteristics of reservoirs tend differ from those of lakes (Johnson et al., 2008). While reservoirs, themselves, may not create dispersal barriers to lacustrine-adapted species, dams often block upstream, and sometimes downstream, movements.

As elsewhere, European dams can imperil crayfishes by fragmenting stream systems. In an English stream, a flat-plate weir with a 25-cm drop during low flow blocked upstream, but not downstream, movements by *Austropotamobius pallipes* (white-clawed crayfish), illustrating that even small obstacles can fragment populations and should be assessed during conservation planning (Bubb et al., 2008). A population viability analysis on a German population of *Astacus astacus* (noble crayfish) provided a rare, population-level insight into conservation risks exacerbated by stream fragmentation (Meyer et al., 2007). *Astacus astacus* in a 400 m stream reach was bound by a dam upstream and a ford downstream (Meyer et al., 2007). The dam presented a complete barrier to upstream movements and the ford minimized downstream movements. The results suggested that the only path to long term population viability was increasing the population carrying capacity, achievable only by expanding available habitat or improving habitat quality. The most obvious solution was to remove the barriers to crayfish movements; however, such an action could increase risks of exposure to crayfish plague (see Positives below).

Other detrimental effects of European dams are likely to result from: water extraction facilitated by dams; alteration of downstream conditions (e.g., hydrology, temperature, water quality, channel morphology, and substrate); species introductions to reservoirs; or invasions facilitated by altered downstream conditions. For example, in some Mediterranean countries, water extraction is an important stressor, and in Latvia, damming, which is not regulated, dewaters streams to the detriment of some crayfish populations (Füreder, 2016). In Serbia, *Astacus leptodactylus* (narrow clawed crayfish), a European species with wide ecological tolerances, has been introduced to reservoirs through fish stocking (Simić et al., 2008). These unintentional introductions and the species' ability to outcompete other native species has allowed *A. leptodactylus* to replace *A. astacus* as the dominant crayfish in some river systems (Simić et al., 2008).

Healthy European crayfish populations tend to be associated with low levels of human activities such as fishing, swimming, and release of aquarium pets (Füreder, 2016), but human activities and access are often higher in reservoirs relative to rivers and even natural lakes (Roell and Orth, 1992; Johnson et al., 2008; Adams et al., 2015). To reduce the spread of invasive crayfishes and the crayfish plague, many European countries have banned the use of crayfishes as bait (Magnuson et al., 1975; DiStefano et al., 2009a; Peay, 2010) and the European Union has banned the sale, trade, transport, and release of five invasive, North American crayfishes (Regulation No 1143/2104, Commission Implementing Regulation 2016/1141). Given that crayfish plague is often introduced to water bodies via fishing gear, boats, rubber boots, or anything that remains moist (Briede, 2011), recreational boating and fishing in reservoirs is of special concern for crayfish conservation in Europe. A crayfish plague outbreak in a Czech Republic river probably originated downstream in a dense population of non-native *Faxonius limosus* (spiny-cheek crayfish) occupying a reservoir on the River Vlava, although it was unclear how the population was established (Kozubíková-Balcarová et al., 2014). In Ireland and Norway, crayfish plague occurred in popular angling reservoirs that lacked North American crayfishes (Reynolds, 1988; Taugbøl et al., 1993). Contaminated fishing gear or contaminated water associated with fish stocking may have caused the outbreak in Ireland (Reynolds, 1988; Gerrard et al., 2003).

Positive Effects

Dams can have benefits as well as negative effects on native, European crayfish fauna. We grouped potential benefits into two classes: (1) creation of expanded habitat for some species, and (2) creation of barriers to reduce the spread of invasive crayfishes and crayfish plague (Krieg and Zenker, 2020).

Given that the European crayfish fauna is adapted to lakes, it is unsurprising that several examples illustrate some native European species thriving in reservoirs. *Austropotamobius torrentium* (stone crayfish) occurred in "relatively high density" in Dospat Dam, Bulgaria (Zaikov et al., 2011). *Astacus leptodactylus* is native to Turkish lakes and was also widely stocked in reservoirs, where it often thrived (Yuksel et al., 2013; Kokko et al., 2018). *Astacus leptodactylus* stocked in the 68,731 ha Keban Dam Lake, Turkey, became the most economically important species commercially fished, yielding annual harvests of 3,000–35,000 kg from 1994 to 2013 (Yuksel et al., 2013). The population in that reservoir also appeared to be more fecund than other populations in the country (Harlioglu et al., 2004). A small sample from Keban Lake Dam taken in 2011–12 revealed no crayfish plague (Kokko et al., 2018). Nonetheless, crayfish plague was introduced to Turkey in the 1980's and is now widespread, causing periodic crayfishery collapses; however, some resistance is evidenced by latent infections (Kokko et al., 2018).

Krieg and Zenker (2020) reviewed the use of physical barriers to stop crayfish movement and concluded that barriers were the best method to stop the non-anthropogenic spread of invasive crayfishes. Thus, using dams as a barrier can benefit native crayfishes by blocking the upstream spread of invasive crayfishes and the crayfish plague pathogen (Krieg and Zenker, 2020). In three instances in the Czech Republic, dams ranging from 0.5 to 2.0 m high potentially limited the upstream spread of acute crayfish plague outbreaks (Kozubíková-Balcarová et al., 2014). In one case, boards were added to successfully fortify the barrier created by a low dam during an outbreak. Similarly in a English stream, a low-head, flow-gauging weir with relatively low water velocity reduced upstream movements by *P. leniusculus* (Rosewarne et al., 2013). In several Norwegian rivers, the upstream spread of the crayfish plague appeared to be stopped or slowed by dams or waterfalls (Taugbøl et al., 1993). In one example, a lock appeared to block the upstream spread of the crayfish plague until the lock was opened to allow boat passage during a plague outbreak downstream. All authors noted that dams may reduce the spread of invasive crayfishes and diseases, but they are unlikely to permanently prevent upstream invasions (Dana et al., 2011; Rosewarne et al., 2013; Kozubíková-Balcarová et al., 2014). Of course, intentional movement of crayfishes around obstructions by people will foil the best-designed barrier (Dana et al., 2011).

In Spain, three small (1.5–2.0 m high) dams were constructed in a mountain stream with the intent of protecting the "southernmost population of the endangered *Austropotamobius pallipes*" from invasion by *P. clarkii* and crayfish plague, both present downstream (Dana et al., 2011). As of publication, the dams had blocked the invasion for 4 years. In addition, the small population of *P. clarkii* between the two downstream dams dropped below detectable limits, facilitated by removals during sampling.

Barriers intended to block upstream movements by crayfishes, but not fishes (Frings et al., 2013), may have high potential for conservation success where crayfish plague is absent. However, in the presence of the plague, movements by fishes, water birds, mammals, boats, anglers, swimmers, etc. may spread the plague (Taugbøl et al., 1993; Oidtmann et al., 2002; Frings et al., 2013), so providing fish passage may allow transport of the plague upstream of a selective barrier, even without non-native crayfishes passing it (Frings et al., 2013).

ADDITIONAL PREDICTED EFFECTS OF DAMS ON CRAYFISHES AND FUTURE RESEARCH NEEDS

Potential Effects on Burrowing Crayfishes

Fluvial floodplains are unique habitats created, in part, by their temporary connections with open water during river flooding (Opperman et al., 2010; Helms et al., 2013). Burrowing crayfishes spend all or part of their lives in burrows, and some are strictly associated with fluvial floodplains (Helms et al., 2013). When floodplains are either inundated by reservoirs or isolated from rivers due to channel incision and reduced overbank flooding downstream of dams, primary burrower populations are likely negatively affected. Channel incision may further affect such species by locally lowering the water table (Scott et al., 2000; Schilling et al., 2004), thereby requiring crayfishes to burrow

deeper to reach groundwater—at least for species that tend to burrow from the floodplain rather than within stream channels. The extent of such impacts and the population responses remain unexplored. Although such effects would likely be considered detrimental in the context of conserving native burrowers, they could perhaps be beneficial in reducing population sizes of some non-native burrowing species.

Inhibition of Headcutting

The tendency of dams to inhibit upstream headcutting is potentially beneficial to upstream crayfishes in regions with highly erodible soils, lack of bedrock stream channel controls, and certain types of disturbances (e.g., channelization) that foster extreme channel incision and subsequent channel widening and headcutting (upstream spread of incision) (Simon and Rinaldi, 2006). These processes can lead to even small streams being deeply entrenched and incised, a common occurrence in the Coastal Plain of the southeastern US (Patrick et al., 1991; Hartfield, 1993). While the effects of such incision on crayfishes are poorly studied, incised streams are marked by flashy hydrographs, extremely unstable substrates, and a paucity of instream cover, all of which seem to translate to reduced crayfish densities (Adams, 2014). Dams can retard upstream channel incision, thereby helping to minimize stream headcutting and retain instream cover and stream interactions with their floodplains in ways that should benefit crayfish assemblages. Experimental weirs and small check dams have been constructed explicitly to retard channel incision and headcutting (Shields et al., 1995), but the effects on crayfish populations have not been investigated.

Managing Flows to Prevent the Spread of Invasive Species

Modification of dam operations to create bankfull or greater flows downstream may be one tool to reduce numbers of invasive crayfish downstream (Light, 2003). High water velocities or discharges have reduced densities or eliminated both *P. leniusculus* and *P. clarkii* from study stream segments (Light, 2003; Kerby et al., 2005). A similar approach used for fishes indicated that mimicking high spring flows benefitted native fish recruitment but had little impact on non-native fishes (Propst and Gido, 2004). Modifying flows in concert with vertical barriers to crayfish movements warrants further research as a tactic for halting invasions and reducing populations of some widely introduced, invasive crayfishes. However, floods may also negatively affect some native crayfishes, and increased flooding reduced modeled persistence time for an isolated population of *A. astacus* (Meyer et al., 2007).

Additional Research Needs

In striving to slow or halt biodiversity losses and lotic community alterations caused by anthropogenic environmental changes, more research is needed that explicitly examines the effects of dams and dam removals on both native and invasive crayfishes (Johnson et al., 2008). Research should include understanding crayfish habitat preferences, ecological tolerances, and dispersal capabilities to aid in predictions of how species will be impacted by dams. Also, assessing how various dam types (e.g., purpose, operation, size, and age) in different regional contexts affect crayfishes is key to understanding their costs and benefits to crayfish populations.

Further research on dam characteristics that block upstream crayfish migrations is needed, especially in low gradient systems. Understanding the interaction of temperature and barrier characteristics is needed (Frings et al., 2013) and may lead to barriers that could be seasonally adjusted to facilitate fish but not crayfish passage. Other non-permanent barriers (e.g., push-up or inflatable dams) could possibly be pre-installed but not raised until an invasion is imminent, such as when a downstream barrier is passed, but such an approach would require extensive testing. Frings et al. (2013) suggested that existing dams with fish ladders provide potential locations for crayfish barriers, but given that crayfish ascended an eel ladder (Welsh and Loughman, 2015), more research is needed on characteristics of fish barriers to exclude crayfishes. A fish ladder with relatively high water velocities (up to 2 ms^{-1}) at a hydropower dam on the Elbe River prevented upstream movement of *F. limosus* during short-term trials, but a shipping channel in the same dam may have facilitated their upstream migration (Kozák et al., 2004). Studies are needed that evaluate alternatives to dam removals, such as reducing dam sizes—and thus, ecological impacts—while maintaining their functions as barriers to invasive crayfishes. Finally, further evaluation of the trade-offs between dams as beneficial barriers to upstream invasions versus their roles in fragmenting populations and increasing risks of novel introductions will facilitate decision making about barrier construction and dam removals.

CONCLUSIONS

In summary, this review highlights the challenges and opportunities dams create for crayfish conservation. Dams affect crayfishes differently in three zones: the reservoir and the up- and downstream fluvial river segments. Detrimental effects to native crayfishes, especially small-bodied or habitat-specialist species, can occur in each zone, with larger dams often causing greater changes to crayfish communities than smaller dams. The reduction of suitable habitats, fragmentation of crayfish populations, and reduction of a species' ability to recolonize upstream habitats may further negatively affect native populations. In addition, dams create conditions conducive to introduction of, and invasion by, non-native species that often threaten native crayfishes. Conversely, dams frequently create barriers that slow or halt upstream invasions by non-native animals, and therefore, can serve a vital role in protecting isolated, remnant populations of native crayfishes upstream. Both the ecological costs and benefits should be considered when assessing removal or installation of barriers. Although crayfish conservation concerns vary greatly between Europe and North America due to the differential effects of the crayfish plague between the continents, lessons can be learned from effects of dams on crayfishes in North America and other continents and applied to

European crayfish conservation. On both continents, additional research is needed to better understand both the beneficial and detrimental effects of various types of dams on native crayfishes and is essential for effective conservation of many crayfish species.

AUTHOR CONTRIBUTIONS

Both authors contributed to the conception and design of the manuscript, conducted literature reviews, wrote sections of the manuscript, conducted manuscript revisions, read and approved the submitted version.

ACKNOWLEDGMENTS

We are grateful to the two reviewers for comments that improved our manuscript. Work was supported by the USDA Forest Service, Southern Research Station. This is publication number 021 of the Center for Biodiversity & Conservation Research at the University of Mississippi.

REFERENCES

Abernathy, E., McCombs, E., Siefferman, L., and Gangloff, M. M. (2013). Effect of small dams on freshwater mussel population genetics in two southeastern USA streams. *Walkerana* 16, 21–28. doi: 10.31931/fmbc.v16i1.2013.21-28

Adams, S. B. (2013). Effects of small impoundments on downstream crayfish assemblages. *Freshw. Sci.* 32, 1318–1332. doi: 10.1899/12-161.1

Adams, S. B. (2014). Crayfish use of trash versus natural cover in incised, sand-bed streams. *Environ. Manage.* 53, 382–392. doi: 10.1007/s00267-013-0197-3

Adams, S. B., Roghair, C., Krause, C., Warren, M. L. Jr, Cochran, J. A., Dolloff, A., et al. (2015). New crayfish species records from the sipsey fork drainage, including lewis smith reservoir (Alabama, USA): native or introduced species? *Freshw. Crayfish* 21, 17–32. doi: 10.5869/fc.2015.v21-1.17

Anderson, E. P., Freeman, M. C., and Pringle, C. M. (2006). Ecological consequences of hydropower development in central America: impact of small dams and water diversion on neotropical stream fish assemblages. *River Res. Appl.* 22, 397–411. doi: 10.1002/rra.899

Armitage, P. D. (1978). Downstream changes in the composition, numbers, and biomass of bottom fauna in the tees beelow cow green reservoir and in an unregulated tributary maize beck, in the first five years after impoundment. *Hydrobiologia* 58, 145–156. doi: 10.1007/BF00007996

Banha, F., and Anastácio, P. M. (2014). Desiccation survival capacities of two invasive crayfish species. *Knowl. Manag. Aquat. Ecosyst.* 413, 1–5. doi: 10.1051/kmae/2013084

Banha, F., and Anastácio, P. M. (2015). Live bait capture and crayfish trapping as potential vectors for freshwater invasive fauna. *Limnologica* 51, 63–69. doi: 10.1016/j.limno.2014.12.006

Barnett, Z. C. (2017). Habitat use and life history of the vernal crayfish, procambarus viaeviridis (Faxon, 1914), a secondary burrowing crayfish in Mississippi, USA. *J. Crustacean Biol.* 37, 544–555. doi: 10.1093/jcbiol/rux073

Barnett, Z. C., Adams, S. B., Ochs, C. A., and Garrick, R. C. (2020). Crayfish populations genetically fragmented in streams impounded for 36-104 years. *Freshw. Biol.* 65, 768–785. doi: 10.1111/fwb.13466

Barnett, Z. C. (2019). Effects of impoundments on the community assemblage and gene flow of sream crayfishes. Ph.D. thesis, Barnett: University of Mississippi.

Bates, J. M. (1962). The impact of impoundments on the mussel fauna of kentucky reservoir, tennessee river. *Am. Midl. Nat.* 68, 232–236. doi: 10.2307/2422648

Baumgartner, M. T., Piana, P. A., Baumgartner, G., and Gomes, L. C. (2020). Storage or run-of-river reservoirs: exploring the ecological effects of dam operation on stability and species interactions of fish assemblages. *Environ. Manage.* 65, 220–231. doi: 10.1007/s00267-019-01243-x

Baxter, R. M. (1977). Environmental effects of dams and impoundments. *Annu. Rev. Ecol. Syst.* 8, 255–283. doi: 10.1146/annurev.es.08.110177.001351

Bobeldyk, A. M., and Lamberti, G. A. (2008). A decade after invasion: evaluating the continuing effects of rusty crayfish on a michigan River. *J. Great Lakes Res.* 34, 265–275. doi: 10.3394/0380-1330(2008)34[265:ADAIET]2.0.CO;2

Bohl, E. (1987). Compariative studies on crayfish brooks in bavaria (*Astacus astacus* L., *Austropotamobius torrentium* Schr.). *Freshw. Crayfish* 7, 287–294.

Branco, P., Segurado, P., Santos, J. M., Pinheiro, P., and Ferreira, M. T. (2012). Does longitudinal connectivity loss affect the distribution of freshwater fish? *Ecol. Eng.* 48, 70–78. doi: 10.1016/j.ecoleng.2011.05.008

Briede, I. (2011). Crayfish in latvia. *Acta Biol. Univ. Daugavp*. 11, 83–87.

Bubb, D. H., Thom, T. J., and Lucas, M. C. (2008). Spatial ecology of the white-clawed crayfish in an upland stream and implications for the conservation of this endangered species. *Aquat. Conserv.* 18, 647–657. doi: 10.1002/aqc.862

Bunn, S. E., and Arthington, A. H. (2002). Basic principles and ecological consequences of altered flow refimes for aquatic biodiversity. *Environ. Manage.* 30, 492–507. doi: 10.1007/s00267-002-2737-0

Caiola, N., Ibáñez, C., Verdú, J., and Munné, A. (2014). Effects of flow regulation on the establishment of alien fish species: a community structure approach to biolgical validation of environmental flows. *Ecol. Indic.* 45, 598–604. doi: 10.1016/j.ecolind.2014.05.012

Camargo, J. A., and Voelz, N. J. (1998). Biotic and abiotic changes along the recovery gradient of two impounded rivers with different impoundment use. *Environ. Monit. Assess.* 50, 143–158. doi: 10.1023/A:1005712024049

Cassidy, R. A. (1989). "Water temperature, dissolved oxygen, and turbidity control in reservoir releases," in *Alternatives in Regulated River Management, (Boca Raton, FL: CRC Press)*, 28–62.

Chien, N. (1985). Changes in river regime after the construction of upstream reservoirs. *Earth Surface Process. Landforms* 10, 143–159. doi: 10.1002/esp.3290100207

Chucholl, C., and Wendler, F. (2017). Positive selection of beautiful invaders: long-term persistence and bio0invasion risk of freshwater crayfish in the pet trade. *Biol. Invasions* 19, 197–208. doi: 10.1007/s10530-016-1272-5

Cole, E., Keller, R. P., and Garbach, K. (2019). Risk of invasive species spread by recreational boaters remains high despite widespread adoption of conservation behaviors. *J. Environ. Manag.* 229, 112–119. doi: 10.1016/j.jenvman.2018.06.078

Cowart, D. A., Breedveld, K. G. H., Ellis, M. J., Hull, J. M., and Larson, E. R. (2018). Environmental DNA (eDNA) applications for the conservation of imperiled crayfish (*Decapoda: Astacidea*) through monitoring of invasive species barriers and relocated populations. *J. Crustacean Biol.* 38, 257–266. doi: 10.1093/jcbiol/ruy007

Crook, D. A., Lowe, W. H., Allendorf, F. W., Erös, T., Finn, D. S., Gillanders, B. M., et al. (2015). Human effects on ecological connectivity in aquatic ecosystems: integrating scientific approaches to support management and mitigation. *Sci. Total Environ.* 534, 52–64. doi: 10.1016/j.scitotenv.2015.04.034

Cumming, G. S. (2004). The impact of low-head dams on fish species richness in Wisconsin, USA. *Ecol. Appl.* 14, 1495–1506. doi: 10.1890/03-5306

Dana, E. D., García-de-Lomas, J., González, R., and Ortega, F. (2011). Effectiveness of dam construction to contain the invasive crayfish Procambarus clarkii in a mediterranean mountain stream. *Ecol. Eng.* 37, 1607–1613. doi: 10.1016/j.ecoleng.2011.06.014

Davies, G. D., Gozlan, R. E., and Britton, J. R. (2013). Can accidental introduction of non-native species be prevented by fish stocking audits? *Aquat. Conserv.* 23, 366–373. doi: 10.1002/aqc.2341

Diéguez-Uribeondo, J. (2010). Current techniques, approaches and knowledge in diagnosis of crayfish plague and other crayfish diseases. *Knowl. Manage. Aquat. Ecosyst.* 394–395. doi: 10.1051/kmae/2010004

DiStefano, R. J., Litvan, M. E., and Horner, P. T. (2009a). The bait industry as a potential vector for alien crayfish introductions: problems recognition y fisheries agencies and a Missouri evaluation. *Fisheries* 34, 586–597. doi: 10.1577/1548-8446-34.12.586

DiStefano, R. J., Magoulick, D. D., Imhoff, E. M., and Larson, E. R. (2009b). Imperiled crayfishes use hyporheic zone during seasonal drying of an intermittent stream. *J. North Am. Benthol. Soc.* 28, 142–152. doi: 10.1899/08-072.1

Dixo, M., Metzger, J. P., Morgante, J. S., and Zamudio, K. R. (2009). Habitat fragmentation reduces genetic diversity and connectivity among populations in the Brazilian Atlantic Coastal forest. *Biol. Conserv.* 142, 1560–1569. doi: 10.1016/j.biocon.2008.11.016

Dorn, N. J., and Mittelbach, G. G. (1999). More than predator and prey: a review of interactions between fish and crayfish. *Vie Milieu* 49, 229–237.

Ellis, L. E., and Jones, N. E. (2013). Longitudinal trends in regulated rivers: a review and synthesis within the context of the serial discontinuity concept. *Environ. Rev.* 21, 136–148. doi: 10.1139/er-2012-0064

Fagan, W. F. (2002). Connectivity, fragmentation, and extinction risk in dendritic metapopulations. *Ecology* 83, 3243–3249. doi: 10.1890/0012-9658(2002)083[3243:CFAERI]2.0.CO;2

Falke, J. A., and Gido, K. B. (2006). Effects of reservoir connectivity on stream fish assemblages in the great plains. *Can. J. Fish. Aquat. Sci.* 63, 480–493. doi: 10.1139/f05-233

Finstad, A. G., Einum, S., Forseth, T., and Ugedal, O. (2007). Shelter availability affects behviour, size-dependent and mean growth of juvenile Atlantic salmon. *Freshw. Biol.* 52, 1710–1718. doi: 10.1111/j.1365-2427.2007.01799.x

Flinders, C. A., and Magoulick, D. D. (2003). Effects of stream permanence on crayfish community structure. *Am. Midl. Nat.* 149, 134–147. doi: 10.1674/0003-0031(2003)149[0134:EOSPOC]2.0.CO;2

Fluker, B. L., Kuhajda, B. R., and Harris, P. M. (2014). The effects of riverine impoundment on genetic structure and gene flow in two stream fishes in the mobile river basin. *Freshw. Biol.* 59, 526–543. doi: 10.1111/fwb.12283

Frings, R. M., VaeBen, S. C. K., Grob, H., Roger, S., Schuttrumpf, H., and Hollert, H. (2013). A fish-passable barrier to stop the invasion of non-indigenous crayfish. *Biol. Conserv.* 159, 521–529. doi: 10.1016/j.biocon.2012.12.014

Fuller, M. R., Doyle, M. W., and Strayer, D. L. (2015). Causes and consequences of habitat fragmentation in river networks. *Ann. N. Y. Acad. Sci.* 1355, 31–51. doi: 10.1111/nyas.12853

Fullerton, A. H., Burnett, K. M., Steel, E. A., Flitcroft, R. L., Pess, G. R., Feist, B. E., et al. (2010). Hydrological connectivity for riverine fish: measurement challenges and research opportunities. *Freshw. Biol.* 55, 2215–2237. doi: 10.1111/j.1365-2427.2010.02448.x

Füreder, L. (2016). "Crayfish in Europe," in *Freshwater Crayfish: A Global Overview*, eds T. Kawai, Z. Faulkes and G. Scholtz (Boca Raton, FL: CRC Press), 594–627. doi: 10.1201/b18723-26

Gehrke, P. C., Gilligan, D. M., and Barwick, M. (2002). Changes in fish communities of the shoalhaven river 20 years after construction of Tallowa Dam, Australia. *River Res. Appl.* 18, 265–286. doi: 10.1002/rra.669

Gerrard, C., Addy, C., Tero, C., and Kite, N. (2003). "White-clawed crayfish in the river Ise, northhamptonshire," in *Management and Conservation of Crayfish*, Proceedings of a conference held in Nottingham on 7th November 2002, eds D. M. Holdich and P. J. Sibley (Bristol: Environment Agency), 112-120.

Gherardi, F., Aquiloni, L., Diéguez-Uribeondo, J., and Tricarico, E. (2011). Managing invasive crayfish: is there a hope? *Aquat. Sci.* 73, 185–200. doi: 10.1007/s00027-011-0181-z

Gillespie, J., Nyaupane, N., and Boucher, R. (2012). Changes in crawfish production costs and current production practices used. *Aquac. Econ. Manage.* 16, 250–265. doi: 10.1080/13657305.2012.704619

Gordon, N. D., McMahon, T. A., Finlayson, B. L., Gippel, C. J., and Nathan, R. J. (2004). *Stream Hydrology: an Introduction for Ecologists, 2nd Edn*. Chichester: John Wiley and Sons.

Graf, W. L. (2005). Geomorphology and American dams: the scientific, social, and economic context. *Geomorphology* 71, 3–26. doi: 10.1016/j.geomorph.2004.05.005

Graf, W. L. (2006). Downstream hydrologic and geomorphic effects of large dams on American rivers. *Geomorphology* 79, 336–360. doi: 10.1016/j.geomorph.2006.06.022

Grill, G., Lehner, B., Lumsdon, A. E., MacDonald, G. K., Zarfl, C., and Reidy Liermann, C. (2015). An index-based framework for assessing patterns and trends in river fragmentation and flow regulation by global dams at multiple scales. *Environ. Res. Lett.* 10, 1–15. doi: 10.1088/1748-9326/10/1/015001

Haas, T. C., Blum, M. J., and Heins, D. C. (2010). Morphological responses of a stream fish to water impoundment. *Biol. Lett.* 6, 803–806. doi: 10.1098/rsbl.2010.0401

Hall, C. J., Jordaan, A., and Frisk, M. G. (2011). The historic influence of dams on diadromous fish habitat with a focus on river herring and hydrologic longitudinal connectivity. *Landsc. Ecol.* 26, 95–107. doi: 10.1007/s10980-010-9539-1

Hanks, R. D., and Hartman, K. J. (2019). Evaluation of the influences of dam release types, land use, and habitat affecting abundance, richness, diversity, andcommunity structure of larval and juvenile fish. *Can. J. Fish. Aquat. Sci.* 76, 1388–1397. doi: 10.1139/cjfas-2017-0107

Harlioglu, M. M., Barim, Ö., Türkgülü, I., and Harlioglu, A. G. (2004). Potential fecundity of an introduced population, keban dam lake, Elazig, Turkey, of freshwater crayfish, *Astacus leptodactylus* leptodactylus (Esch., 1852). *Aquaculture* 230, 189–195. doi: 10.1016/S0044-8486(03)00430-7

Hartfield, P. (1993). "Headcuts and their effects on freshwater mussels," in *Conservation and Management of Freshwater Mussels*, eds K. S. Cummings, A. C. Buchanan, and L. M. Kock (Rock Island, IL: Upper Mississippi River Conservation Committee), 131–141.

Hartfield, E. E. (2010). Consequences of low-head dams on crayfish distribution and gene flow in Alabama streams. (M.S. thesis). Auburn: Auburn University.

Havel, J. E., Lee, C. E., and Zanden, M. J. V. (2005). Do reservoirs facilitate invasions into landscapes? *Bioscience* 55, 518–525. doi: 10.1641/0006-3568(2005)055[0518:DRFIIL]2.0.CO;2

Helms, B. S., Budnick, W., Pecora, P., Skipper, J., Kosnicki, E., Feminella, J., et al. (2013). The influence of soil type, congeneric cues, and floodplain connectivity on the local distribution of the devil crayfish (*Cambarus diogenes* Girard). *Freshw. Sci.* 32, 1333–1344. doi: 10.1899/12-160.1

Herbert, M. E., and Gelwick, F. P. (2003). Spatial variation of headwater fish assemblages explained by hydrologic variability and upstream effects of impoundment. *Copeia* 2003, 273–284. doi: 10.1643/0045-8511(2003)003[0273:SVOHFA]2.0.CO;2

Hicks, B. J. (2003). Distribution and abundance of fish and crayfish in a waikato stream in relation to basin area. *NZ. J. Zool.* 30, 149–160. doi: 10.1080/03014223.2003.9518333

Hladík, M., and Kubečka, J. (2003). Fish migration between temperate reservoir and its main tributary. *Hydrobiologia* 504, 251–266. doi: 10.1023/B:HYDR.0000008525.46939.42

Hobbs, H. H. III., Jass, J. P., and Huner, J. V. (1989). A review of global crayfish introductions with particular emphasis on two North American species (Decapoda, Cambaridae). *Crustaceana* 56, 299–316. doi: 10.1163/156854089X00275

Hu, B., Yang, Z., Wang, H., Sun, X., Bi, N., and Li, G. (2009). Sedimentation in the three gorges dam and the future trend of changjiang (Yangtze River) sediment flux to the sea. *Hydrol. Earth Syst. Sci.* 13, 2253–2264. doi: 10.5194/hess-13-2253-2009

Johnson, P. T. J., Olden, J. D., and Zanden, M. J. V. (2008). Dam invaders: impoundments facilitate biological invasions into freshwaters. *Front. Ecol. Environ.* 6, 357–363. doi: 10.1890/070156

Jones, J. I., Murphy, J. F., Collins, A. L., Sear, D. A., Naden, P. S., and Armitage, P. D. (2012). The impact of fine sediment on macro-invertebrates. *River Res. Appl.* 28, 1055–1071. doi: 10.1002/rra.1516

Joy, M. K., and Death, R. G. (2001). Control of freshwater fish and crayfish community structure in Taranaki, New Zealand: dams, diadromy or habitat structure? *Freshw. Biol.* 46, 417–429. doi: 10.1046/j.1365-2427.2001.00681.x

Júnior, H. F. J., Tós, C. D., Agostinho, Â. A., and Pavanelli, C. S. (2009). A massive invasion of fish species after eliminating a natural barrier in the upper rio Paraná basin. *Neotrop. Ichthyol.* 7, 709–718. doi: 10.1590/S1679-62252009000400021

Kanno, Y., and Vokoun, J. C. (2010). Evaluating effects of water withdrawals and impoundments on fish assemblages in southern New England streams, USA. *Fish. Manag. Ecol.* 17, 272–283. doi: 10.1111/j.1365-2400.2009.00724.x

Kerby, J. L., Riley, S. P. D., Kats, L. B., and Wilson, P. (2005). Barriers and flow as limiting factors in the spread of an invasive crayfish (*Procambarus clarkii*) in southern California streams. *Biol. Conserv.* 126, 402–409. doi: 10.1016/j.biocon.2005.06.020

Kilian, J. V., Klauda, R. J., Widman, S., Kashiwagi, M., Bourquin, R., Weglein, S., et al. (2012). An assessment of a bait industry and angler behavior as a vector of invasive species. *Biol. Invasions* 14, 1469–1481. doi: 10.1007/s10530-012-0173-5

Kim, J., and Mandrak, N. E. (2016). Assessing the potential movement of invasive fishes through the Welland Canal. *J. Great Lakes Res.* 42, 1102–1108. doi: 10.1016/j.jglr.2016.07.009

Kinsolving, A. D., and Bain, M. B. (1993). Fish assemblage recovery along a riverine disturbance gradient. *Ecol. Appl.* 3, 531–544. doi: 10.2307/1941921

Kokko, H., Harlioglu, M. M., Aydin, H., Makkonen, J., Gökmen, G., Aksu, Ö., et al. (2018). Observations of crayfish plague infections in commercially important narrow-clawed crayfish populations in Turkey. *Knowl. Manag. Aquat. Ecosyst.* 419, 1–4. doi: 10.1051/kmae/2018001

Kozák, P., Policar, T., and Duriš, Z. (2004). Migratory ability of orconectes limosus through a fishpass and notes on its occurrence in the czech republic. *Bull. Français Pêche Piscic.* 372–373, 367–373. doi: 10.1051/kmae:2004010

Kozubíková, E., Petrusek, A., Duriš, Z., Martín, M. P., Diéguez-Uribeondo, J., and Oidtmann, B. (2008). The old menace is back: recent crayfish plague outbreaks in the czech republic. *Aquaculture* 274, 208–217. doi: 10.1016/j.aquaculture.2007.11.015

Kozubíková-Balcarová, E., Beran, L., Duriš, Z., Fischer, D., Horká, I., Svobodová, J., et al. (2014). Status and recovery of indigenous crayfish populations after recent crayfish plague outbreaks in the czech republic. *Ethol. Ecol. Evol.* 26, 299–319. doi: 10.1080/03949370.2014.897652

Krieg, R., and Zenker, A. (2020). A review of the use of physical barriers to stop the spread of non-indigenous crayfish species. *Rev. Fish Biol. Fish.* 30, 423–435. doi: 10.1007/s11160-020-09606-y

Kunz, M. J., Wüest, A., Wehrli, B., Landert, J., and Senn, D. B. (2011). Impact of a large tropical reservoir on riverine transport of sediment, carbon, and nutrients to downstream wetlands. *Water Resour. Res.* 47:W12531. doi: 10.1029/2011WR010996

Lacy, R. C. (1987). Loss of genetic diversity from managed populations: interacting effects of drift, mutation, immigration, selection, and population subdivision. *Conserv. Biol.* 1, 143–158. doi: 10.1111/j.1523-1739.1987.tb00023.x

Lande, R., and Barrowclough, G. (1987). "Effective population size, genetic variation, and their use in population management," in *Viable Populations for Conservation*, ed M. E. Soule (Great Britain: University Press, Cambridge) 87–124. doi: 10.1017/CBO9780511623400.007

Lessard, J. L., and Hayes, D. B. (2003). Effects of elevated water temperature on fish and macroinvertebrate communities below small dams. *River Res. Appl.* 19, 721–732. doi: 10.1002/rra.713

Lieb, D. A., Bouchard, R. W., Carline, R. F., Nuttall, T. R., Wallace, J. R., and Burkholder, C. L. (2011). Conservation and management of crayfishes: lessons from pennsylvania. *Fisheries* 36, 489–507. doi: 10.1080/03632415.2011.607080

Liermann, C. R., Nilsson, C., Robertson, J., and Ng, R. Y. (2012). Implications of dam obstruction for global freshwater fish diversity. *Bioscience* 62, 539–548. doi: 10.1525/bio.2012.62.6.5

Light, T. (2003). Success and failure in a lotic crayfish invasion: the roles of hydrologic variability and habitat alteration. *Freshw. Biol.* 48, 1886–1897. doi: 10.1046/j.1365-2427.2003.01122.x

Lodge, D. M., Stein, R. A., Brown, K. M., Covich, A. P., Brönmark, C., Garvey, J. E., et al. (1998). Predicting impact of freshwater exotic species on native biodiversity: challenges in spatial scaling. *Austr. J. Ecol.* 23, 53–67. doi: 10.1111/j.1442-9993.1998.tb00705.x

Lodge, D. M., Taylor, C. A., Holdich, D. M., and Skurdal, J. (2000). Nonindigenous crayfishes threaten North American freshwater biodiversity: lessons from Europe. *Fisheries* 25, 7–20. doi: 10.1577/1548-8446(2000)025<0007:NCTNAF>2.0.CO;2

Longshaw, M. (2011). Diseases of crayfish: a review. *J. Invertebrate Pathal.* 106, 54–70. doi: 10.1016/j.jip.2010.09.013

Lowery, R. S. (1988). "Growth, moulting, and reproduction," in *Freshwater Crayfish: Biology, Management and Exploitation*, eds D. M. Holdich and R. S. Lowery (Portland, OR: Croom Helm Ltd), 83–113.

Lynch, D. T., Leasure, D. R., and Magoulick, D. D. (2019). Flow alteration-ecology relationships in Ozark Highland streams: consequences for fish, crayfish and macroinvertebrate assemblages. *Sci. Total Environ.* 672, 680–697. doi: 10.1016/j.scitotenv.2019.03.383

Maavara, T., Dürr, H. H., and Van Cappellen, P. (2014). Worldwide retention of nutrient silicon by river damming: from sparse data set to global estimate. *Glob. Biogeochem. Cycles* 28, 842–855. doi: 10.1002/2014GB004875

Madzivanzira, T. C., South, J., Wood, L. E., Nunes, A. L., and Weyl, O. L. F. (2020). A review of freshwater crayfish introductions in Africa. *Rev. Fish. Sci. Aquac.* 1–24. doi: 10.1080/23308249.2020.1802405

Magilligan, F. J., Nislow, K. H., Kynard, B. E., and Hackman, A. M. (2016). Immediate changes in stream channel geomorphology, aquatic habitat, and fish assemblages following dam removal in a small upland catchment. *Geomorphology* 252, 158–170. doi: 10.1016/j.geomorph.2015.07.027

Magnuson, J. J., Capelli, G. M., Lorman, J. G., and Stein, R. A. (1975). "Consideration of crayfish for macrophyte control," in *Symposium on Water Quality Management Through Biological Control*, eds P. L. Brezonik and J. L. Fox (Gainesville: University of Florida), 66–74.

Maneux, E., Probst, J. L., Veyssy, E., and Etcheber, H. (2001). Assessment of dam trapping efficiency from water residence time: application to fluvial sediment transport in the Adour, Dordogne, and Garonne River basins (France). *Water Resour. Res.* 37, 801–811. doi: 10.1029/2000WR900195

Manfrin, C., Souty-Grosset, C., Anastácio, P. M., Reynolds, J., and Giulianini, P. G. (2019). Detection and control of invasive freshwater crayfish: from traditional to innovative methods. *Divers. Distrib.* 11, 1–16. doi: 10.3390/d11010005

Matthews, W. J. (2012). *Patterns in Freshwater Fish Ecology*. New York, NY: Springer Science and Business Media.

McAllister, D. E., Craig, J. F., Davidson, N., Delany, S., and Seddon, M. (2001). "Biodiversity impacts of large dams.", in: IUCN, UNEP, WCD: Background paper No.1.).

McGregor, S. W., and Garner, J. T. (2003). Changes in the freshwater mussel (*Bivalvia: Unionidae*) fauna of the bear creek system of northwest Alabama and northeast Mississippi. *Am. Malacol. Bull.* 18, 61–70. Available online at: https://pubs.er.usgs.gov/publication/70025377

Mead, K. S. (2008). Do antennule and aesthetasc structure in the crayfish *Orconectes virilis* correlate with flow habitat? *Intergr. Comp. Biol.* 48, 823–833. doi: 10.1093/icb/icn067

Mejia, F., Torgersen, C. E., Berntsem, E. K., Maroney, J. R., Connor, J. M., Fullerton, A. H., et al. (2020). Longitudinal, lateral, vertical, and temporal thermal heterogeneity in a large impounded river: implications for cold-water refuges. *Remote Sens.* 12:1386. doi: 10.3390/rs12091386

Meyer, K. M., Gimpel, K., and Brandl, R. (2007). Viability analysis of endangered crayfish populations. *J. Zool.* 273, 364–371. doi: 10.1111/j.1469-7998.2007.00336.x

Morita, K., and Yamamoto, S. (2002). Effects of habitat fragmentation by damming on the persistence of stream-dwelling charr populations. *Conserv. Biol.* 16, 1318–1323. doi: 10.1046/j.1523-1739.2002.01476.x

Mrugala, A., Kawai, T., Kozubáková-Balcarová, E., and Petrusek, A. (2017). Aphanomyces astaci presence in Japan: a threat to the endemic and endangered crayfish species in Cambaroides japonicus? *Aquat. Conserv.* 27, 103–114. doi: 10.1002/aqc.2674

Muirhead, J. R., and Macisaac, H. J. (2005). Development of inland lakes as hubs in an invasion network. *J. Appl. Ecol.* 42, 80–90. doi: 10.1111/j.1365-2664.2004.00988.x

Mulligan, M., van Soesbergen, A., and Saenz, L. (2020). GOODD, a global dataset of more than 38,000 georeferenced dams. *Sci Data* 7, 1–8. doi: 10.1038/s41597-020-0362-5

Nakata, K., and Goshima, S. (2003). Competition for shelter of preferred sizes between the native crayfish species *Cambaroides japonicus* and the alien crayfish species *Pacifastacus leniusculus* in Japan in relation to prior residence, sex difference, and body size. *J. Crustacean Biol.* 23, 897–907. doi: 10.1651/C-2411

Nunes, A. L., Zengeya, T. A., Hoffman, A. C., Measey, G. J., and Weyl, O. L. F. (2017). Distribution and establishment of the alien Australian redclaw crayfish, *Cherax quadricarinatus*, in South Africa and Swaziland. *PeerJ* 5, 1–21. doi: 10.7717/peerj.3135

Oidtmann, B., Heitz, E., Rogers, D., and Hoffmann, R. W. (2002). Transmission of crayfish plague. *Dis. Aquat. Organ.* 52, 159–167. doi: 10.3354/dao052159

Opperman, J. J., Luster, R., McKenney, B. A., Roberts, M., and Meadows, A. W. (2010). Ecologically functional floodplains: connectivitiy, flow regime, and scale. *J. Am. Water Resourc. Assoc.* 46, 211–226. doi: 10.1111/j.1752-1688.2010.00426.x

Parkyn, S. M., and Collier, K. J. (2004). Interaction of press and pulse disturbance on crayfish populations: flood impacts in pasture and forest streams. *Hydrobiologia* 527, 113–124. doi: 10.1023/B:HYDR.0000043189.91134.94

Parkyn, S. M., DiStefano, R. J., and Imhoff, E. M. (2011). Comparison of constructed microhabitat and baited traps in table rock reservoir, Missouri, USA. *Freshw. Crayfish* 18, 69–74. doi: 10.5869/fc.2011.v18.69

Patrick, D. M., Mao, L., and Ross, S. T. (1991). *The Impact of Geomorphic Change on the Distribution of Bayou Darters in the Bayou Pierre System*. Jackson, MS: Mississippi Department of Wildlife, Fisheries and Parks, Museum of Natural Science.

Peay, S. (2010). Invasive non-indigenous crayfish species in Europe: recommendations on managing them. *Knowl. Manag. Aquat. Ecosyst.* 3, 394–395. doi: 10.1051/kmae/2010009

Perkin, J. S., Gido, K. B., Cooper, A. R., Turner, T. F., Osborne, M. J., Johnson, E. R., et al. (2015). Fragmentation and dewatering transform great plains stream fish communities. *Ecol. Monogr.* 85, 73–92. doi: 10.1890/14-0121.1

Perry, W. L., Jacks, A. M., Fiorenza, D., Young, M., and Kuhnke, R. (2013). Effects of water velocity on the size and shape of rusty crayfish, *Orconectes rusticus*. *Freshw. Sci.* 32, 1398–1409. doi: 10.1899/12-166.2

Phillips, B. W., and Johnston, C. E. (2004a). Changes in the fish assemblage of bear creek (Tennessee River Drainage) Alabama and Mississippi: 1968-2000. *Southeastern Nat.* 3, 205–218. doi: 10.1656/1528-7092(2004)003[0205:CITFAO]2.0.CO;2

Phillips, B. W., and Johnston, C. E. (2004b). Fish assemblage recovery and persistence. *Ecol. Freshw. Fish.* 13, 145–153. doi: 10.1111/j.1600-0633.2004.00047.x

Poff, N. L., Allan, J. D., Bain, M. B., Karr, J. R., Prestegaard, K. L., Richter, B. D., et al. (1997). The natural flow regime. *Bioscience* 47, 769–784. doi: 10.2307/1313099

Poff, N. L., and Ward, J. V. (1989). Implications of streamflow variability and predictability for lotic community structure: a regional analysis of streamflow patterns. *Can. J. Fish. Aquat. Sci.* 46, 1805–1818. doi: 10.1139/f89-228

Poff, N. L., and Zimmerman, J. K. H. (2010). Ecological responses to altered flow regimes: a literature review to inform the science and management of environmental flows. *Freshw. Biol.* 55, 194–205. doi: 10.1111/j.1365-2427.2009.02272.x

Power, M. E., Parker, M. S., and Dietrich, W. E. (2008). Seasonal reassembly of a river food web: floods, droughts, and impacts of fish. *Ecol. Monogr.* 78, 263–282. doi: 10.1890/06-0902.1

Pringle, C. M. (1997). Exploring how disturbance is transmitted upstream: going against the flow. *J. North Am. Benthol. Soc.* 16, 425–438. doi: 10.2307/1468028

Pringle, C. M. (2000). Threats to U.S. public lands from cumulative hydrologic alterations outside of their boundaries. *Ecol. Appl.* 10:971. doi: 10.1890/1051-0761(2000)010[0971:TTUSPL]2.0.CO;2

Propst, D. L., and Gido, K. B. (2004). Responses of native and nonnative fishes to natural flow regime mimicry in the San Juan River. *Trans. Am. Fish. Soc.* 133, 922–931. doi: 10.1577/T03-057.1

Puth, L. M., and Allen, T. F. H. (2004). Potential corridors for the rusty crayfish, *Orconectes rusticus*, in northern Wisconsin (USA) lakes: lessons for exotic invasions. *Lansc. Ecol.* 20, 567–577. doi: 10.1007/s10980-004-5649-y

Rabeni, C. F. (1985). Resource partitioning by stream-dwelling crayfish: the influence of body size. *Am. Midl. Nat.* 113, 20–29. doi: 10.2307/2425344

Rahel, F. J. (2002). Homogenization of freshwater faunas. *Annu. Rev. Ecol. Syst.* 33, 291–315. doi: 10.1146/annurev.ecolsys.33.010802.150429

Rahel, F. J. (2013). Intentional fragmentation as a management strategy in aquatic systems. *Bioscience* 63, 362–372. doi: 10.1525/bio.2013.63.5.9

Rahel, F. J., and Stein, R. A. (1988). Complex predator-prey interactions and predator intimidation among crayfish piscivorous fish, and small benthic fish. *Oecologia* 75, 94–98. doi: 10.1007/BF00378819

Reynolds, J. D. (1988). Crayfish extinctions and crayfish plague in central Ireland. *Biol. Conserv.* 45, 279–285. doi: 10.1016/0006-3207(88)90059-6

Reynolds, J., and Souty-Grosset, C. (2011). *Management of Freshwater Biodiversity: Crayfish as Bioindicators*. Reynolds, GA: Cambridge University Press. doi: 10.1017/CBO9781139031790

Richman, N. I., Böhm, M., Adams, S. B., Alvarez, F., Bergey, E. A., Bunn, J. J. S., et al. (2015). Multiple drivers of decline in the global status of freshwater crayfish (Decapoda: Astacidea). *Philos. Trans. R. Soc. B Biol. Sci.* 370:20140060. doi: 10.1098/rstb.2014.0060

Richmond, J. Q., Reid, D. T., Ashton, K. G., and Zamudio, K. R. (2009). Delayed genetic effects of habitat fragmentation on the ecologically specialized Florida sand skink (*Plestiodon reynoldsi*). *Conserv. Genetics* 10, 1281–1297. doi: 10.1007/s10592-008-9707-x

Richter, B. D., and Thomas, G. A. (2007). Restoring environmental flows by modifying dam operations. *Resilience Alliance* 12:12. doi: 10.5751/ES-02014-120112

Roell, M. J., and Orth, D. J. (1992). Production of three crayfish populations in the new river of West Virginia, USA. *Hydrobiologia* 228, 185–194. doi: 10.1007/BF00006585

Rogers, W. D., and Holdich, D. M. (1995). Crayfish production in Britain. *Freshw. Crayfish* 10, 583–596.

Rosewarne, P. J., Piper, A. T., Wright, R. M., and Dunn, A. M. (2013). Do low-head riverine structures hinder the spread of invasive crayfish? Case study of signal crayfish (Pacifastacus leniusculus) movements at a flow gauging weir. *Manag. Biol. Invasions* 4, 273–282. doi: 10.3391/mbi.2013.4.4.02

Rothlisberger, J. D., Chadderton, W. L., McNulty, J., Lodge, D. M. (2010). Aquatic invasive species transport via trailered boats: what is being moved, who is moving it, and what can be done. *Fisheries* 35, 121–132. doi: 10.1577/1548-8446-35.3.121

Ruhr, C. E. (1956). Effect of stream impoundment in Tennessee on the fish populations of tributary streams. *Trans. Am. Fish. Soc.* 86, 144–157. doi: 10.1577/1548-8659(1956)86[144:EOSIIT]2.0.CO;2

Santos, R. M. B., Sanches Fernandes, L. F., Cortes, R. M. V., Varandas, S. G. P., Jesus, J. J. B., and Pacheco, F. A. L. (2017). Integrative assessment of river damming impacts on aquatic fauna in a Portuguese reservoir. *Sci. Total Environ.* 601–602, 1108–1118. doi: 10.1016/j.scitotenv.2017.05.255

Schilling, K., Zhang, Y., and Drobney, P. (2004). Water table fluctuations near an incised stream, Walnut Creek, Iowa. *J. Hydrol.* 286, 236–248. doi: 10.1016/j.jhydrol.2003.09.017

Schleiss, A. J., Franca, M. J., Juez, C., and Cesare, G. D. (2016). Reservoir sedimentation. *J. Hydr. Res.* 54, 595–614. doi: 10.1080/00221686.2016.1225320

Schwarz (2019). *Hydropower Pressure on European Rivers: The Story in Numbers*. FLUVIUS, WWF, RiverWatch, EuroNatur, GEOTA.

Scott, M. L., Lines, G. C., and Auble, G. T. (2000). Channel incision and patterns of cottonwood stress and mortality along the mojave river, California. *J. Arid Environ.* 44, 399–414. doi: 10.1006/jare.1999.0614

Sheldon, A. (1989). *A Reconnaissance of Crayfish Populations in Western Montana*. Missoula, MT: The University of Montana. doi: 10.5962/bhl.title.51422

Shields, F. D. Jr., Knight, S. S., and Cooper, C. M. (1995). Rehabilitation of watersheds with incising channels. *Water Resourc. Bull.* 31, 971–982. doi: 10.1111/j.1752-1688.1995.tb03413.x

Simić, V., Petrović, M., and Paunović, M. (2008). Crayfish of serbia and montenegro-the population status and the level of endangerment. *Crustaceana* 81, 1153–1179. doi: 10.1163/156854008X374496

Simon, A., and Rinaldi, M. (2006). Disturbance, stream incision, and channel evolution: the roles of excess transport capacity and boundary materials in controlling channel response. *Geomorphology* 79, 361–383. doi: 10.1016/j.geomorph.2006.06.037

Skurdal, J., and Taugbøl, T. (2002). "Astacus," in *Biology of Freshwater Crayfish*, ed D. M. Holdich (Ames, IA: Blackwell Scientific, Ltd), 467–510.

Smith, G. R. T., Learner, M. A., Slater, F. M., and Foster, J. (1996). Habitat features important for the conservation of the native crayfish Austropotamobius pallipes in Britain. *Biol. Conserv.* 75, 239–246. doi: 10.1016/0006-3207(95)00073-9

Souty-Grosset, C., Holdich, D. M., Noël, P. Y., Reynolds, J. D., and Haffner, P. (2006). *Atlas of Crayfish in Europe*. Paris: Muséum National d'Histoire Naturelle.

Stanford, J. A., and Ward, J. V. (2001). Revisiting the serial discontinuity concept. *Regul. Rivers* 17, 303–310. doi: 10.1002/rrr.659

Stein, R. A., and Magnuson, J. J. (1976). Behavioral response of crayfish to a fish predator. *Ecology* 57, 751–761. doi: 10.2307/1936188

Swink, W. D., and Jacobs, K. E. (1983). Influence of a kentucky flood-control reservoir on the tailwater and headwater fish populations. *North Am. J. Fish. Manage.* 3, 197–203. doi: 10.1577/1548-8659(1983)3<197:IOAKFR>2.0.CO;2

Taugbøl, T., Skurdal, J., and Håstein, T. (1993). Crayfish plague and management strategies in Norway. *Biol. Conserv.* 63, 75–82. doi: 10.1016/0006-3207(93)90076-D

Taylor, C. A., Knouft, J. H., and Hiland, T. M. (2001). Consequences of stream impoundment on fish communities in a small North American drainage. *Regul. Rivers* 17, 687–698. doi: 10.1002/rrr.629

The Heinz Center (2002). *Dam Removal: Science and Decision Making*. Washington, DC: The H. John Heinz III Center for Science, Economics, and the Environment.

Tilman, D., May, R. M., Lehman, C. L., and Nowak, M. A. (1994). Habitat desruction and the extinction debt. *Lett. Nat.* 371, 65–66. doi: 10.1038/371065a0

Vaughn, C. C., and Taylor, C. M. (1999). Impoundments and the decline of freshwater mussels: a case study of an extinction gradient. *Conserv. Biol.* 13, 912–920. doi: 10.1046/j.1523-1739.1999.97343.x

Voelz, N. J., and Ward, J. V. (1991). Biotic responses along the recovery gradient of a regulated stream. *Can. J. Fish. Aquat. Sci.* 48, 2477–2490. doi: 10.1139/f91-289

Ward, J. V. (1976). "Effects of flow patterns below large dams on stream benthos," in *Instream Flow Needs,* eds J. F. Orsborn and C. H. Allman (Bethesda, MD: American Fisheries Society), 235–253.

Ward, J. V., and Stanford, J. A. (1983). "The serial discontinuity concept of lotic ecosystems," in *Dynamics of Lotic Ecosystems*, eds T. D. Fontaine III and S. M. Bartell (Ann Arbor, MI: Ann Arbor Science), 29–42.

Watters, G. T. (1996). Small dams as barriers to freshwater mussels (Bivalvia, *Unionoida*) and their hosts. *Biol. Conserv.* 75, 79–85. doi: 10.1016/0006-3207(95)00034-8

Welsh, S. A., and Loughman, Z. J. (2015). Upstream dispersal of an invasive crayfish aided by a fish passage facility. *Manage. Biol. Invasions* 6, 287–294. doi: 10.3391/mbi.2015.6.3.07

Westhoff, J. T., Guyot, J. A., and DiStefano, R. J. (2006). Distribution of the imperiled williams' crayfish (*Orconectes williamsi*) in the white river drainage of missouri: associations with multi-scale environmental variables. *Am. Midl. Nat.* 156, 273–288. doi: 10.1674/0003-0031(2006)156[273:DOTIWC]2.0.CO;2

Winston, M. R., Taylor, C. M., and Pigg, J. (1991). Upstream extirpation of four minnow species due to damming of a prairie stream. *Trans. Am. Fish. Soc.* 120, 98–105. doi: 10.1577/1548-8659(1991)120<0098:UEOFMS>2.3.CO;2

Winton, R. S., Calamita, E., and Wehrli, B. (2019). Reviews and syntheses: dams, water quality and tropical reservoirstratification. *Biogeosciences* 16, 1657–1671. doi: 10.5194/bg-16-1657-2019

Wood, P. J., and Petts, G. E. (1994). Low flows and recovery of macroinvertebrates in a small regulated chalk stream. *Regul. Rivers* 9, 303–316. doi: 10.1002/rrr.3450090410

Worth, D. F. (1995). Gradient changes in water quality during low flows in run-of-the-river and reservoir impoundments, lower snake River, Idaho. *Lake Reserv. Manag.* 11, 217–224. doi: 10.1080/07438149509354202

Yuksel, F., Demirol, F., and Gunduz, F. (2013). Leslie population estimation for Turkish crayfish (*Astacus leptodactylus* Esch., 1823) in the Keban Dam Lake, Turkey. *Turkish J. Fish. Aquat. Sci.* 13, 835–839. doi: 10.4194/1303-2712-v13_5_07

Zaikov, A., Hubenova, T., Iliev, I., Vasileva, P., and Piskov, I. (2011). Study on the stone crayfish Austropotamobius torrentium (Schrank, 1803) (Crustacea: Decapoda: Astacidae) population in the dospat dam (Western Rhodope Mountains, Bulgaria). *ZooNotes* 18, 1–5.

Zeng, Y., Chong, K. Y., Grey, E. K., Lodge, D. M., and Yeo, D. C. J. (2015). Disregarding human pre-introduction selection can confound invasive crayfish risk assessments. *Biol. Invasions* 17, 2373–2385. doi: 10.1007/s10530-015-0881-8

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Genetics

ORIGINAL RESEARCH
published: 21 January 2021
doi: 10.3389/fgene.2020.611745

OPEN ACCESS

Edited by:
Gabriel Luz Wallau, Aggeu Magalhães Institute (IAM), Brazil

Reviewed by:
Geyner Cruz, Universidade de Pernambuco, Brazil
Lukas Kratochvil, Charles University, Czechia
Adriana Ludwig, Carlos Chagas Institute (ICC), Brazil

***Correspondence:**
Jelena Mlinarec
jelena@biol.pmf.hr

†*These authors have contributed equally to this work and share first authorship*

‡**ORCID:**
Višnja Besendorfer
orcid.org/0000-0001-9706-4921
Ivana Maguire
orcid.org/0000-0001-7456-8449
Carola Greve
orcid.org/0000-0003-4993-1378
Jelena Mlinarec
orcid.org/0000-0002-2627-5374
Frederick Grandjean
orcid.org/0000-0002-8494-0985
Ljudevit Luka Boštjančić
orcid.org/0000-0001-8941-9753

Specialty section:
This article was submitted to Evolutionary and Population Genetics, a section of the journal Frontiers in Genetics

Received: *29 September 2020*
Accepted: *21 December 2020*
Published: *21 January 2021*

Citation:
Boštjančić LL, Bonassin L, Anušić L, Lovrenčić L, Besendorfer V, Maguire I, Grandjean F, Austin CM, Greve C, Hamadou AB and Mlinarec J (2021) The Pontastacus leptodactylus (Astacidae) Repeatome Provides Insight Into Genome Evolution and Reveals Remarkable Diversity of Satellite DNA. Front. Genet. 11:611745. doi: 10.3389/fgene.2020.611745

The *Pontastacus leptodactylus* (Astacidae) Repeatome Provides Insight Into Genome Evolution and Reveals Remarkable Diversity of Satellite DNA

Ljudevit Luka Boštjančić [1†‡], ***Lena Bonassin*** [1†], ***Lucija Anušić*** [1], ***Leona Lovrenčić*** [2], ***Višnja Besendorfer*** [1‡], ***Ivana Maguire*** [2‡], ***Frederic Grandjean*** [3‡], ***Christopher M. Austin*** [4], ***Carola Greve*** [5‡], ***Alexander Ben Hamadou*** [5] ***and Jelena Mlinarec*** [1*‡]

[1] *Division of Molecular Biology, Department of Biology, University of Zagreb, Zagreb, Croatia,* [2] *Division of Zoology, Department of Biology, University of Zagreb, Zagreb, Croatia,* [3] *Laboratoire Ecologie Biologie des Interactions-UMR CNRS 7267, University of Poitiers, Poitiers, France,* [4] *Centre of Integrative Ecology, School of Life and Environmental Sciences Deakin University, Geelong, VIC, Australia,* [5] *LOEWE Centre for Translational Biodiversity Genomics (LOEWE-TBG), Frankfurt, Germany*

Pontastacus leptodactylus is a native European crayfish species found in both freshwater and brackish environments. It has commercial importance for fisheries and aquaculture industries. Up till now, most studies concerning *P. leptodactylus* have focused onto gaining knowledge about its phylogeny and population genetics. However, little is known about the chromosomal evolution and genome organization of this species. Therefore, we performed clustering analysis of a low coverage genomic dataset to identify and characterize repetitive DNA in the *P. leptodactylus* genome. In addition, the karyogram of *P. leptodactylus* ($2n = 180$) is presented here for the first time consisting of 75 metacentric, 14 submetacentric, and a submetacentric/metacentric heteromorphic chromosome pair. We determined the genome size to be at ∼18.7 gigabase pairs. Repetitive DNA represents about 54.85% of the genome. Satellite DNA repeats are the most abundant type of repetitive DNA, making up to ∼28% of the total amount of repetitive elements, followed by the Ty3/*Gypsy* retroelements (∼15%). Our study established a surprisingly high diversity of satellite repeats in *P. leptodactylus*. The genome of *P. leptodactylus* is by far the most satellite-rich genome discovered to date with 258 satellite families described. Of the five mapped satellite DNA families on chromosomes, PlSAT3-411 co-localizes with the AT-rich DAPI positive probable (peri)centromeric heterochromatin on all chromosomes, while PlSAT14-79 co-localizes with the AT-rich DAPI positive (peri)centromeric heterochromatin on one chromosome and is also located subterminally and intercalary on some chromosomes. PlSAT1-21 is located intercalary in the vicinity of the (peri)centromeric heterochromatin on some chromosomes, while PlSAT6-70 and PlSAT7-134 are located intercalary on some *P. leptodactylus* chromosomes. The FISH results reveal amplification of interstitial telomeric

repeats (ITRs) in *P. leptodactylus*. The prevalence of repetitive elements, especially the satellite DNA repeats, may have provided a driving force for the evolution of the *P. leptodactylus* genome.

Keywords: FISH, genome size, interstitial telomeric repeats, karyotype, narrow-clawed crayfish, (peri)centromeric heterochromatin

INTRODUCTION

Freshwater crayfish constitute a monophyletic group of over 640 described species, arranged into four families: Astacidae, Cambaridae, Cambaroididae, and Parastacidae (Crandall and De Grave, 2017). These species are distributed across all but the Antarctic continent, the Indian subcontinent, and African mainland, with centers of diversity in the southeastern Appalachian Mountains in the North America and southeastern Australia (Crandall and Buhay, 2008). The Northern (Astacidae, Cambaroididae, and Cambaridae) and Southern (Parastacidae) hemisphere families form deeply divergent reciprocally monophyletic clades (Bracken-Grissom et al., 2014). The crayfish species of the family Astacidae belong to four genera of which *Pacifastacus* is native to North America, while *Astacus*, *Pontastacus*, and *Austropotamobius* are native to the European continent (Crandall and De Grave, 2017). In the last decades numbers and sizes of native European crayfish populations have been in decline due to climate change, degraded water quality, negative anthropogenic pressure on freshwater habitats, and the introduction of alien invasive crayfish species and their pathogens (e.g., *Aphanomyces astaci*) (Holdich et al., 2009; Kouba et al., 2014). One of the native European crayfish species is *Pontastacus leptodactylus* (Eschscholtz, 1823), found both in freshwater and brackish environments with a nowadays distribution encompassing Europe, eastern Russia, and the Middle East (Kouba et al., 2014). Up till now, the majority of studies on this species have focused on morphology, phylogeny and population genetics (Maguire and Dakić, 2011; Akhan et al., 2014; Maguire et al., 2014; Gross et al., 2017; Khoshkholgh and Nazari, 2019). Analyses of phylogenetic relationships among *P. leptodactylus* populations, using mtDNA, revealed three well-supported divergent lineages; one distributed in Europe (Croatia, Bulgaria, Poland, and Turkey) (European lineage *sensu* Maguire et al., 2014), another in Asia (Armenia, Russia) (Asian lineage *sensu* Maguire et al., 2014), and the third endemic to Turkey (Clade III *sensu* Akhan et al., 2014). While genomic information has started to accumulate for North American and Australian species (Gutekunst et al., 2018; Tan et al., 2020; Van Quyen et al., 2020), so far few studies have focused on cytogenetic and genome organization of European freshwater crayfish species (Mlinarec et al., 2011, 2016), and therefore the general aim of this study was to increase knowledge on genome evolution and diversity focusing on repetitive DNAs in *P. leptodactylus*.

Abbreviations: FISH, Fluorescence *in situ* hybridization; HOR, higher order structures; HSs, heterochromatin segments; ITRs, interstitial telomeric repeats; LTRs, long terminal repeats; rDNA, ribosomal DNA; SFs, superfamilies; SSRs, simple sequence repeats; TEs, transposable elements.

The majority of animal and plant genomes contain a substantial portion of repetitive DNA, collectively referred to as the repeatome of a species, which is considered largely responsible for genome size variation. The repeatome is comprised of dispersed (DNA transposons and retrotransposons) and tandemly arranged sequences (such as nuclear ribosomal RNA genes and satellite DNAs) (Garrido-Ramos, 2017). Satellite DNAs (satDNAs) are organized in large tandem arrays of highly repetitive non-coding short sequences. SatDNAs are one of the most rapidly evolving DNAs in the genome (Garrido-Ramos, 2017). Their evolution is mainly marked by amplification and homogenization processes (both decreasing divergence) and point mutations (increasing divergence) (Ruiz-Ruano et al., 2019). Considering the differences in the size of the repeating units, satDNAs are classified into microsatellites (repeat units $<$10 bp), minisatellites (repeat units in the range 10–100 bp), and conventional satellites (repeat units larger than 100 bp) (Garrido-Ramos, 2017). Conventional satellites are found specifically at pericentromeric and subtelomeric locations of the chromosomes, but might be found occupying interstitial positions of the chromosomes constituting heterochromatin segments (HSs) (Garrido-Ramos, 2017). The satDNAs perform functions in the regulation of gene expression and play an important structural role in the vital functions including among others, chromosome segregation and the preservation of genetic material (Blackburn, 2005; Louis and Vershinin, 2005; Riethman et al., 2005; Kuo et al., 2006).

The characterization of repetitive DNAs from poorly characterized genomes or species lacking a reference genome can be a challenging task (Ávila Robledillo et al., 2018). Up to now, only a few satDNAs have been reported in crustaceans, manly using traditional methods such as centrifugation through sequential CsCl gradients (Chambers et al., 1978; Wang et al., 1999). Today, repetitive DNAs can now be analyzed more easily owing to the recent advances in next generation sequencing (NGS) and high-throughput *in silico* analysis of the information contained in the NGS reads (Weiss-Schneeweiss et al., 2015; Ruiz-Ruano et al., 2019). Development of the RepeatExplorer software tool allows for *de novo* repeat identification using analyses of short sequences, randomly sampled from the genome (Novák et al., 2010, 2013). The Tandem Repeat Analyzer (TAREAN) further improved the RepeatExplorer pipeline allowing for the automatic identification and reconstruction of monomer sequences for each satDNA family in the species, collectively referred to as satellitome (Novák et al., 2017).

Decapod crustaceans present an attractive study model due to the existence of polyploidy, a large quantity of AT-rich HSs as well as the adaptation to a broad range of environments (Mlinarec et al., 2011; Martin et al., 2015; Tan et al., 2019). However, the

majority of crustaceans have been poorly investigated at the genomic and cytogenomic level (Tan et al., 2020; Van Quyen et al., 2020). To a large extent, this is reflective of the fact that decapod crustaceans, and freshwater crayfish in particular, have a low mitotic index, a high diploid chromosome number, small chromosomes, and highly repetitive genomic elements (Tan et al., 2004, 2019, 2020; Mlinarec et al., 2011; Gutekunst et al., 2018; Van Quyen et al., 2020). Therefore, cytogenetic studies on freshwater crayfish species are rare, often limited to the report of chromosome number and structure, with very few reports on molecular cytogenetics (Tan et al., 2004; Indy et al., 2010; Scalici et al., 2010; Mlinarec et al., 2011, 2016; Kostyuk et al., 2013; Salvadori et al., 2014) (**Table 1**).

Keeping in mind the lack of research in the field for European freshwater crayfish, this study aims to: (i) identify and characterize repetitive sequences in the *P. leptodactylus* genome in order to get better insight into genome organization and evolution of this species, and (ii) analyze the chromosomal distribution patterns of major tandem repetitive DNA families to contribute with the chromosome organization and evolution. In addition, *COI* barcoding was used to place the samples used in this study within the context of patterns of diversity to determine the phylogenetic placement of *P. leptodactylus* individuals from Lake Maksimir.

MATERIALS AND METHODS

Samples and DNA Extraction

Seven individuals (four males and three females) of narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823) were collected from the Third Maksimir Lake (Zagreb, Croatia); 45.82972°N 16.02056°E.

One pereiopod from each individual was removed and stored in 96% ethanol at 4°C until DNA extraction. Genomic DNA was isolated from muscle tissue using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer's protocol and stored at −20°C.

DNA Barcoding and Phylogenetic Network Reconstruction

Mitochondrial cytochrome oxidase subunit I (*COI*) barcode region was amplified and sequenced from genomic DNA of two individuals taken from Lake Maksimir using primer pairs LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO-2198 (5′-TAAACTTCAGGGTGACCAAAAAAT CA-3′) described in Folmer et al. (1994). PCR reaction conditions and purification of PCR product followed the protocols described in Maguire et al. (2014). Sequencing of purified PCR products was performed by Macrogen Inc. (Amsterdam, Netherlands). Phylogenetic analysis included a total of 129 *COI* gene sequences of which 127 were downloaded from GenBank (accession KX279350), while the other two were obtained from Lake Maksimir individuals obtained in this study (**Supplementary Table 1**). Sequences were edited using SEQUENCHER 5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA) and aligned using MAFFT (Katoh and Standley, 2013). Sequences were collapsed to unique *COI* haplotypes using the software DnaSP 6.12.03 (Rozas et al., 2017). A median joining network was constructed on *COI* haplotype dataset using PopArt (Bandelt et al., 1999) to visualize non-hierarchical haplotype relationships and their geographical distribution. Sites containing ambiguities were excluded from network reconstruction. This approach is recommended as a standard for cytogenetic studies as it links karyotypes with DNA barcodes (Lukhtanov and Iashenkova, 2019).

Flow Cytometry Analysis

The genome size was estimated following a flow cytometry protocol with propidium iodide-stained nuclei described in Hare and Johnston (2011). Different tissue (tail muscle, vascular tissue, and gills) of −80°C frozen adult samples of *P. leptodactylus* and neural tissue of the internal reference standard *Acheta domesticus* (female, 1C = 2Gb) was each mixed and chopped with a razor blade in a petri dish containing 2 ml of ice-cold Galbraith buffer. The suspension was filtered through a 42-μm nylon mesh and stained with the intercalating fluorochrome propidium iodide (PI, Thermo Fisher Scientific) and treated with RNase II A (Sigma-Aldrich), each with a final concentration of 25 μg/ml. The mean red PI fluorescence of stained nuclei was quantified using a Beckman-Coulter CytoFLEX flow cytometer with a solid-state laser emitting at 488 nm. Fluorescence intensities of 5000 nuclei per sample were recorded. We used the CytExpert 2.3 software for histogram analyses. The total quantity of DNA in the sample was calculated as the ratio of the mean fluorescence signal of the 2C peak of the stained nuclei of the crayfish sample divided by the mean fluorescence signal of the 2C peak of the stained nuclei of the reference standard times the 1C amount of DNA in the reference standard. Three individuals were scored to produce biological replicates. For one individual we prepared different tissues to make sure that we have not used polyploid tissue. The genome size is reported as 1C, the mean amount of DNA in Mb in a haploid nucleus.

Next Generation Sequencing, Data Pre-processing, and Clustering Analysis

Raw Illumina pair-end reads 150 bp long obtained from low coverage DNA-seq experiments on *Pontastacus leptodactylus* are available from the European Nucleotide Archive (NGS run accession: SRR7698976). After the quality filtering (quality cut-off value: 10 according to Novák et al., 2020b; percent of bases in sequence that must have quality equal to/higher than the cut-off value: 95 and filtered against a costomized database containing *P. leptodactylus* mitochondrial sequences), the reads were subjected to similarity-based clustering analysis using RepeatExplorer2 (Novák et al., 2010, 2013). We used a subset of reads (2 × 125,000) representing coverage of 0.002×. Genome coverage was calculated as follows: coverage = $(r \times l)/g$, where r corresponds to number of reads used in our analysis, l to read length and g to haploid genome size of *P. leptodactylus*. The clustering was performed using the default settings of 90% similarity over 55% of the read length. To confirm the results obtained through the RepeatExplorer pipeline, reconstruction of monomer sequences of individual satellite DNA families was performed using TAREAN analysis, specific for identification of satellite DNA repeats (Novák et al., 2017).

TABLE 1 | Chromosomal and cytogenetic characteristics of freshwater crayfish species of families Astacidae, Cambaridae, and Parastacidae.

Family/species	2*n*	45S rDNA	Karyotype formula	IHSs	Reference
Family Astacidae					
Astacus astacus	176	2	52m+35sm+1a	22 pairs of chromosomes	Mlinarec et al., 2011
*Pontastacus leptodactylus**(1)	180	2		6 pairs of chromosomes	Mlinarec et al., 2011
*Pontastacus leptodactylus**(2)	180	1	75m+14sm+1sm/m	10 pairs of chromosomes	This study
Austropotamobius torrentium	176	2	76m+11sm+1a		Mlinarec et al., 2016
Austropotamobius pallipes	176	2	76m+11sm+1a		Mlinarec et al., 2016
Pacifastacus leniusculus	376				Niiyama et al., 1962
Family Cambaroididae					
Cambaroides japonicus	194				Komagata and Komagata, 1992
Family Cambaridae					
Procambarus clarkii	188	2			Salvadori et al., 2014
Procambarus llamasi	120		120t		Indy et al., 2010
Procambarus digueti	102		35M+15m+1st		Diupotex Chong et al., 1997
Procambarus alleni	188				Martin et al., 2015
Procambarus fallax	184				Martin et al., 2015
*Procambarus virginalis**(3)	276		171m+39sm+3st+63t		Martin et al., 2015
Faxonius virilis	200				Fasten, 1914
Faxonius immunis	208				Fasten, 1914
Family Parastacidae					
Cherax destructor	188		70m+42sm+48st+28t		Scalici et al., 2010
Cherax quadricarinatus	200		33m+25sm+14st+28t		Tan et al., 2004

2n, diploid chromosome number; 45SrDNA, number of 45S rDNA loci; IHSs, number of homologous chromosome pairs possessing probable interstitial heterochromatic segments. m, metacentric; ms, metacentric-submetacentric; sm, submetacentric; a, acrocentric; t, telocentric; st, subtelocentric chromosome. Species classification according to Crandall and De Grave (2017).

*(1) *Euroasian lineage.*

*(2) *Asian lineage I.*

*(3) *autotriploid species.*

Repeat Classification

Repeat cluster classification of the top 0.01% clusters identified in comparative analysis was implemented in RepeatExplorer through which similarity searches with DNA and protein databases. After *de novo* identification of contigs that make up repetitive elements in RepeatExplorer, contigs were further classified using two homology-based approaches applied in LTRClassifier (Monat et al., 2016), specific for LTR retrotransposons, and Censor (Jurka et al., 1996) for all repetitive elements. This was followed by manual examination of individual clusters graph shapes, similarity searches using BLASTN and BLASTX against public databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi), inspection for the presence of sub-repeats using program dotmatcher (https://www.bioinformatics.nl/cgi-bin/emboss/dotmatcher) with parameters specific to individual monomer length (10% of length as window size and sequence specific similarity cut off), for the final manual annotation and quantification of repeats.

Putative satellite repeats were identified based on the properties of cluster graphs obtained by similarity-based clustering of low coverage genome sequencing Illumina reads, as implemented in the TAREAN pipeline (Novák et al., 2017). All satellite repeats with an abundance exceeding 0.1% of the *P. leptodactylus* genome were subjected to detailed sequence analysis (**Supplementary Table 2**). This analysis focused on AT content, genomic abundance, and presence of telomeric $(TTAGG)_n$ repeats and detection of sequence similarities (**Supplementary Table 2**). Individual satellite DNA clusters were further classified into the satellite groups via h-CD-HIT-EST (Fu et al., 2012) in two consecutive runs, with sequence identity cut-off set at 90% followed by 80% cut-off. Algorithm parameters were kept at default value. Furthermore, we classified tandem repeats as minisatellites (10–100 bp) and conventional satellites (>100 bp) depending on the monomer size (Garrido-Ramos, 2017).

To explore the relation between the repeat length and the %GC of satellite DNA we first performed Shapiro–Wilk's test to access the normality of both length and the %GC variable. Because the length variable did not follow normal distribution, we used non-parametric Spearman's rank correlation test to access correlation between two variables. Bioinformatic and statistical analysis were conducted in the R software environment (R Core Team, 2016).

Primer Design, PCR Amplification, and Cloning of Satellite DNA Families

From the *P. leptodactylus* reference monomers, outward facing primers were designed (**Table 2**, **Supplementary Figure 1**).

TABLE 2 | Characterization of selected satellite DNA families in *P. leptodactylus*.

Satellite family	Genome abundance (%)	Monomer (bp)	GC (%)	Localization	Primer Sequence (5′-3′)
PlSAT1–21	10.91	21	47.62	Interstitial	F-AGTTTCAATCGTCCCTGCTG
					R-TCAGCAGGGACGATTGAAAC
PlSAT3–411	1.29	411	26.76	(Peri)centromeric	F-TGTCTATTTTCCGTATATTGTAATGA
					R-ATCAACCATTTGCATTTCGTTC
PlSAT6–70	0.40	70	35.71	Interstitial	F-GACATGTTTTACATTAGACTTGTGA
					R-TATATGTGCCTGCAAGGTAAGT
PLSAT7–134	0.35	134	29.10	Interstitial	F-GGCAAGCCCAATTGGGTCTGA
					R-TCCGTAACGAAAGTAGAC
PLSAT14–79	0.17	79	44.30	Subtelomeric, interstitial, (peri)centromeric, the whole arm	F-GGTCAGTAAGCTATTGTGTGT
					R-CAACCTATGGAAGGTTATTAAGG

Repeat unit lengths, G+C content (%), abundances (%), chromosomal localization, and primer pairs used for satellite repeat amplification.

Specific primer pairs have been used for amplification of satellite DNA probes for FISH. All PCRs were performed using GoTaq® Green Master Mix (Promega, Madison, WI, USA): 1X GoTaq® Green Master Mix, 10 pmol of each primer (Macrogen, Amsterdam, The Netherlands) and 1 μl of template DNA (16 ng), in a 50 μl final reaction volume. PCR program consisted of 35 cycles, each with 1 min denaturation at 95°C, 10 s annealing at 56°C, 1 min extension at 72°C, and a final extension of 20 min.

The sequences of the amplified monomers were verified by cloning of the PCR product into pGEM-T Easy vector according to the manufacturer's instruction (Promega, Madison, WI, USA). Amplicons were extracted and purified using ReliaPrep™ DNA Clean-Up and Concentration System and cloned into pGEM-T Easy vector according to the manufacturer's instruction (Promega, Madison, WI, USA). The individual clones (from one to four per sample) were sequenced by Macrogen (Amsterdam, The Netherlands).

Preparation of Chromosome Spreads, Chromosome Measurements, and Idiogram Reconstruction

Four adult males (m = 17.01, 16.10, 32.21, and 16.27 g) were used for the cytogenetic study. Chromosome spreads were prepared according to the method described in Mlinarec et al. (2011). Individual chromosomes in karyotype were measured using LEVAN plug-in (Sakamoto and Zacaro, 2009) for the program ImageJ (Schneider et al., 2012) to obtain the relative chromosomal length (RCL) data. RCL were then imported into the RIdiogram package (Hao et al., 2020) of R programing environment for the ideogram reconstruction. Idiogram was further modified in the Inkscape vector graphics software (Inkscape Project, 2020) to include the 45S rDNA and DAPI-positive bands.

Fluorescence *in situ* Hybridization (FISH)

The 2.4 kb *Hind*III fragment of the partial 18S rDNA and ITS1 from *Cucurbita pepo*, cloned into the pUC19 vector, was used as the 45S rDNA probe (Torres-Ruiz and Hemleben, 1994). Telomeric DNA was generated by PCR amplification in the absence of template using primers $(TTAGG)_4$ and $(CCTAA)_4$ according to Ijdo et al. (1991). Probes used to map satDNAs in the chromosomes were DNA fragments cloned into the plasmid vector. Plasmids containing the monomer sequence were directly labeled with either Aminoallyl-dUTP-Cy3 (Jena Bioscience GmbH, Jena, Germany) or Green-dUTP (Abbott Molecular Inc., USA) using Nick Translation Reagent Kit according to the manufacturer's instructions (Abbott Molecular Inc., USA) with some modifications: Plasmid DNA (700 ng) was labeled in a total volume of reaction of 25 μl using 2.5 μl of enzyme mixture for 6 h at 15°C. FISH was performed according to Mlinarec et al. (2019) with slight modification: chromosome preparations were denatured at 72°C for 5 min after applying the hybridization mix. The preparations were mounted in Dako Fluorescence Mounting Medium (Dako North America Inc., USA) and stored at 4°C overnight. Signals were visualized and photographs captured using an Olympus BX51 microscope, equipped with a cooled CCD camera (Olympus DP70). Single channel images were overlaid and contrasted using Adobe Photoshop 6.0 with only those functions that apply to the whole image. An average of 10 well-spread metaphases was analyzed per each individual.

Accession Codes

Cloned sequences of satellite repeats were deposited in genBank under accession numbers MW044674 for PlSAT1-21, MW044678 for PlSAT3-411, MW044675 for PlSAT6-70, MW044677 for PlSAT7-134, and MW044676 for PlSAT14-79. *COI* gene sequences were deposited in GenBank under accession numbers MW045515 for Hap1 and MW045516 for Hap2.

RESULTS

DNA Barcoding and Phylogenetic Network Reconstruction

Phylogenetic tree was constructed to place samples used in this study within the context of patterns of diversity across the range of *P. leptodactylus*. Final alignment consisted of *COI* barcode sequences 487 bp long and included 91 unique haplotypes from across 10 countries. Haplotype relatedness and geographical haplotype distribution is presented in the

Supplementary Figure 2. Three distinct lineages were observed in the median joining network, separated by 8–24 mutational steps. DNA barcoding showed that the samples from the lake Maksimir (Zagreb, Croatia) belong to the Asian lineage *sensu* Maguire et al. (2014) and formed two haplotypes (Hap 1 and Hap 2) closely related to haplotypes from Armenia.

Pontastacus leptodactylus Karyotype and Genomic Organization of 45S rDNA and Telomeric $(TTAGG)_n$ Repeats

The karyogram of *P. leptodactylus* ($2n = 180$) is presented here for the first time (**Figures 1A,B**). The karyotype consists of 75 metacentric, 14 submetacentric, and 1 submetacentric/metacentric heteromorphic chromosome pair. Thus, the proposed diploid formula is $2n = 75m+14sm+1sm/m$. The probable HSs revealed after DAPI staining were found in the (peri)centromeric region of all chromosome pairs as well as in the intercalary regions of 10 chromosome pairs. FISH performed with the 45S rDNA probe revealed two signals positioned on the entire longer arm of the submetacentric/metacentric heteromorphic chromosome pair (**Figures 1A,B**). Chromosome size and morphology of each chromosome pair within the complement is presented in **Supplementary Table 3**, while idiogram with position DAPI-positive bands and 45S rDNA loci is presented in **Supplementary Figure 3**.

FISH experiments using the probe $(TTAGG)_n$ revealed strong and consistent signals in the terminal ends of both chromosomal arms of all *P. leptodactylus* chromosomes. The telomeric probe also hybridized to interstitial regions (ITRs) of eight chromosome pairs (**Figures 1C,D**). The ITR signals were of different sizes and intensity and the majority of ITR signals were more intense than the signals in the terminal chromosome ends. All ITRs were devoid of microscopically recognizable heterochromatic regions and did not co-localize with 45S rDNA loci.

Pontastacus leptodactylus Repeatome Characterization and Identification of Tandem Repeats

The genome size of *P. leptodactylus* was measured in three individuals from a single population. Results showed that the average 1C DNA value was 18.7 Gbp (**Figure 2**). Clustering of $2 \times 125{,}000$ paired-end reads resulted in 19,092 clusters. The nuclear repetitive DNA constituted 54.85% of the genome (**Table 3**). Of all the repetitive elements, 84.1% were classified to the known repetitive element groups (belonging to 37 major categories), while 4.48% remained unclassified as "other." Satellite repeats were the most abundant elements, representing 27.52% of the genome, of which minisatellites (10–100 bp) comprised 24.7%, while conventional satellites (>100 bp) comprised 2.87% of the genome. Transposable elements (TEs) contributed 22.67% to the *P. leptodactylus* nuclear genome. Repeats classified as LTR retrotransposons represented the major fraction of the TEs of *P. leptodactylus*, comprising 15.32% (71 clusters) of nuclear DNA, followed by DIRS, LINE, and Penelope elements that comprised 3.57% (4 clusters), 2.23% (33 clusters), 1.00% (2 clusters) of nuclear DNA, respectively. LTR retrotransposons were mostly represented by Ty3/*gypsy* elements (14.95%, 55 clusters), followed by Ty1/*copia* (0.1%, 4 clusters), BEL (0.05%, 2 clusters), and ERV (0.03%, 1 cluster). DNA transposons constituted 0.51% (23 clusters) of the nuclear genome, with Helitrons as the most abundant (0.15%, 6 clusters). Ribosomal RNA genes (45S rDNA) represented 0.01% (1 cluster) of the genome (**Table 3**).

Based on the RepeatExplorer pipeline, 258 satellite DNA families have been identified. Satellite DNA families have been designated as PlSAT1-21, through PlSAT258-57 (stands for *Pontastacus leptodactylus* satellite 1 through to 258 in decreasing genomic abundance, with the respective monomer length separated by a dash; **Supplementary Table 2**). Their unit lengths ranged from 14 to 664 bp (median value 59 bp; **Supplementary Table 2**). The distribution of the lengths was biased due to the predominance of short satellite repeats, with more than half (240) being classified as minisatellites. The A+T content of the consensus satDNA sequences varied between 29.17 and 73.14% among the families, with a median value of 54.34%, which indicated a slight bias toward A+T rich satellites. Spearman's rank correlation test showed no significant correlation between satellite length and A+T content (p-value: 0.368, correlation coefficient: 0.056) (**Figure 3**). Only one monomer of the perfect telomeric sequence motif (TTAGG/CCTAA) was present within the consensus sequence of 13 satellite elements, while monomers of other satDNAs contained no telomeric sequence motifs. Based on BLAST searches the satDNA sequences showed no similarity with any other DNA sequence deposited in non-redundant databases. **Supplementary Table 2** shows the reconstruction of representative monomer sequences for each satDNA family. Genomic abundance of satellite DNAs ranged from 0.01% up to 10.91% of the genome (**Supplementary Table 2**). SatDNA family PlSAT1-21 showed the highest abundance (10.91%), followed by PlSAT2-21 (3.79%) and PlSAT3-411 (1.29%).

Detailed Characterization and Chromosomal Localization of PlSAT3-411, PlSAT6-70, PlSAT7-134, and PlSAT14-79 Satellite DNA Families

Five satellite DNA families, PlSAT1-21, PlSAT3-411, PlSAT6-70, PlSAT7-134, and PlSAT14-79 were selected for further analysis (**Table 2**, **Supplementary Figures 1**, **4**–**6**). Firstly, to confirm their tandem arrangement the predicted monomer sequences of selected satellite DNA families have been validated by performing PCR with *P. leptodactylus* genomic DNA as a template using primers designed to face outwards from the reconstructed monomer consensus (**Table 2**, **Supplementary Figure 1**). In this arrangement, the amplification can occur only between the primer pairs located in adjacent tandemly repeated arrays. All five putative repeats tested using this assay produced the expected amplification products, and their cloned sequences (from one to four per satellite) matched the predicted consensus with 82–100% similarity. The lowest similarity (82%) was observed between

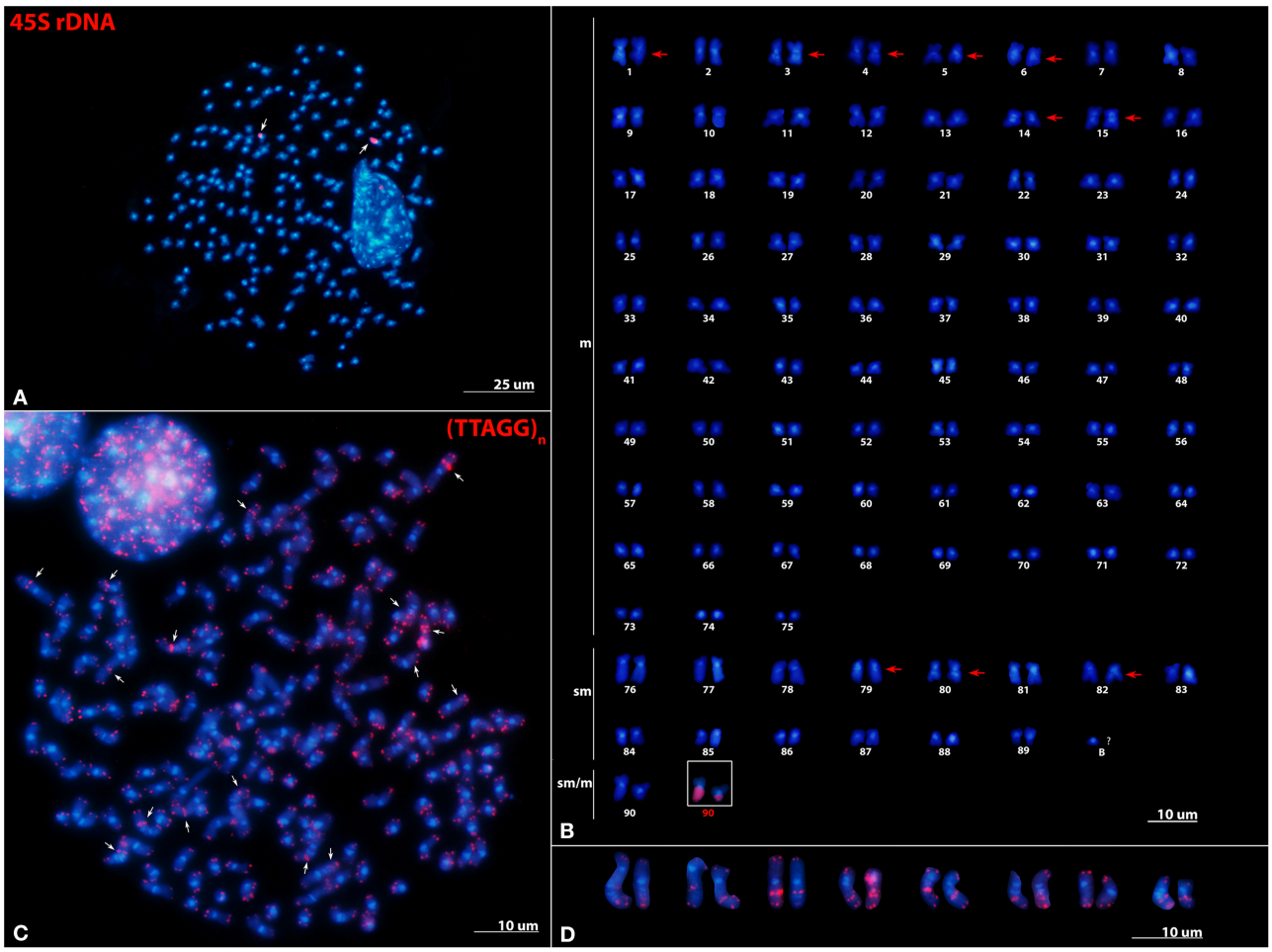

FIGURE 1 | Distribution of 45S rDNA and telomeric repeats on metaphase chromosomes of *Pontastacus leptodactylus*. **(A)** Mitotic metaphase and **(B)** karyogram of *P. leptodactylus* after FISH with 45S rDNA probe (red signals). m, metacentric chromosomes; sm, submetacentric chromosomes; sm/m, submetacentric/metacentric heteromorphic chromosome pair. Arrows point to interstitial HSs. 45S rDNA bearing heteromorphic chromosome pair is framed. **(C)** Mitotic metaphase of *P. leptodactylus* after FISH with telomeric repeats $(TTAGG)_n$ (red signals). ITRs are marked with arrows. **(D)** Eight chromosome pairs possessing ITRs. Chromosomes are counterstained with DAPI. Scale bar = 10 μm.

cloned and predicted consensus PlSAT7-134 repeat, while the other four satellite families exhibited 95–100% similarity between cloned and predicted consensus sequence. We selected the one with the highest identity to the reference monomer as the probe for subsequent hybridizations.

Dot plot analysis of PlSAT1-21, PlSAT3-411, PlSAT6-70, PlSAT7-134, and PlSAT14-79 did not reveal any consecutive tandem sub-repeats, although multiple poly-A and poly-T repetitions were observed in GC poor satellite repeat families PLSAT3-411 and PLSAT7-134 (**Supplementary Figure 6**).

Chromosome mapping of the PlSAT1-21, PlSAT3-411, PlSAT6-70, PlSAT7-134, and PlSAT14-79 satellites revealed distinct hybridization sites, with reproducible and unambiguous markings for all analyzed mitotic metaphases (**Figure 4**). PlSAT1-21 satellite family hybridized to the interstitial positions in the vicinity to the probable (peri)centromeric HSs on some chromosomes (**Figure 4A**). The PlSAT3-411 satellite hybridized in the (peri)centromeric regions, labeling all probable (peri)centromeric HSs on all *P. leptodactylus* chromosomes (**Figure 4B**). The PlSAT7-134 and PlSAT6-70 satellite families hybridized to the interstitial positions of some chromosomes (**Figures 4C,D**). The PlSAT14-79 satellite family co-localized with the AT-rich DAPI-positive probable (peri)centromeric heterochromatin on some chromosomes and is also located subterminally and intercalary on some chromosomes (**Figure 4D**). Besides, the PlSAT14-79 probe marked the whole shorter arm of one chromosome pair. The PlSAT6-70, PlSAT7-134, and PlSAT14-79 signals co-localized with some interstitial probable HSs.

Similarity Between satDNA Families of *P. leptodactylus*

Some longer satDNA families showed similarity to other shorter families. Of 258 satellite repeats characterized in

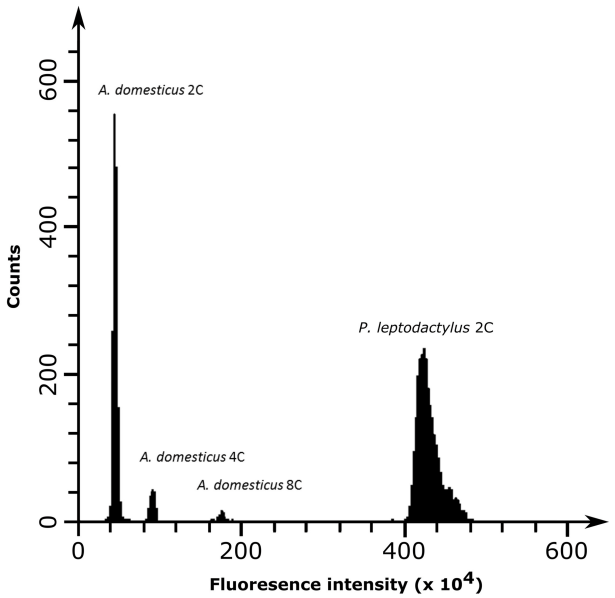

FIGURE 2 | Flow cytometry histograms of neural tissue from house cricket *Acheta domesticus* 2C (first peak), *A. domesticus* 4C (second peak), *A. domesticus* 8C (third peak), and vascular tissue from *P. leptodactylus* (fourth peak) obtained by PI fluorescence dye excitation and counts representing the cell population.

P. leptodactylus, 39 repeats showed similarities, forming 18 groups. Each group consisted of two or three satellite repeats. Similarity within each group ranged from 55 to 78%, average similarity is 63%. Only one satDNA family, PlSAT75-664, showed complex units including sub-repeats with high percentages of similarity to other shorter family, PlSAT3-411 (**Figure 5).** Detailed analysis showed that PlSAT75-664 unit includes the complete PlSAT3-411 unit and four direct sub-repeats, each ∼70 bp long, each showing high similarity (79.9, 80.6, 70.21, and 56.82%) to 3′ end of the core of the PLSAT3-411 unit (**Figure 5**).

TABLE 3 | Major types of repetitive DNA in *P. leptodactylus* (classification according to Wicker et al., 2007).

Categories/Superfamilies	Abundance (%)	Clusters (n)
Satellites	27.57	258
Minisatellites	24.70	240
Satellites	2.87	18
Class I (retrotransposons)		
LTR elements	15.32	71
Ty3/gypsy	14.95	55
Ty1/copia	0.10	4
BEL	0.05	2
ERV	0.03	1
Integrated virus DNA	0.19	9
DIRS	3.57	4
Penelope	1.00	2
LINE	2.23	33
R1	0.04	2
R2	0.03	2
RTE	0.01	1
RTEX	0.03	1
Jockey	0.10	7
I	0.21	1
L1	0.02	1
Ingi	0.05	1
CRE	0.01	1
CR1	1.32	6
CR2	0.03	2
Nimb	0.03	2
Kiri	0.02	1
Daphne	0.01	1
Unclassified LINE	0.32	4
SINE	0.07	1
Class II (DNA transposons)—Subclass 1		
TIR	0.27	14
Mariner	0.05	4
hAT	0.05	2
CACTA	0.03	1
Harbinger	0.03	1
piggyBAC	0.03	1
Dada	0.02	1
Ginger2	0.02	1
Sola	0.02	1
Ginger3	0.01	1
Transisb	0.01	1
Class II (DNA transposons)-Subclass 2		
Helitron	0.15	6
Polinton	0.09	3
rDNA	0.01	1
Unclassified repetitive	4.48	97
Total repetitive DNA	54.85	490

DISCUSSION

Phylogenetic Placement of *P. leptodactylus* Individuals Used in This Study

Although, *Pontastacus leptodactylus* is naturally distributed across Europe, previous study by Njegovan et al. (2017) indicated that its presence in Lake Maksimir is the consequence of human mediated translocation. Phylogenetic reconstruction indicated that the individuals of *P. leptodactylus* belong to the Asian lineage *sensu* Maguire et al. (2014), specifically they are closely related to haplotypes originating from Armenia. Although, we observed two haplotypes within the Lake Maksimir population, they differed only in one base (site 378: Hap1-C, Hap2-A), collapsed to a single haplotype in the network reconstruction. This supports a theory by Njegovan et al. (2017) that the crayfish were introduced into lakes from the local market, supplied from the Armenian breeders. Further sampling and population studies, coupled with a multigene approach may help in resolving the taxonomic status of the three lineages within the *P. leptodactylus* species complex.

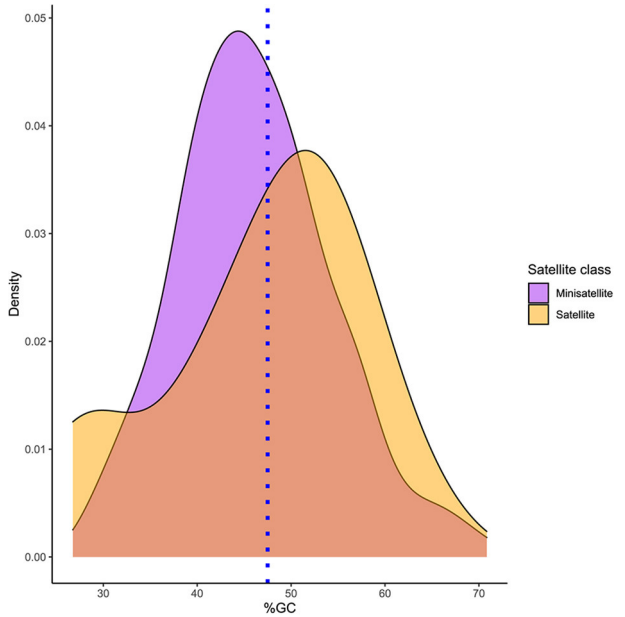

FIGURE 3 | Density plot representing the %GC distribution of *P. leptodactylus* minisatellite and satellite repeats. Blue line represents average %GC percentage of *P. leptodactylus* reported from the NGS reads.

P. leptodactylus Karyotype and Genomic Organization of 45S rDNA and Telomeric $(TTAGG)_n$ Repeats

In this study, FISH results showed one 45S rDNA locus and ten probable interstitial HSs in the studied *P. leptodactylus*, which is different from the previous work on *P. leptodactylus* that reported two 45S rDNA loci and six interstitial HSs (Mlinarec et al., 2011). The observed discrepancy suggests the presence of intraspecific variability within *P. leptodactylus*, and we could speculate that differences in rDNA loci number as well as in the number of interstitial HSs could possibly be lineage specific. In particular, samples analyzed in Mlinarec et al. (2011) belonged to European lineage *sensu* Maguire et al. (2014), while samples used in the present study belong to the Asian lineage *sensu* Maguire et al. (2014). Intraspecific variability has been reported in other groups of organisms such as two fish species from genus *Schistura* (Sember et al., 2015), as well as in plants *Phaseolus vulgaris* and *Tanacetum cinerariifolium* (Pedrosa-Harand et al., 2006; Mlinarec et al., 2019). Different mechanisms can lead to intrachromosomal variability such as unequal crossing-over, non-homologous recombination and movement mediated by transposons (Liu et al., 2003; Nguyen et al., 2010; Pereira et al., 2013; Vershinina et al., 2015; Mlinarec et al., 2019).

Large AT-rich probable HSs positioned in the (peri)centromeric position on all chromosomes and interstitially on some chromosomes suggest a high amount of repetitive DNA in the genome of *P. leptodactylus* (this study; Mlinarec et al., 2011). Large (peri)centromeric HSs have been found in different crustacean families such as Astacidae (Mlinarec et al., 2011, 2016), Nephropidae (Deiana et al., 1996; Coluccia et al., 2001; Salvadori et al., 2002), Scyllaridae (Deiana et al., 2007), Palinuridae (Coluccia et al., 1999, 2005; Cannas et al., 2004), Cambaridae (Salvadori et al., 2014), and Palaemonidae (González-Tizón et al., 2013; Torrecilla et al., 2017; Molina et al., 2020).

In this study it was observed that telomeres of *P. leptodactylus* consist of $(TTAGG)_n$ pentameric repeats, same as in all decapod crustaceans studied until now and in most arthropods (Vítková et al., 2005; Salvadori et al., 2012, 2014). However, this study showed that a significant part of telomeric repeats is located interstitially in the chromosomes of *P. leptodactylus*. ITRs were also observed in other crustaceans such as *Jasus lalandii* and *Procambarus clarkii* (Salvadori et al., 2012, 2014). In *J. lalandii*, ITRs are associated with rDNA (Salvadori et al., 2012), while in *P. leptodactylus* and *P. clarkii* co-localization of ITRs with rDNA loci has not been observed (Salvadori et al., 2014). The occurrence of ITRs outside of the chromosomal termini is not fully understood. ITRs in (peri)centromeric regions could represent remnants of structural chromosome fusions (Ruiz-Herrera et al., 2008; Bolzán, 2012). This is unlikely in *P. leptodactylus* as there were no ITRs in (peri)centromeric positions. ITRs might have originated from the transposition of telomeric repeats by transposable elements or during repair of double stranded breaks (Aksenova and Mirkin, 2019) or might simply reflect the fact that telomeric sequences are present within repetitive DNA components like in some plants (Tek and Jiang, 2004; Mlinarec et al., 2009; Emadzade et al., 2014). The last case is unlikely as in *P. leptodactylus*, satellite repeats do not contain stretches of telomeric repeats.

Pontastacus leptodactylus Repeatome

This work represents the most comprehensive characterization of the repetitive elements in any species belonging to the family Astacidae. In this study, we showed that *P. leptodactylus* harbors a large variety of repetitive elements, accounting for about 54.85% of its genome. As repeats may escape their detection by degradation, we consider this value as an underrepresentation. Degraded repeats arise from point mutations, indels and rearrangements, and they may be so substantial that they contribute repeats into tracks of unique or low-copy sequences. This is supported by recent studies on 101 species showing that in the large genomes, such as the genome of *P. leptodactylus*, the proportion of single and low-copy (up to 20 copies) sequences significantly increases with genome size, which is accompanied by a significant decrease in the genome proportion of medium-copy repeats (Novák et al., 2020a).

The analyses of draft genomes of *C. quadricarinatus* and *P. virginalis* showed that they have a significantly lower amount of repetitive DNA, 33.73 and 27.52%, respectively (Gutekunst et al., 2018; Tan et al., 2020), in comparison with *P. leptodactylus*. Furthermore, in *P. leptodactylus* satellite repeats and Ty3/*gypsy* elements are the most abundant, while in *C. quadricarinatus* and *P. virginalis*, LINE elements are the most abundant repetitive elements in the genome (Tan et al., 2020). However, comparison of the results of this study with those of Tan et al. (2020)

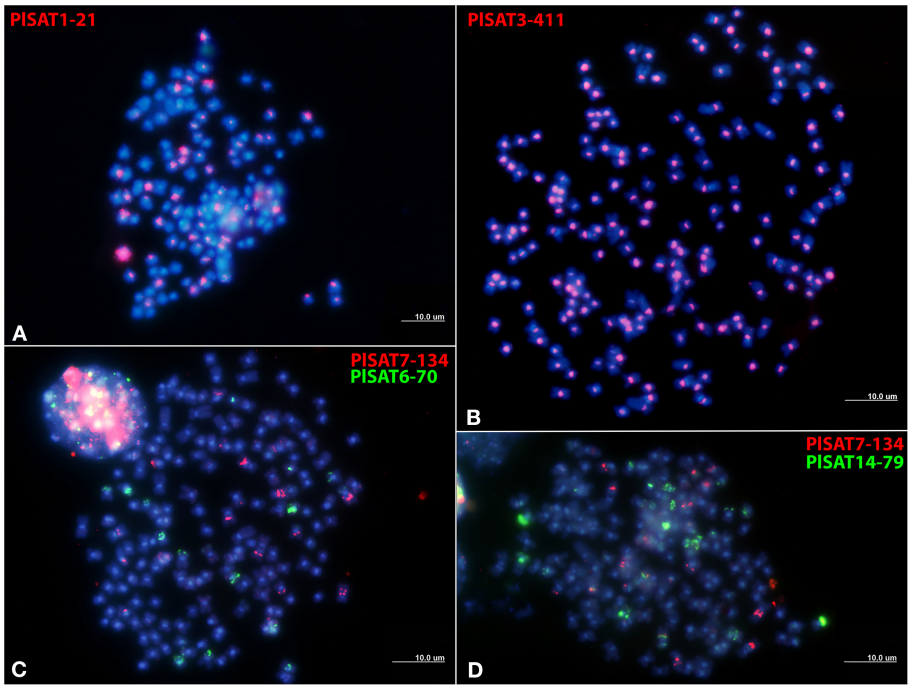

FIGURE 4 | Distribution of satellite repeat families on metaphase chromosomes of *Pontastacus leptodactylus*. **(A)** PISAT1-21 (in red), **(B)** PISAT3-411 (in red), **(C)** PISAT7-134 (in red) and PISAT6-70 (in green), and **(D)** PISAT7-134 (in red) and PISAT6-70 (in green) as probes. Chromosomes are counterstained with DAPI. Scale bar = 10 μm.

should be taken with caution since different methods have been applied for repeat identification. Estimation of the repeat abundance form the *de novo* genome assemblies generated by short-read sequencing as in Tan et al. (2020), can lead to the underrepresentation of the highly repetitive elements. These elements are often clustered into a single contig or fragmented across multiple short contigs due to the inherited characteristics of the *de novo* genome assembly tools, therefore misrepresenting the abundance of the repetitive elements in the genome (Chu et al., 2016). The flow cytometry method estimated 1C = 18.7 Gbp size for the *P. leptodactylus* genome, providing the first report on genome size for any species within the family Astacidae. However, there is still a general lack of genome sizes for the infraorder Astacidea. As far as we are aware, genome size is available for several members of the familiy Cambaridae (5 species) and Parastacidae (1 species) ranging from 3.82 to 6.06 Gbp (Gregory, 2020; Tan et al., 2020). This makes *P. leptodactylus* (1C = 18.7 Gbp) species with the highest genome size of all known members of the infraorder Astacidea. In *P. leptodactylus*, genome expansion can be a result of the accumulation of short tandem repeats and retroelements as it is shown in this study that the genome of this species is rich in satellite DNA and retroelements. A large genome size as well as a highly repetitive genome explains difficulties generated during the genome assembly process, which limit the generation of available genomic resources from crustacean species (Tan et al., 2020; Van Quyen et al., 2020).

In *P. leptodactylus*, satellite repeats are the most abundant group of repetitive elements, accounting for 27.52% of its genome. Although, the knowledge about repetitive DNA composition in the genomes of decapod crustaceans is scarce, it is likely that a great expansion of satellites occurred in the genome of *P. leptodactylus*. The large amount of satellite repeats has been reported in other organisms such as insects *Drosophila virilis* and *Triatoma infestans* (Wei et al., 2014; Pita et al., 2018). In *D. virilis* nearly 50% of the genome is composed of satDNA, while in *T. infestans* satellite repeats make up 25-33% of the genome and are arranged into at least 42 satellite DNA families (Wei et al., 2014; Pita et al., 2018). Furthermore, we found great diversity of satDNA repeats in the genome of *P. leptodactylus* with a total of 258 satellite families which is by far the most satellite-rich species discovered to date. A large number of different satDNA elements is found in other organisms such as the fish *Megaleporinus macrocephalus* (Teleostei, Anostomidae) where 164 satellite repeats have been described (Utsunomia et al., 2019). Similar to *P. leptodactylus,* in *M. macrocephalus*, short satellites dominate in the genome. Among plants, *Luzula elegans* (Poaceae) has the highest number of satellites, 37, constituting 9.9% of the genome (Heckmann et al., 2013). The species *Vicia faba* (Fabaceae) is another example of the plant species with a high number of satellites, over 30, that together constitutes 935 Mbp (7%) of its genome (Ávila Robledillo et al., 2018). Large satDNA abundance and diversity is not a common characteristic for all animal and plant genomes, as there are, as far as we know, many more reports on the organisms poor in satellite DNA using

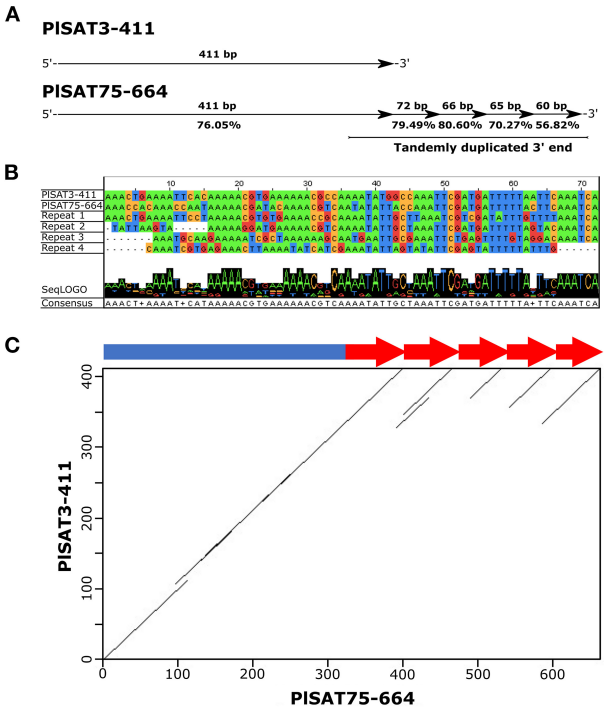

FIGURE 5 | (A) Detailed analysis of satellite family PlSAT75-664 and its similarity with satellite family PlSAT3-411, **(B)** Alignment of PlSAT75-664 subrepeats visualized within Jalview (Waterhouse et al., 2009) and **(C)** Dot plot of the satellite family PlSAT75-664 obtained in the RepeatExplorer analysis of the *P. leptodactylus* genome, that shows similarity with the satellite family PlSAT3-411 satDNA of *P. leptodactylus*, revealing subrepeats with a periodicity of about 70 bp (arrows).

similar approaches. In *Tanacetum cinerariifolium* (Asteraceae), only three among the 58,204 clusters obtained were classified as satellites, representing 1.04% of the genome (Mlinarec et al., 2019). Similarly, after the investigation of *Passiflora edulis* by RepeatExplorer, only two of the 233 repetitive elements were satellites, representing less than 0.1% of the genome (Pamponét et al., 2019).

It is tempting to speculate where the diversity of *P. leptodactylus* satellites originate from. Novel satellite DNA families may arise from the independent duplication of different genomic sequences, such as intergenic spacers, or even from those derived from other satellite DNAs (Garrido-Ramos, 2017). The satDNA sequences can interact with transposable elements to create new repetitive DNA (Pita et al., 2018). It is suggested that transposable elements provide the mechanism by which satDNA repeats could propagate in the genome through dispersed short repeat arrays (Macas et al., 2011; Bardella et al., 2014). The *P. leptodactylus* genome is rich in the LTR retrotransposons.

Minisatellites (monomer size 10–100 bp) were found to be surprisingly numerous in the *P. leptodactylus* genome, accounting for about 24.7% of the genome. High content of minisatellites in the *P. leptodactylus* genome might indicate a high level of DNA polymerase slippage as it is generally considered that short tandem repeats (<100 bp) expand through DNA polymerase slippage (Garrido-Ramos, 2017). The most abundant satDNA in the genome of *P. leptodactylus* is a minisatellite PlSAT1-21. Its short monomer size of 21 bp is unusual for a tandem repeat of high abundances, which generally consist of 160–180 or 320–360 bp monomers (Garrido-Ramos, 2017). This underpins that satellites with short monomer lengths can form very large arrays as observed here for PlSAT1-21. In the hermit crab *Pagurus pollicaris*, a minisatellite AGTGCAG(CTG)$_n$ constitutes a large fraction of its genome (Chambers et al., 1978). An exceptional abundance of microsatellite and SSR sequences has also been found in the genome of freshwater prawns of the genus *Macrobrachium* (Palaemonidae) as well as in the penaeid shrimp *Litopenaeus vannamei* (Zhang et al., 2019; Molina et al., 2020), suggesting that short tandem repeats are a significant component of decapod crustaceans genomes.

In *P. leptodactylus*, 171 (66.27%) satellite DNA families showed A+T content higher than 50%, and could be classified as AT-rich (**Figure 3**). Furthermore, there is no correlation between A+T content and satellite length. The high A+T content could be a consequence of satDNA being subject to epigenetic modifications such as the methylation of cytosines, consequently deamination of 5-methylcytosines forming more AT base pairs in *P. leptodactylus* satDNAs. In the fish *Megaleporinus microcephalus* short (<100 bp) and long (>100 bp) satellites had a similar amount of A+T content (Utsunomia et al., 2019). In the fern *V. speciosa* satDNAs longer unit length showed a higher A+T content (Ruiz-Ruano et al., 2019). In *V. faba*, most of the satellite sequences had an elevated A+T content (65–80%) (Ávila Robledillo et al., 2018).

In *P. leptodactylus*, the satellites are abundant in the (peri)centromeric region, on both ends of the chromosomes and some of them are distributed on the interstitial regions of the chromosomes. This is in line with previous results which show that subtelomere and centromere regions contain large parts of satellite repeats (Melters et al., 2013; Garrido-Ramos, 2017). Conventional satellites (monomer size>100 bp) and minisatellites (monomer size 10–100 bp) are conventionally differentiated by their location (Garrido-Ramos, 2017). While classic satDNAs are usually located as long arrays at the heterochromatin segments, minisatellites are generally proper of euchromatic regions (Garrido-Ramos, 2017). In *P. leptodactylus*, the classic satellite family PlSAT3-411 constitutes (peri)centromeric HSs, while minisatellites PlSAT6-70 and PlSAT14-79 as a part of euchromatic regions are located along the chromosome arms.

(Peri)centromeric Satellite Family PLSAT3-411

Centromeres are often packaged into heterochromatin, containing large amounts of repetitive DNA (Wang et al., 2009; Mehta et al., 2010). Here we showed that the probable (peri)centromeric heterochromatic segments located on all *P. leptodactylus* chromosomes are formed by a specific highly amplified satellite family PlSAT3-411. The arrangement of the (peri)centromeric satDNA family PlSAT3-411 can be

explained by the principle of equilocality, according to which, heterochromatin accumulates at equivalent positions in each chromosome within a genome (Garrido-Ramos, 2017). The most consistent form of equilocality relates to the heterochromatin in the vicinity of centromeres (John et al., 1985), which is true for PlSAT3-411 being present in the (peri)centromeric regions of all chromosomes. Following the survey of tandem satellite repeats in 282 species from various kingdoms (Melters et al., 2013), PlSAT3-411 is an ideal candidate for centromeric repeat sequences. It is one of the most abundant satellite repeats accounting for 1.29% of the genome and it is A+T-rich. It has been found that centromeric satDNAs are generally A+T rich (Garrido-Ramos, 2015; Yuan et al., 2018). Most animal species investigated so far have a single or only a few centromeric satellites with monomers hundreds of nucleotides long that are shared by all chromosomes, an observation that is explained by their coevolution with kinetochore proteins (Garrido-Ramos, 2015). The (peri)centromeric satellite family PlSAT3-411 is common in that respect. (Peri)centromere composition of *P. leptodactylus* calls for the investigation of additional species from different genera to get a more representative insight into the evolution of the (peri)centromeric satellite family. The frequent accumulation of satDNA in centromeric regions is explained by its role in centromere functions, such as kinetochore assembly and chromosome segregation during mitosis or meiosis, or even some epigenetic regulations, or simply by passive accumulation due to the absence of recombination-based elimination mechanisms (McFarlane and Humphrey, 2010; Plohl et al., 2014; Catania et al., 2015). To fully confirm that PlSAT3-411 is a true centromeric satellite family, underlying the functional kinetochore CENH3-ChIP followed by sequencing is needed.

Similarity Between satDNA Families

Most of the satDNA families described in this study did not show any conserved features or sequence similarities between each other suggesting their independent origin. Only 39 of the 258 satDNA repeats described in *P. leptodactylus*, showed similarities, however, their similarity is not high, ranging from 55 to 78%, average similarity is 63%. Two satellites, PlSAT3-411 and PlSAT75-664, were among the most interesting. The longer unit PlSAT75-664 is organized into HOR (higher order repeat) structures that consist of PlSAT3-411 basic monomer and four times directly repeated ∼70 bp long sequence that shows high similarity to PlSAT3-411 (**Figure 5**). The similarity between PlSAT3-411and PlSAT75-664 indicates the existence of a satDNA superfamily (SF), derived from a common ancestor satDNA. In the most parsimonious scenario, HOR structure might have formed after a ∼70 bp fragment was four times amplified within the satDNA, resulting in a new repeat unit of 664. It is known that the simultaneous amplification and homogenization of two or more adjacent monomers leads to the formation of HORs (Garrido-Ramos, 2017). Furthermore, it is generally considered that shorter repeats originate by replication slippage, while longer units originate by unequal crossing over (Garrido-Ramos, 2017). Therefore in *P. leptodactylus*, replication slippage might be the mechanism for the origin of the four times tandemly repeated ∼70 bp subunits within PlSAT75-664. The combination of short repeat units into longer units constituting HORs is a common trend in satDNA evolution (Plohl et al., 2008; Garrido-Ramos, 2017). Regular HORs, usually dimeric, have been found in several species of beetles (Palomeque and Lorite, 2008; Vlahović et al., 2017). Complex HORs, shaped from interspersed and/or inversely oriented monomers and frequently with extraneous sequence elements, have been found in non-human mammals, such as mouse, pig, bovid, horse, dog, elephant, insect, and fish (Palomeque and Lorite, 2008; Vlahović et al., 2017; Utsunomia et al., 2019).

The present study is the first one focusing on the repeatome of *P. leptodactylus* and enables a new perspective into the evolution of this complex species. *P. leptodactylus* repeatome serves as an important and valuable resource to support ongoing comparative genomic, cytogenomic, fundamental, and applied biology studies. To gain a more comprehensive understanding of chromosome evolution and genomic compositions of freshwater crustaceans, chromosome and genome resources are much needed for more species across taxonomic groups.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ebi.ac.uk/ena/browser/view/SRR7698976, SRR7698976; https://www.ncbi.nlm.nih.gov/nuccore/KX279350.1, KX279350.

AUTHOR CONTRIBUTIONS

The study was conceived by IM, VB, JM, LLB, and LB, while LLB, LB, and JM designed the experimental part of the study. Field work was carried out by LLB, LA, LB, and LL, while lab work (DNA isolation, cytogenetic experiments) by LLB, LA, LB, LL, and IM. CG and AH conducted flow cytometry analysis. LLB carried out the bioinformatic analyses and JM designed the primers. JM, LLB, LB, IM, VB, FG, and CA discussed and interpreted the results. JM wrote the paper with the help of LLB and LB. The initial version of the manuscript was drafted by IM, VB, LLB, and LB. All authors read, edited, enhanced original version of the manuscript, and approved its final version.

FUNDING

This work was supported by the institutional project financed by the University of Zagreb, by the University of Zagreb Student union grant for the project Development of new karyotypization techniques of freshwater crayfish (family: Astacidae), and LL by ESF-DOK-2018-01-9589.

ACKNOWLEDGMENTS

We would like to thank Lana Jelić and JU Park Maksimir for providing us with their support in conducting this research and raising awareness of the presence of native freshwater crayfish species in the City of Zagreb.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.611745/full#supplementary-material

Supplementary Table 1 | *COI* gene sequences from *P. leptodactylus* used to construct a phylogenetic tree.

Supplementary Table 2 | *Pontastacus leptodactylus* satellitome. Repeat unit lengths, G+C content (%), abundances (%), and consensus sequence.

Supplementary Table 3 | Morphometric measurements of *P. leptodacylus* chromosomes ($n = x = 90$).

Supplementary Figure 1 | Consensus sequences of PlSAT1-21, PlSAT3-411, PlSAT6-70, PlSAT7-134, and PlSAT14-79 repeats in fasta format. Primer sequences for amplification of tandem repeat specific probes are underlined and bolded.

Supplementary Figure 2 | Median-joining network of the *COI* barcode region haplotypes. Haplotype from the lake Maksimir is marked with an arrow. Haplotype size reflects relative frequency. Each branch represents one mutational step, unless otherwise noted (numbers in red above branches). Black circles represent missing intermediate haplotypes. Different colored circles denote the share of distinct haplotypes within countries (legend is shown in the upper-left corner).

Supplementary Figure 3 | An idiogram of *P. leptodactylus* chromosomes with marked localization of 45S rDNA and DAPI positive AT-rich heterochromatin bands This idiogram was generated based on the FISH information from **Figures 1A,B**.

Supplementary Figure 4 | The graph layout corresponding to read clusters of **(A)** PlSAT1-21, **(B)** PlSAT3-411, **(C)** PlSAT6-70, **(D)** PlSAT7-134, and **(E)** PlSAT14-79. The percentage indicates the genome proportion of each cluster.

Supplementary Figure 5 | Sequence logos showing the level of sequence divergence.

Supplementary Figure 6 | Dot plot analysis of **(A)** PlSAT1-21, **(B)** PlSAT3-411, **(C)** PlSAT6-70, **(D)** PlSAT7-134, and **(E)** PlSAT14-79 obtained in TAREAN analysis of *P. leptodactylus*.

REFERENCES

Akhan, S., Bektas, Y., Berber, S., and Kalayci, G. (2014). Population structure and genetic analysis of narrow-clawed crayfish (*Astacus leptodactylus*) populations in Turkey. *Genetica* 142, 381–395. doi: 10.1007/s10709-014-9782-5

Aksenova, A. Y., and Mirkin, S. M. (2019). At the beginning of the end and in the middle of the beginning: structure and maintenance of telomeric DNA repeats and interstitial telomeric sequences. *Genes* 10:118. doi: 10.3390/genes10020118

Ávila Robledillo, L., KoblíŽková, A., Novák, P., Böttinger, K., Vrbová, I., Neumann, P., et al. (2018). Satellite DNA in Vicia faba is characterized by remarkable diversity in its sequence composition, association with centromeres, and replication timing. *Sci. Rep*. 8:5838. doi: 10.1038/s41598-018-24196-3

Bandelt, H. J., Forster, P., and Röhl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Mol. Biol. Evol*. 16, 37–48. doi: 10.1093/oxfordjournals.molbev.a026036

Bardella, V. B., Aristeu, J. A., and Vanzela, A. L. L. (2014). Origin and distribution of AT-rich repetitive DNA families in Triatoma infestans (Heteroptera). *Infect. Genet. Evol*. 23, 106–114. doi: 10.1016/j.meegid.2014.01.035

Blackburn, E. H. (2005). Telomeres and telomerase: their mechanisms of action and the effects of altering their functions. *FEBS Lett*. 579, 859–862. doi: 10.1016/j.febslet.2004.11.036

Bolzán, A. D. (2012). Chromosomal aberrations involving telomeres and interstitial telomeric sequences. *Mutagenesis* 27, 1–15. doi: 10.1093/mutage/ger052

Bracken-Grissom, H. D., Ahyong, S., Wilkinson, R. D., Feldmann, R. M., Schweizer, C. E., Breinholt, J. W., et al. (2014). The emergence of lobsters: phylogenetic relationships, morphological evolution and divergence time comparisons of an ancient group (Decapoda: Achelata, Astacidea, Glypheidea, Polychelida). *Syst. Biol*. 63, 457–479. doi: 10.1093/sysbio/syu008

Cannas, R., Deiana, A. M., Salvadori, S., and Coluccia, E. (2004). Preliminary data on the karyology of *Panulirus regius* De Brito Capello, 1864 (Crustacea Decapoda). *Biol. Mar. Mediterr*. 11, 721–723.

Catania, S., Pidoux, A. L., and Allshire, R. C. (2015). Sequence features and transcriptional stalling within centromere DNA promote establishment of CENP-A chromatin. *PLoS Genet*. 11:e1004986. doi: 10.1371/journal.pgen.1004986

Chambers, C. A., Schell, M. S., and Skinner,. D. M. (1978). The primary sequence of a crustacean satellite containing a family of repeats. *Cell* 13, 97–110. doi: 10.1016/0092-8674(78)90141-1

Chu, C., Nielsen, R., and Wu, Y. (2016). REPdenovo: inferring *de novo* repeat motifs from short sequence reads. *PLoS One* 11:e0150719. doi: 10.1371/journal.pone.0150719

Coluccia, E., Cannas, R., Deiana, A. M., Milia, A., Salvadori, S., and Libertini, A. (1999). Genome size and A-T base content in five Palinuridae species (Crustacea Decapoda). *Biol. Mar. Mediterr*. 6, 688–691.

Coluccia, E., Cau, R. A., Cannas, R., Milia, A., Salvadori, S., and Deiana, A. M. (2001). Mitotic and meiotic chromosomes of the American lobster *Homarus americanus* (Nephropidae, Decapoda). *Hydrobiologia* 449, 149–152. doi: 10.1023/A:1017557523022

Coluccia, E., Salvadori, S., Cannas, R., Milia, A., and Deiana, A. M. (2005). First data on the karyology of *Palinurus gilchristi* (Crustacea Decapoda). *Biol. Mar. Mediterr*. 12, 661–663.

Crandall, K. A., and Buhay, J. E. (2008). Global diversity of crayfish (Astacidae, Cambaridae, and Parastacidae-Decapoda) in freshwater. *Hydrobiologia* 595, 295–301. doi: 10.1007/s10750-007-9120-3

Crandall, K. A., and De Grave, S. (2017). An updated classification of the freshwater crayfishes (Decapoda: Astacidea) of the world, with a complete species list. *J. Crust. Biol*. 37, 615–653. doi: 10.1093/jcbiol/rux070

Deiana, A. M., Cau, A., Cannas, R., Coluccia, E., and Salvadori, S. (2007). "Genetics of Slipper Lobsters," in *The Biology and Fisheries of the Slipper Lobster*, eds K. L. Lavalli and E. Spanier (Boca Raton, FL: CRC Press), 53–67.

Deiana, A. M., Coluccia, E., Milia, A., and Salvadori, S. (1996). Supernumerary chromosomes in *Nephrops norvegicus* L. (Crustacea, Decapoda). *Heredity* 76, 92–99. doi: 10.1038/hdy.1996.12

Diupotex Chong, M. E., Foster, N. R., and Zarate, L. A. (1997). A cytogenetic study of the crayfish *Procambarus digueti* (Bouvier, 1897) (Decapoda Cambaridae) from Lake Camecuaro, Michoacan, Mexico. *Crustaceana* 70, 875–885. doi: 10.1163/156854097X00492

Emadzade, K., Jang, T. S., Macas, J., Kovarík, A., Novák, P., Parker, J., et al. (2014). Differential amplification of satellite PaB6 in chromosomally hypervariable Prospero autumnale complex (Hyacinthaceae). *Ann. Bot*. 114, 1597–1608. doi: 10.1093/aob/mcu178

Eschscholtz, F. F. (1823). Descriptio novae *Astacorum speciei* Rossicae. *Mem Soc Imper Nat Moscu* 6, 109–110, 1 plate.

Fasten, N. (1914). Spermatogenesis of the American crayfish, *Cambarus virilis* and *C. immunis*, with special reference to synapsis and the chromatoid bodies. *J. Morphol*. 25, 587–649. doi: 10.1002/jmor.1050250403

Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol*. 3, 294–299.

Fu, L., Niu, B., Zhu, Z., Wu, S., and Li, W. (2012). CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics* 28, 3150–3152. doi: 10.1093/bioinformatics/bts565

Garrido-Ramos, M. A. (2015). Satellite DNA in plants: more than just rubbish. *Cytogenet. Genome Res*. 146, 153–170. doi: 10.1159/000437008

Garrido-Ramos, M. A. (2017). Satellite DNA: an evolving topic. *Genes*. 8:230. doi: 10.3390/genes8090230

González-Tizón, A. M., Rojo, V., Menini, E., Torrecilla, Z., and Martínez-Lage, A. (2013). Karyologycal analysis of the shrimp *Palaemon serratus* (Decapoda: Palaemonidae). *J. Crust. Biol.* 33, 843–848. doi: 10.1163/1937240X-00002185

Gregory, T. R. (2020). *Animal Genome Size Database*. Available online at: http://www.genomesize.com.

Gross, R., Kõiv, K., Pukk, L., and Kaldre, K. (2017). Development and characterization of novel tetranucleotide microsatellite markers in the noble crayfish (*Astacus astacus*) suitable for highly multiplexing and for detecting hybrids between the noble crayfish and narrow-clawed crayfish (*A. leptodactylus). Aquaculture* 472, 50–56. doi: 10.1016/j.aquaculture.2016.04.015

Gutekunst, J., Andriantsoa, R., Falckenhayn, C., Hanna, K., Stein, W., Rasamy, J., et al. (2018). Clonal genome evolution and rapid invasive spread of the marbled crayfish. *Nat. Ecol. Evol.* 2, 567–573. doi: 10.1038/s41559-018-0467-9

Hao, Z., Lv, D., Ge, Y., Shi, J., Weijers, D., Yu, G., and Chen, J. (2020). RIdeogram: drawing SVG graphics to visualize and map genome-wide data on the idiograms. *PeerJ Comput. Sci.* 6:e251. doi: 10.7717/peerj-cs.251

Hare, E. E., and Johnston, J. S. (2011). "Genome size determination using flow cytometry of propidium iodide-stained nuclei," in *Molecular Methods for Evolutionary Genetics*, *Methods in Molecular Biology*, eds V. Orgogozo and M. V. Rockman (Totowa, NJ: Humana Press), 3–12.

Heckmann, S., Macas, J., Kumke, K., Fuchs, J., Schubert, V., Ma, L., et al. (2013). The holocentric species Luzula elegans shows interplay between centromere and large-scale genome organization. *Plant J.* 73, 555–565. doi: 10.1111/tpj.12054

Holdich, D. M., Reynolds, J. D., Souty-Grosset, C., and Sibley, P. J. (2009). A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. *Knowl Manag. Aquat. Ecosyst.* 11, 394–395. doi: 10.1051/kmae/2009025

Ijdo, J. W., Wells, R. A., Baldini, A., and Reeders, S. T. (1991). Improved telomere detection using a telomere repeat probe (TTAGGG)n generated by PCR. *Nucleic Acids Res.* 19:4780. doi: 10.1093/nar/19.17.4780

Indy, J. R., Arias-Rodriguez, L., Páramo-Delgadillo, S., Hernández-Vidal, U., Álvarez-González, C. A., and Contreras-Sánchez, W. M. (2010). Mitotic karyotype of the tropical freshwater crayfish Procambarus (Austrocambarus) llamasi (Decapoda, Cambaridae). *Rev. Biol. Trop.* 58, 655–662. doi: 10.15517/rbt.v58i2.5236

Inkscape Project. (2020). *Inkscape*. Retrieved from: https://inkscape.org.

John, B., King, M., Schweizer, D., and Mendelak, M. (1985). Equilocality of heterochromatin distribution and heterochromatin heterogeneity in acridid grasshoppers. *Chromosoma* 91, 185–200. doi: 10.1007/BF00328216

Jurka, J., Klonowski, P., Dagman, V., and Pelton, P. (1996). CENSOR - a program for identification and elimination of repetitive elements from DNA sequences. *Comput. Chem.* 20, 119–121. doi: 10.1016/S0097-8485(96)80013-1

Katoh, K. and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Khoshkholgh, M., and Nazari, S. (2019). The genetic diversity and differentiation of narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823) (Decapoda: Astacidea: Astacidae) in the Caspian Sea Basin, Iran as determined with mitochondrial and microsatellite DNA markers. *J. Crust. Biol.* 2, 112–120. doi: 10.1093/jcbiol/ruy113

Komagata, N., and Komagata, K. (1992). Chromosome numbers in three species of Japanese fresh-water crayfishes. *Chromosome Inf. Serv.* 53, 15–16.

Kostyuk, V. S., Garbar, A. V., and Mezhzherin, S. V. (2013). Karyotypes and morphological variability of crayfish *Pontastacus leptodactylus* and *P. Angulosus* (Malacostraca, Decapoda). *Vestn. Zool.* 47, 11–16. doi: 10.2478/vzoo-2013-0020

Kouba, A., Petrusek, A., and Kozák, P. (2014). Continental-wide distribution of crayfish species in Europe: update and maps. *Knowl. Manag. Aquat. Ecosyst.* 413, 5. doi: 10.1051/kmae/2014007

Kuo, H. F., Olsen, K. M., and Richards, E. J. (2006). Natural variation in a subtelomeric region of Arabidopsis: implications for the genomic dynamics of a chromosome end. *Genetics* 173, 401–417. doi: 10.1534/genetics.105.055202

Liu, Z. L., Zhang, D., Hong, D. Y., and Wang, X. R. (2003). Chromosomal localization of 5S and 18S-5.8S-25S ribosomal DNA sites in five Asian pines using fluorescence in situ hybridization. *Theor. Appl. Genet.* 106, 198–204. doi: 10.1007/s00122-002-1024-z

Louis, E. J., and Vershinin, A. V. (2005). Chromosome ends: different sequences may provide conserved functions. *Bioessays* 27, 685–697. doi: 10.1002/bies.20259

Lukhtanov, V. A., and Iashenkova, Y. (2019). Linking karyotypes with DNA barcodes: proposal for a new standard in chromosomal analysis with an example based on the study of Neotropical Nymphalidae (Lepidoptera). *Comp. Cytogenet.* 13, 435–449. doi: 10.3897/CompCytogen.v13i4.48368

Macas, J., Kejnovský, E., Neumann, P., Novák, P., KoblíŽková, A., and Vyskot, B. (2011). Next generation sequencing-based analysis of repetitive DNA in the model dioecious plant Silene latifolia. *PLoS One* 6:e27335. doi: 10.1371/annotation/4ccaacb2-92d7-445a-87da-313cedf18feb

Maguire, I., and Dakić, L. (2011). Comparative analyses of *Astacus leptodactylus* morphological characteristics from Croatia and Armenia. *Biol. Sect. Zool.* 66, 491–498. doi: 10.2478/s11756-011-0045-y

Maguire, I., Podnar, M., Jelić, M., Štambuk, A., Schrimpf, A., Schulz, H., and Klobučar G (2014). Two distinct evolutionary lineages of the *Astacus leptodactylus* species-complex (Decapoda : Astacidae) inferred by phylogenetic analyses. *Invertebr. Syst.* 28, 117–123. doi: 10.1071/IS13030

Martin, P., Thonagel, S., and Scholtz, G. (2015). The parthenogenetic Marmorkrebs (Malacostraca: Decapoda: Cambaridae) is a triploid organism. *J. Zool. Syst. Evol. Res.* 54, 13–21. doi: 10.1111/jzs.12114

McFarlane, R. J., and Humphrey, T. C. (2010). A role for recombination in centromere function. *Trends Genet.* 26, 209–213. doi: 10.1016/j.tig.2010.02.005

Mehta, G. D., Agarwal, M. P., and Ghosh, S. K. (2010). Centromere identity: a challenge to be faced. *Mol. Genet. Genomics* 284, 75–94. doi: 10.1007/s00438-010-0553-4

Melters, D. P., Bradnam, K. R., Young, H. A., Telis, N., May, M. R., Ruby, J. G., et al. (2013). Comparative analysis of tandem repeats from hundreds of species reveals unique insights into centromere evolution. *Genome Biol.* 14, 1–20. doi: 10.1186/gb-2013-14-1-r10

Mlinarec, J., Chester, M., Siljak-Yakovlev, S., Papes, D., Leitch, A. R., and Besendorfer, V. (2009). Molecular structure and chromosome distribution of three repetitive DNA families in *Anemone hortensis* L. (Ranunculaceae). *Chromosome Res.* 17, 331–346. doi: 10.1007/s10577-009-9025-2

Mlinarec, J., Mužić, M., Pavlica, M., Šrut, M., Klobučar, G., and Maguire, I. (2011). Comparative karyotype investigations in the European crayfish *Astacus astacus* and *A. leptodactylus* (Decapoda, Astacidae). *Crustaceana* 84, 1497–1510. doi: 10.1163/156854011X607015

Mlinarec, J., Porupski, I., Maguire, I., and Klobučar, G. (2016). Comparative karyotype investigations in the white-clawed crayfish *Austropotamobius pallipes* (Lereboullet, 1858) species complex and stone crayfish *A. torrentium* (Schrank, 1803) (Decapoda: Astacidae). *J. Crustacean Biol.* 36, 87–93. doi: 10.1163/1937240X-00002390

Mlinarec, J., Skuhala, A., Jurković, A., Malenica, N., McCann, J., Weiss-Schneeweiss, H., et al. (2019). The repetitive DNA composition in the natural pesticide producer *Tanacetum cinerariifolium*: interindividual variation of subtelomeric tandem repeats. *Front. Plant Sci.* 10:613. doi: 10.3389/fpls.2019.00613

Molina, W. F., Costa, G., Cunha, I., Bertollo, L., Ezaz, T., Liehr, T., et al. (2020). Molecular cytogenetic analysis in freshwater prawns of the genus *Macrobrachium* (Crustacea: Decapoda: Palaemonidae). *Int. J. Mol. Sci.* 21:2599. doi: 10.3390/ijms21072599

Monat, C., Tando, N., Tranchant-Dubreuil, C., and Sabot, F. (2016). LTRclassifier: a website for fast structural LTR retrotransposons classification in plants. *Mob. Genet. Elements* 6:6. doi: 10.1080/2159256X.2016.1241050

Nguyen, P., Sahara, K., Yoshido, A., and Marec, F. (2010). Evolutionary dynamics of rDNA clusters on chromosomes of moths and butterflies (Lepidoptera). *Genetica* 138, 343–354. doi: 10.1007/s10709-009-9424-5

Niiyama, H. (1962). On the unprecedently large number of chromosomes of the crayfish *Astacus trowbridgii* Stimpson. *Annot. Zool. Japon.* 35, 229–233.

Njegovan, V., Orešković, M., Hudina, S., and Maguire, I. (2017). Crayfish fauna of the Maksimir lakes in the urban area of Zagreb. *Freshwater Crayfish* 23, 1–11 doi: 10.5869/fc.2017.v23-1.1

Novák, P., Ávila Robledillo, L., KoblíŽková, A., Vrbová, I., Neumann, P., and Macas, J. (2017). TAREAN: a computational tool for identification and characterization of satellite DNA from unassembled short reads. *Nucleic Acids Res.* 45:e111. doi: 10.1093/nar/gkx257

Novák, P., Guignard, M. S., Neumann, P., Kelly, L. J., Mlinarec, J., KoblíŽková, A., et al. (2020a). Repeat sequence turnover shifts fundamentally in species with large genomes. *Nature Plants* 6, 1325–1329. doi: 10.1038/s41477-020-00785-x

Novák, P., Neumann, P., and Macas, J. (2010). Graph-based clustering and characterization of repetitive sequences in next-generation sequencing data. *BMC Bioinform*. 11:378. doi: 10.1186/1471-2105-11-378

Novák, P., Neumann, P., and Macas, J. (2020b). Global analysis of repetitive DNA from unassembled sequence reads using RepeatExplorer2. *Nat. Protoc.* 15, 3745–3776. doi: 10.1038/s41596-020-0400-y

Novák, P., Neumann, P., Pech, J., Steinhaisl, J., and Macas, J. (2013). RepeatExplorer: a Galaxy-based web server for genome-wide characterization of eukaryotic repetitive elements from next-generation sequence reads. *Bioinformatics* 29, 792–793. doi: 10.1093/bioinformatics/btt054

Palomeque, T., and Lorite, P. (2008). Satellite DNA in insects: a review. *Heredity* 100, 564–573. doi: 10.1038/hdy.2008.24

Pamponét, V. C. C., Souza, M. M., Gonçalo, S. S., Micheli, F., Ferreira de Melo, C. A., Gomes de Oliveira, S., et al. (2019). Low coverage sequencing for repetitive DNA analysis in Passiflora edulis Sims: citogenomic characterization of transposable elements and satellite DNA. *BMC Genomics* 20:262. doi: 10.1186/s12864-019-5576-6

Pedrosa-Harand, A., de Almeida, C. C., Mosiolek, M., Blair, M. W., Schweizer, D., and Guerra, M. (2006). Extensive ribosomal DNA amplification during Andean common bean (Phaseolus vulgaris L.) evolution. *Theor. Appl. Genet.* 112, 924–933. doi: 10.1007/s00122-005-0196-8

Pereira, C. S. A., Aboim, M. A., Ráb, P., and Collares-Pereira, M. J. (2013). Introgressive hybridization as a promoter of genome reshuffling in natural homoploid fish hybrids (Cyprinidae, Leuciscinae). *Heredity* 112, 343–350. doi: 10.1038/hdy.2013.110

Pita, S., Mora, P., Vela, J., Palomeque, T., Sánchez, A., Panzera, F., et al. (2018). Comparative analysis of repetitive DNA between the main vectors of chagas disease: *Triatoma infestans* and *Rhodnius prolixus*. *Int. J. Mol. Sci.* 19:1277. doi: 10.3390/ijms19051277

Plohl, M., Luchetti, A., Mestrović, N., and Mantovani, B. (2008). Satellite DNAs between selfishness and functionality: structure, genomics and evolution of tandem repeats in centromeric (hetero)chromatin. *Gene* 409, 72–82. doi: 10.1016/j.gene.2007.11.013

Plohl, M., Meštrović, N., and Mravinac, B. (2014). Centromere identity from the DNA point of view. *Chromosoma* 123, 313–325. doi: 10.1007/s00412-014-0462-0

R Core Team. (2016). *R: A Language and Environment for Statistical Computing*. Vienna. Retrieved from: https://www.R-project.org/.

Riethman, H., Ambrosini, A., and Paul, S. (2005). Human subtelomere structure and variation. *Chromosome Res.* 13, 505–515. doi: 10.1007/s10577-005-0998-1

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., et al. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Mol. Biol. Evol.* 34, 3299–3302. doi: 10.1093/molbev/msx248

Ruiz-Herrera, A., Nergadze, S. G., Santagostino, M., and Giulotto, E. (2008). Telomeric repeats far from the ends: mechanisms of origin and role in evolution. *Cytogenet. Genome Res.* 122, 219–228. doi: 10.1159/000167807

Ruiz-Ruano, F. J., Navarro-Domínguez, B., Camacho, J., and Garrido-Ramos, M. A. (2019). Characterization of the satellitome in lower vascular plants: the case of the endangered fern *Vandenboschia speciosa*. *Ann. Bot.* 123, 587–599. doi: 10.1093/aob/mcy192

Sakamoto, Y., and Zacaro, A. A. (2009). *LEVAN, An ImajeJ Plugin for Morphological Cytogenetic Analysis of Mitotic and Meiotic Chromosomes*. Initial version. An Open Source Java Plugin Distributed Over the Internet from: http://rsbweb.nih.gov/ij/.

Salvadori, S., Coluccia, E., Deidda, F., Cau, A., Cannas, R., and Deiana, A. M. (2012). Comparative cytogenetics in four species of Palinuridae: B chromosomes, ribosomal genes and telomeric sequences. *Genetica* 140, 429–437. doi: 10.1007/s10709-012-9691-4

Salvadori, S., Coluccia, E., Deidda, F., Cau, A., Cannas, R., Lobina, C., et al. (2014). Karyotype, ribosomal genes, and telomeric sequences in the crayfish *Procambarus clarkii* (Decapoda: Cambaridae). *J. Crust. Biol.* 34, 525–531. doi: 10.1163/1937240X-00002247

Salvadori, S., Coluccia, E., Milia, A., Cannas, R., and Deiana,. A. M. (2002). Study of mitotic and meiotic chromosomes of *Homarus gammarus* (Crustacea Decapoda). *Biol. Mar. Mediterr.* 9, 889–891.

Scalici, M., Solano, E., and Gibertini, G. (2010). Karyological analyses on the Australian crayfish Cherax destructor (Decapoda: Parastacidae). *J. Crust. Biol.* 30, 528–530. doi: 10.1651/09-3200.1

Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 9, 671–675. doi: 10.1038/nmeth.2089

Sember, A., Bohlen, J., Šlechtová, V., Altmanová, M., Symonová, R., and Ráb, P. (2015). Karyotype differentiation in 19 species of river loach fishes (Nemacheilidae, Teleostei): extensive variability associated with rDNA and heterochromatin distribution and its phylogenetic and ecological interpretation. *BMC Evol. Biol.* 15:251. doi: 10.1186/s12862-015-0532-9

Tan, M. H., Gan, H. M., Lee, Y. P., Bracken-Grissom, H., Chan, T. Y., Miller, A. D., et al. (2019). Comparative mitogenomics of the Decapoda reveals evolutionary heterogeneity in architecture and composition. *Sci. Rep.* 9:3617. doi: 10.1038/s41598-019-47145-0

Tan, M. H., Gan, H. M., Lee, Y. P., Grandjean, F., Croft, L. J., and Austin, C. M. (2020). A giant genome for a giant crayfish (*Cherax quadricarinatus*) with insights into cox1 *Pseudogenes* in decapod genomes. *Front. Genet.* 11:201. doi: 10.3389/fgene.2020.00201

Tan, X., Qin, J. G., Chen, B., Chen, L., and Li, X. (2004). Karyological analyses on red-claw crayfish *Cherax quadricarinatus* (Decapoda: Parastacidae). *Aquaculture* 234, 65–76. doi: 10.1016/j.aquaculture.2003.12.020

Tek, A. L., and Jiang, J. (2004). The centromeric regions of potato chromosomes contain megabase-sized tandem arrays of telomere-similar sequence. *Chromosoma* 113, 77–83. doi: 10.1007/s00412-004-0297-1

Torrecilla, Z., Martínez-Lage, A., Perina, A., González-Ortegón, E., and González-Tizón, A. M. (2017). Comparative cytogenetic analysis of marine *Palaemon* species reveals a X1X1X2X2/X1X2Y sex chromosome system in Palaemon elegans. *Front. Zool.* 14:47. doi: 10.1186/s12983-017-0233-x

Torres-Ruiz, R. A., and Hemleben, V. (1994). Pattern and degree of methylation in ribosomal RNA genes of *Cucurbita pepo* L. *Plant Mol. Biol.* 26, 1167–1179. doi: 10.1007/BF00040697

Utsunomia, R., Silva, D. M. Z. A., Ruiz-Ruano, F. J., Goes, C. A. G., Melo, S., Ramos, L. P., et al. (2019). Satellitome landscape analysis of *Megaleporinus macrocephalus* (Teleostei, Anostomidae) reveals intense accumulation of satellite sequences on the heteromorphic sex chromosome. *Sci. Rep.* 9:5856. doi: 10.1038/s41598-019-42383-8

Van Quyen, D., Gan, H. M., Lee, Y. P., Nguyena, D. D., Nguyena, T. H., Tran, X. T., et al. (2020). Improved genomic resources for the black tiger prawn (Penaeus monodon). *Marine Genomics* 52:100751. doi: 10.1016/j.margen.2020.100751

Vershinina, A., Anokhin, B., and Lukhtanov, V. (2015). Ribosomal DNA clusters and telomeric (TTAGG)n repeats in blue butterflies (Lepidoptera, Lycaenidae) with low and high chromosome numbers. *Comp. Cytogenet.* 9, 161–171. doi: 10.3897/CompCytogen.v9i2.4715

Vítková, M., Král, J., Traut, W., Zrzavý, J., and Marec, F. (2005). The evolutionary origin of insect telomeric repeats, $(TTAGG)_n$. *Chromosome Res.* 13, 145–156. doi: 10.1007/s10577-005-7721-0

Vlahović, I., Glunčić, M., Rosandić, M., Ugarković, Đ*., and Paar, V. (2017). Regular higher order repeat structures in beetle *Tribolium castaneum* genome. *Genome Biol. Evol.* 9, 2668–2680. doi: 10.1093/gbe/evw174

Wang, G., Zhang, X., and Jin, W. (2009). An overview of plant centromeres. *J. Genet. Genomics* 36, 529–537. doi: 10.1016/S1673-8527(08)60144-7

Wang, S. Y., Biesiot, P. M., and Skinner, D. M. (1999). Toward an understanding of satellite DNA function in Crustacea. *Amer. Zool.* 39, 471–486.

Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M., and Barton, G. J. (2009). Jalview Version 2-a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25, 1189–1191. doi: 10.1093/bioinformatics/btp033

Wei, K. H., Grenier, J. K., Barbash, D. A., and Clark, A. G. (2014). Correlated variation and population differentiation in satellite DNA abundance among lines of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 111, 18793–18798. doi: 10.1073/pnas.1421951112

Weiss-Schneeweiss, H., Leitch, A. R., McCann, J., Jang, T. S., and Macas, J. (2015). "Chapter 5: Employing next generation sequencing to explore the repeat landscape of the plant genome," in *Next-Generation Sequencing in Plant Systematics*, eds E. Hörandl and M. S. Appelhans (Washington, DC: IAPT), 1–25.

Wicker, T., Sabot, F., Hua-Van, A., Bennetzen, J., Capy, P., Chalhoub, B., et al. (2007). A unified classification system for eukaryotic transposable elements. *Nat. Rev. Genet.* 8, 973–982. doi: 10.1038/nrg2165

Yuan, Z., Zhou, T., Bao, L., Liu, S., Shi, H., Yang, Y., et al. (2018). The annotation of repetitive elements in the genome of channel catfish (*Ictalurus punctatus*). *PLoS One* 13:e0197371. doi: 10.1371/journal.pone.0197371

Zhang, X., Yuan, J., Sun, Y., Li, S., Gao, Y., Yu, Y., et al. (2019). Penaeid shrimp genome provides insights into benthic adaptation and frequent molting. *Nat. Commun.* 10:356. doi: 10.1038/s41467-018-08197-4

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 10 February 2021
doi: 10.3389/fevo.2021.581247

Force and Boldness: Cumulative Assets of a Successful Crayfish Invader

Lucian Pârvulescu[1], Dan Ioan Stoia[2], Kristian Miok[3], Mihaela Constanţa Ion[4,5], Adela Estera Puha[1], Melania Sterie[1], Mihajel Vereş[1], Ioan Marcu[6], Mirela Danina Muntean[7] and Oana Maria Aburel[7]*

[1] Department of Biology and Chemistry, Faculty of Chemistry, Biology, Geography, West University of Timisoara, Timisoara, Romania, [2] Department of Mechanics and Strength of Materials, Politehnica University of Timisoara, Timisoara, Romania, [3] Department of Informatics, Faculty of Mathematics and Computer Science, West University of Timisoara, Timisoara, Romania, [4] Institute of Biology Bucharest, Romanian Academy, Bucharest, Romania, [5] Faculty of Biology, University of Bucharest, Bucharest, Romania, [6] Department of Genetics, Evolution and Environment, University College London, London, United Kingdom, [7] Department of Functional Sciences and Pathophysiology, Center for Translational Research and Systems Medicine, "Victor Babeş" University of Medicine and Pharmacy, Timisoara, Romania

Multiple causes can determine the disturbance of natural equilibrium in a population of a species, with a common one being the presence of invasive competitors. Invasives can drive native species to the resettlement of the trophic position, changing reproduction strategies or even daily normal behaviours. Here, we investigated the hypothesis that more effective anatomical features of an intruder (*Faxonius limosus*) come with increased boldness behaviour, contributing to their invasion success in competition against the native species (*Pontastacus leptodactylus*). We tested the boldness of specimens representing the two species by video-based assessment of crayfish individuals' attempts to leave their settlement microenvironment. The experiment was followed by a series of measurements concerning chelae biometry, force and muscle energetics. The native species was less expressive in terms of boldness even if it had larger chelae and better muscular tissue performance. In contrast, because of better biomechanical construction of the chelae, the invasive species was capable of twice superior force achievements, which expectedly explained its bolder behaviour. These findings suggest that, in interspecific agonistic interactions, the behaviour strategy of the invasive crayfish species is based on sheer physical superiority, whereas the native crayfish relies on intimidation display.

Keywords: behaviour, competition, *Faxonius limosus*, invasive species, *Pontastacus leptodactylus*

OPEN ACCESS

Edited by:
Japo Jussila,
University of Eastern Finland, Finland

Reviewed by:
Pierre Horwitz,
Edith Cowan University, Australia
Marco Gamba,
University of Turin, Italy

****Correspondence:***
Lucian Pârvulescu
lucian.parvulescu@e-uvt.ro
orcid.org/0000-0002-1528-1429

Specialty section:
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *08 July 2020*
Accepted: *20 January 2021*
Published: *10 February 2021*

Citation:
Pârvulescu L, Stoia DI, Miok K, Ion MC, Puha AE, Sterie M, Vereş M, Marcu I, Muntean MD and Aburel OM (2021) Force and Boldness: Cumulative Assets of a Successful Crayfish Invader. Front. Ecol. Evol. 9:581247. doi: 10.3389/fevo.2021.581247

INTRODUCTION

Biological invasions are one of the most challenging issues for nowadays conservationists (Nentwig et al., 2018; Flood et al., 2020). The successful establishment of alien species in a new environment is generally driven by intrinsic mechanisms (Sullivan et al., 2017; Pacioglu et al., 2020) and extrinsic pathways (Light, 2003; Essl et al., 2015). Multiple perspectives have been approached to understand and depict the processes behind successful invasions, such as ecological (Lodge, 1993; South et al., 2020), economical (Perrings et al., 2002), or pathological (Strauss et al., 2012) ones. Eco-behavioural perspectives might allow more insights into the complex process of biological invasions. Here, we

addressed the hypothesis that a successful crayfish invader is driven by its own bolder behaviour against shyness of the native resident. To test this, we used behaviour, morpho-mechanical and bioenergetic approaches.

Boldness, considered as the propensity of an animal to engage in risky activities (White et al., 2013), is giving the animals a presumed confidence against the unknown, associated with high diel activity and exploration initiatives (Chapple et al., 2012; Juette et al., 2014). The expression of boldness in agonistic confrontation is aggressivity and bite force superiority (Usio et al., 2001; De Meyer et al., 2019), and this behaviour was found associated with successful biological invasions (Ferrari et al., 2018). Analysing behavioural interactions between invasive and native residents may provide useful information for understanding the mechanisms behind the spreading success of invasives (Carere and Gherardi, 2013).

Relatively equal in body size (Richardson, 2019), the native narrow-clawed crayfish *Pontastacus leptodactylus* Eschscholtz 1823 and the invasive spiny-cheek crayfish *Faxonius limosus* (Rafinesque, 1817) are two crayfish species with a long interaction history in Europe (Holdich et al., 2009). The native species is a typical K-strategist, whereas the invasive crayfish shows characteristics of r-strategists, demonstrating plasticity and significant improvement of reproduction (i.e., increased fecundity) in the active front of invasion (Pârvulescu et al., 2015). Experimentally, it was demonstrated that the invasive species dominates the native one, even in highly imbalanced confrontations (e.g., smaller invasive vs. larger native specimens) for food and shelter (Lele and Pârvulescu, 2017). Crayfish chelae are used for gripping (Ion et al., 2020) and pinching (van der Meijden et al., 2010), or showing sheer superiority (Graham and Angilletta, 2020), hence are important in both intra- and interspecific interactions. Prolonged competition either drove the native species to local extinction or resource partitioning between species; recent investigations point to natural selection of heavier (i.e., stronger and combative) specimens in recovering populations after the invasion is established, compared to the non-invaded ones (Haubrock et al., 2019; Pacioglu et al., 2020).

In this study, we aimed to dig inside the bio-mechanical mechanisms behind a confrontation between two crayfish species, testing the hypothesis that more effective chela weapons came with increased boldness behaviour of the invasive species compared to a native one. The invasive *F. limosus* and the native *P. leptodactylus* were used as a models for multiple comparisons, including muscle metabolic capacity and fundamental for chelae function (Guderley and Couture, 2005).

MATERIALS AND METHODS

Study Specimens and Morphological Measurements

To avoid biases caused by any sexual influences during the experiment, this study involved intermolt adult males only, collected from the most recent known invasion front in the Lower Danube (near Orşova, Romania, GPS 44.689064°N/22.500242°E), where both investigated species have been coexisting for at least 5 years (Pârvulescu et al., 2015; Pacioglu et al., 2020). This study omitted those specimens injured or showing obvious discrepancies between the left and the right chelae.

The geometric dimensions of the specimens were then acquired using a digital calliper of 0.1-mm accuracy. A total of nine dimensions were measured for every individual: the total length of the crayfish [TL (mm)], chela length, thickness and width [CL, CT, and CW (mm)] for left and right cheliped and also the length of the movable finger (dactylus, DL). Using the three dimensions of the chela, its volume was computed (CV) as an approximated elliptical cylinder, were the ellipse semi-axes were the half of the chela length and height, while the height of the cylinder was the chela width.

After measurements, each specimens' chelae were detached for further investigation. The muscle of the propodus was extracted and oven-dried for 48 h at 60°C to obtain the dry muscle mass [DMM (g)]. The chela (propodus and dactylus) cuticle was also collected and dried in the same setup, obtaining the dry cuticle, and dry dactylus mass [DCM and DDM (g)]. These variables were measured by using a precision balance (Kern & Sohn GmbH, Balingen, Germany) of 0.001-g accuracy.

Experimental Set-Up for Boldness Assessment

In total, 73 specimens of *P. leptodactylus* and 78 of *F. limosus* were involved in the experiments. Aiming to obtain best results on the assessment of boldness behaviour, we acclimated crayfish prior to the experiments in the experimental arena (20-L water tank), for each trial, with *ad libitum* food and shelter but lacking the ramp (described below). The arena was placed in a second (larger) tank (60 L) to retain the successfully escaped individuals. Both tanks were equipped with a submersed filtering pump for cleaning and oxygenating water. A ramp, facilitating the escape from the inside of the arena, was built from adherent (ceramic) material inclined at 30°, with a length of 45 cm, with half of this length being submersed and half dry, making the crossing conditions more difficult. Such an aerial ramp simulates an environment that is potentially risky, more prone to predatory attacks; hence, approaching and crossing it by the crayfish can be used as a measure of the individual's boldness. For a better understanding of the experimental setup, we refer readers to a video sample in the **Supplementary Material**. This set-up was placed into the area on the third day of the settlement period, allowing the individuals to freely explore the ramp and opening the way to a new environment in the larger tank. The experiment started once the ramp was inserted and lasted 24 h for each trial and was video recorded. The ethogram for each specimen consisted of the binary variable (0/1) of successful attempts to escape (further referred to as "sa" variable) in both day, and night, and, if the case, the duration (s) of the *sa* (time completed ramp crossing, referred as "tcrs" variable). To avoid any bias in crayfish activity caused by temperature (Seebacher and Wilson, 2006; Zhao and Feng, 2015), we maintained the environmental temperature for all the experiments at ∼20°C.

To obtain the first insights into the differences between the two crayfish species' behaviour, exploratory data analysis was performed. We investigated if there was a difference between the *sa* of the two species. Crayfish individuals that have completed the crossing action were selected and their *tcrs* were compared using the non-parametric Mann-Whitney *U* test. In the same respect, we tested if the TL of the analysed crayfish influenced the crossing success, using a logistic regression model.

Force Measurement Device and Procedure

Pinching force measurements were conducted *in vivo* using a 1,400 g/0.1 g loading cell custom-made for measuring the pressure exerted by the tip of the crayfish chela. The transducer was connected to an Arduino Uno board, and the data were acquired using a sampling frequency of 6 Hz. On the transducer, two rigid metallic levers were attached for providing pinching elements for the chelae. To convert the electrical parameter of the transducer (mV) in mechanical force (N), the device calibration was conducted using five different weights: 100, 250, 500, 750, and 1,000 g, reading the corresponding voltage values. For measurement consistency, the transducer's levers were placed at the tips of the chelae of all individuals. Also, the longitudinal direction of the chela and the levers were always as collinear as possible. The time span of the measurement was an average of 60 s for each investigated chela (a.k.a. claw).

The same 151 (73 *P. leptodactylus* and 78 *F. limosus*) crayfish male specimens used for previous experiments were used. While measuring the force values, some crayfish demonstrated high activity, generating several pinching cycles in the recorded time interval. On the other hand, some individuals manifested less activity and, therefore, fewer pinching cycles or just one was acquired. To obtain a representative biomechanical behaviour for each individual, the cycles were isolated and processed (**Figure 1**).

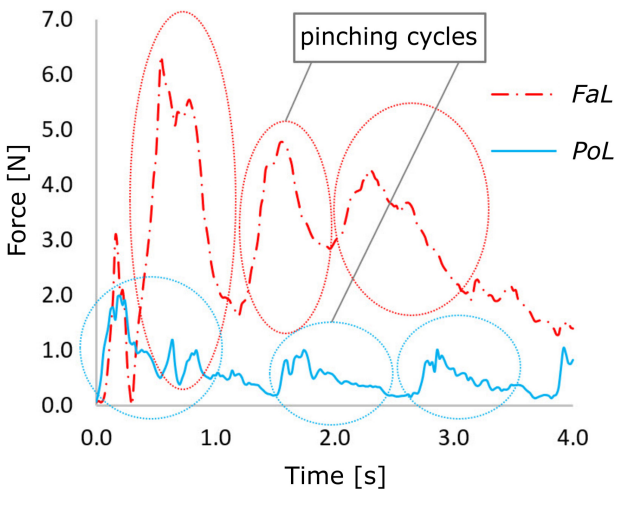

FIGURE 1 | Pinching force cycles of two representative individuals of *Pontastacus leptodactylus* and *Faxonius limosus*.

Based on the pinching cycles, the absolute maximum force [F_{max} (N)] was extracted for each crayfish of both species and used in raw data graphic representation. After extracting the pinching cycles from all active individuals, the force-time series were averaged for each single crayfish. Three parameters were directly extracted: average of maximum forces [F_{Av} (N)], average contraction time [C_t (s)], and average releasing time [R_t (s)]. In addition, average loading velocity [LV (N/s)], average releasing velocity [RV (N/s)], and the energy of the pinching cycle [EN (N·s)] were computed. The LV represents the speed of the closing movement from zero force to maximum force and was computed as a numerical derivative of force in respect to time, using Eq. (1). A similar equation was used for computing RV, which represents the speed of releasing the grip from the maximum force value to zero or to an inflexion point from where the force increases again. The EN is an integrative parameter of force in respect to time and characterises the mechanical effort spent to complete a pinching cycle. It was computed by numerical integration using Eq. (2), were the two terms under the sum represent the area under the curve split in two elemental components of the area: a rectangle and a triangle. Here, Δt represents the time interval according to the sampling rate and F_{i+1} and F_i represent consecutive values of instantaneous force.

$$LV = \frac{F_{k+1} - F_k}{t_{k+1} - t_k} \; [N/s] \tag{1}$$

$$EN = \sum_{i=1}^{n} \left(F_i \cdot \Delta t + \frac{(F_{i+1} - F_i) \cdot \Delta t}{2} \right) \; [N \cdot s] \tag{2}$$

Relationship Between Chelae Forces and Body Properties

To obtain the relationship between the body properties and the forces that crayfish produce, various multiple linear regression architectures were tested. The data variables considered as a continuous model outcome were as follows: F_{Av}, EN, LV, RV, R_t, and C_t, and the predictive variables were modelled in two groups: based on the body size (TL, CL, CT, CW, DL, and CV) and based on the chelae weight (DMM, DCM, and DDM). Because crayfish are ambidextrous in the usage of chelae (Lele and Pârvulescu, 2019), we chose to group the associated data to perform the statistical analyses.

Due to the large multicollinearity among the predictive variables, stepwise regression was performed to remove the redundant ones. The predictors that were found to be significant based on the 0.05 cut-off in the final models were interpreted as those that have an effect on the specific outcome. Finally, the *t*-test was applied to investigate the difference between the two species for the biometric variables that were significant in the stepwise regression model describing the chelae forces outcomes at least for one of the species.

Mitochondrial High-Resolution Respirometry (HRR) Analysis

Aerobic metabolic capacity was measured as oxygen consumption of isolated mitochondria in the two groups of

crayfish, the native crayfish group (*P. leptodactylus*, $N = 6$) and the invasive crayfish group (*F. limosus*, $N = 7$). Mitochondria were isolated by differential centrifugation at 4°C, according to a previously described method (Liu et al., 2013). Briefly, 1.5–2.5 g of muscle collected from the crayfish chela was cut into small pieces and homogenised in 30 mL isolation buffer (320 mM Sucrose, 10 mM Tris, 10 mM EGTA, 0.5% BSA, pH 7.3), using a tissue homogeniser (Glas-Col 099C K5424 CE). Homogenisation was performed at minimal speed (two 40 s periods/1 min interval), and the tissue homogenate was further centrifuged (Rotina centrifuge 38R) for 10 min at 2,000 *g*; the supernatant was centrifuged for 10 min at 12,000 *g*. Finally, the resulting mitochondrial rich pellet was re-suspended in 0.25 mL of isolation buffer in a glass homogeniser, kept on ice and used within 4 h in respiratory rate measurements. The mitochondrial protein concentration was further evaluated according to the Biuret method (Gornall et al., 1949).

Oxygen consumption was measured at 37°C with the Oxygraph-2k (Oroboros Instruments, Austria). The mitochondria (0.1 mg protein/mL) were incubated in 2 mL of incubation medium containing 0.5 mM EGTA, 3 mM MgCl2.6H2O, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH2P04, 20 mM HEPES, 110 mM sucrose, 1 g/L BSA, essentially fatty acid free + 280 U/mL catalase lyophilised powder, 2,000–5,000 units/mg protein (pH 7.1, 37°C). The Substrate-Uncoupler-Inhibitor Titration (SUIT) protocol adapted from Duicu et al. (2013) was as follows: chamber A: GMSTATE 2 + ADPOXPHOS + cyt c + OmySTATE 4 + FCCPUncoupled state + AmaROX and chamber B: S(Rot)STATE 2 + ADPOXPHOS + cyt c + OmySTATE 4 + FCCP Uncoupled state + AmaROX. The protocol consisted of the following steps and the corresponding respiratory states: (i) addition of 10 mM glutamate/G and 2 mM malate/M (CI substrates) and 10 mM succinate/S (CII substrate) + 0.5 μM rotenone/Rot (CI inhibitor): STATE 2 (i.e., basal respiration); (ii) addition of 5 mM ADP as a measure of the maximal oxidative phosphorylation capacity: OXPHOS capacity; (iii) addition of 10 μM cytochrome c/cyt c to assess the intactness of the outer mitochondrial membrane; (iv) inhibition of ATP synthase by oligomycin/Omy (2 μg/ml): STATE 4 (i.e., basal respiration); (v) FCCP titration (0.5 μM steps), to obtain the Uncoupled respiration, (vi) inhibition of respiration with 2.5 μM antimycin A/Ama (Residual OXygen consumption): ROX state.

For high-resolution respirometry (HRR) experiments, the independent two-side Mann-Whitney *U* test was applied. Significance was tested based on the 0.05 cut-off, i.e., if the *p*-value was less than 0.05, the null hypothesis of two groups being equal was rejected. Also, the means and standard deviations (mean ± SD) are reported.

TABLE 1 | Results of the stepwise regression on the body size variables.

	Estimate	Std. Error	*t*-value	*p*-value
Size variables vs. F_{Av} for *P. leptodactylus*				
Intercept	−0.070	1.216	−0.058	0.954
TL	0.04030	0.01866	2.160	0.0324*
CL	−0.03219	0.02275	−1.415	0.1592
Size variables vs. F_{Av} for *F. limosus*				
Intercept	3.9636	1.0366	3.824	0.000199***
CL	−0.1394	0.0591	−2.358	0.019789*
CW	0.4534	0.2084	2.176	0.031275*
Size variables vs. EN for *P. leptodactylus*				
Intercept	2.9033	2.3273	0.127	0.214
CT	0.4131	0.2526	1.636	0.104
Size variables vs. EN for *F. limosus*				
Intercept	18.5249	6.4656	2.865	0.004831**
CL	−2.5244	0.6493	−3.888	0.000158***
CW	4.1484	1.3105	3.166	0.001911**
DL	1.7364	1.0246	1.695	0.09243
Size variables vs. LV for *P. leptodactylus*				
Intercept	−1.33979	1.71410	−0.782	0.43569
TL	0.08027	0.02629	3.053	0.00269**
CL	−0.10313	0.03206	−3.217	0.00159**
Size variables vs. LV for *F. limosus*				
Intercept	4.67336	2.57977	1.812	0.0722
TL	−0.07455	0.04656	−1.601	0.1116
CW	0.43343	0.18527	2.339	0.0208*
Size variables vs. RV for *P. leptodactylus*				
Intercept	−0.58568	1.19834	−0.489	0.62575
TL	0.05430	0.01838	2.954	0.00366**
CL	−0.06978	0.02241	−3.113	0.00222**
Size variables vs. RV for *F. limosus*				
Intercept	2.6732	1.4904	1.794	0.0751
CW	0.4408	0.2787	1.582	0.1160
DL	−0.2266	0.1363	−1.663	0.0986
Size variables vs. C_t for *P. leptodactylus*				
Intercept	1.17833	0.18905	6.233	$4.59e-09$***
CW	0.04688	0.02155	2.176	0.0312*
DL	−0.01928	0.01052	−1.832	0.0690
Size variables vs. C_t for *F. limosus*				
Intercept	1.58713	0.36130	4.393	$2.22e-05$***
CL	−0.03147	0.01382	−2.278	0.0243*
CT	0.13849	0.08555	1.619	0.1078

*TL, total length; CL, chela length; CT, chela thickness; CW, chela width; DL, dactylus length; and force outcome modelling (F_{Av}, maximum force; C_t, contraction time; R_t, releasing time; LV, loading velocity; RV, releasing velocity; EN, energy of the pinching cycle) for the two investigated crayfish species. *Borderline significant ($0.01 < p \leq 0.05$). **Medium significant ($0.001 < p \leq 0.01$). ***Very significant ($p \leq 0.001$).*

RESULTS

Relationship Between Chelae Forces and Body Properties

After applying the stepwise regression for *P. leptodactylus* (**Table 1**), only TL had a significant influence on the F_{Av} outcome. Regarding the outcome EN, no predictor was significant. The TL and CL were significant for the LV outcome. The same predictors, TL and CL, had a significant effect on the RV outcome. A significant relationship with the outcome C_t was found for CW. No significant relationships were found between chela weight and force variables (**Table 2**).

The results for *F. limosus* (**Tables 1**, **2**) obtained after stepwise regression indicate that CL CW, DMM, and DCM have a significant influence on the F_{Av} outcome. For the EN, the same

TABLE 2 | Results of the stepwise regression on the chelae weight.

	Estimate	Std. Error	*t*-value	*p*-value
Chelae weight variables vs. F_{Av} for *P. leptodactylus*				
Intercept	2.6173	0.149	17.46	2e-16***
Chelae weight variables vs. F_{Av} for *F. limosus*				
Intercept	4.4937	0.5227	8.597	1.66e − 14***
DMM	21.99	10.5906	2.076	0.0397*
DCM	−6.7442	3.092	−2.181	0.0309*
Chelae weight variables vs. EN for *P. leptodactylus*				
Intercept	4.878	1.255	3.886	0.000153**
DDM	7.772	5.089	1.527	0.1288
Chelae weight variables vs. EN for *F. limosus*				
Intercept	17.941	3.282	5.466	2.13e − 07***
DMM	261.231	67.795	3.853	0.000179***
DCM	−201.592	81.045	−2.487	0.014077*
DDM	264.755	178.968	1.479	0.141362
Chelae weight variables vs. LV for *P. leptodactylus*				
Intercept	2.0754	0.2151	9.648	2e-16***
Chelae weight variables vs. LV for *F. limosus*				
Intercept	2.6810	0.6674	4.017	9.65e − 05***
DCM	2.3155	1.3734	1.686	0.0941
Chelae weight variables vs. RV for *P. leptodactylus*				
Intercept	1.7238	0.1501	11.49	2e-16***
Chelae weight variables vs. RV for *F. limosus*				
Intercept	3.6171	0.2554	14.16	2e-16***
Chelae weight variables vs. C_t for *P. leptodactylus*				
Intercept	1.2569	0.1078	11.664	2e-16***
DCM	−3.2366	1.9911	−1.626	0.1062
Chelae weight variables vs. C_t for *F. limosus*				
Intercept	1.6098	0.1663	9.681	2e-16***
DMM	7.0442	3.3693	2.091	0.0384*
DCM	−2.4522	0.9837	−2.493	0.0139*

*DMM, dry muscle mass; DCM, dry cuticle mass; DDM, dry dactylus mass; and force outcome modelling (F_{Av}, maximum force; C_t, contraction time; R_t, releasing time; LV, loading velocity; RV, releasing velocity; EN, energy of the pinching cycle) for the two investigated crayfish species. *Borderline significant ($0.01 < p \leq 0.05$). **Medium significant ($0.001 < p \leq 0.01$). ***Very significant ($p \leq 0.001$).*

predictors were significant. The CW was significantly related to outcome LV. For the outcome RV, no predictor was significant, whereas for outcome C_t, the variables CL, DMM, and DCM were significant.

To understand the differences in the results obtained for the two investigated species, the body variables that were significant (TL, CL, CW, DMM, and DCM) for at least one of the force variables were compared using the two-sample *t*-test, as all of the variables are normally distributed. Testing the one-sided hypothesis that the mean values of *P. leptodactylus* are greater than those of *F. limosus,* the values for TL, CL, CW, DMM, and DCM were significant. To visualise those significant differences, we refer readers to **Figures 2A–E**.

Boldness Assessment

Of all tested specimens, 5.8% of *P. leptodactylus* and 29.6% of *F. limosus* completely crossed the arena ramp. The Mann-Whitney *U* test was significant ($P < 0.05$) in the *tcrs* of the two species; *F. limosus* managed to cross the arena ramp much faster than *P. leptodactylus*. Analysis of the TL of the crayfish revealed no influence on the complete crossing success, with the logistic regression model (**Table 3**) showing no significant relationship between TL and the binary *sa* variable (0/1).

The results differed significantly between day and night *sa* of the arena ramp for both species ($P < 0.05$), with successful crossing being encountered exclusively during the night in native species, whereas 7.9% of the successful crossings for the invasive species were recorded in daylight.

Force Assessment

Raw data analysis revealed that the invasive species *F. limosus* exhibited higher F_{max} and F_{Av} compared to *P. leptodactylus*, with some individuals producing more than double values (**Figures 3A,B**). The maximum limit of the force recorded for *P. leptodactylus* represented the lower limit for *F. limosus*. A body size in the range of 90 to 100 mm TL seemed to be suitable for the highest force development in *F. limosus*, while no force-length relation could be observed for *P. leptodactylus*. The LV, a measure of how quickly the maximum force is achieved, appeared to be similar for the two investigated species (**Figure 3C**); for a CV of around 2 cm^3, the maximum LV was achieved in both crayfish species. The average RV (**Figure 3D**) showed a higher releasing rate for *F. limosus*, indicating that muscle relaxation occurs much faster for *F. limosus*. Most individuals of both species exhibited a releasing velocity of around 3 N/s. Larger C_t values could be observed for *F. limosus* (**Figure 3E**). Because no obvious difference in loading velocity was identified between the species, the larger C_t might be determined by the higher force values produced by *F. limosus*. The EN (**Figure 3F**) was also higher for *F. limosus*, a normal evolution of this parameter since the forces and contraction times were also larger. The faster releasing rate does, however, not compensate for the other two parameters. Higher mechanical energy was recorded in both species for smaller CV values.

HRR Assessment

The results of HRR measurements in the analysed groups are presented in **Figure 4**. To calculate the respiratory rates, we used the Oroboros software Datlab. Mitochondrial respiration was corrected for oxygen flux due to the instrumental background and ROX (Gnaiger, 2014).

Based on a previous study, STATE 2 (i.e., basal respiration corresponding to mitochondria oxidising substrates when the membrane is fully energised) is determined by the intrinsic proton conductivity of the inner membrane, which dissipates the $\Delta\Psi$ (Harris and Das, 1991). In mitochondria respiring on glutamate + malate, we found a statistically significant increase in basal respiratory state in *F. limosus* vs. *P. leptodactylus* groups (**Figure 4A**): STATE 2 12.04 ± 2.8 vs. 5.8 ± 2.6 $pmol.s^{-1}.mL^{-1}$ ($P < 0.05$). Addition of ADP stimulates oxidative phosphorylation (OXPHOS capacity), which induces a drop in membrane potential and a subsequent increase in respiration (Harris and Das, 1991). In our results, OXPHOS was significantly lower in the *F. limosus* group energised with CI substrates

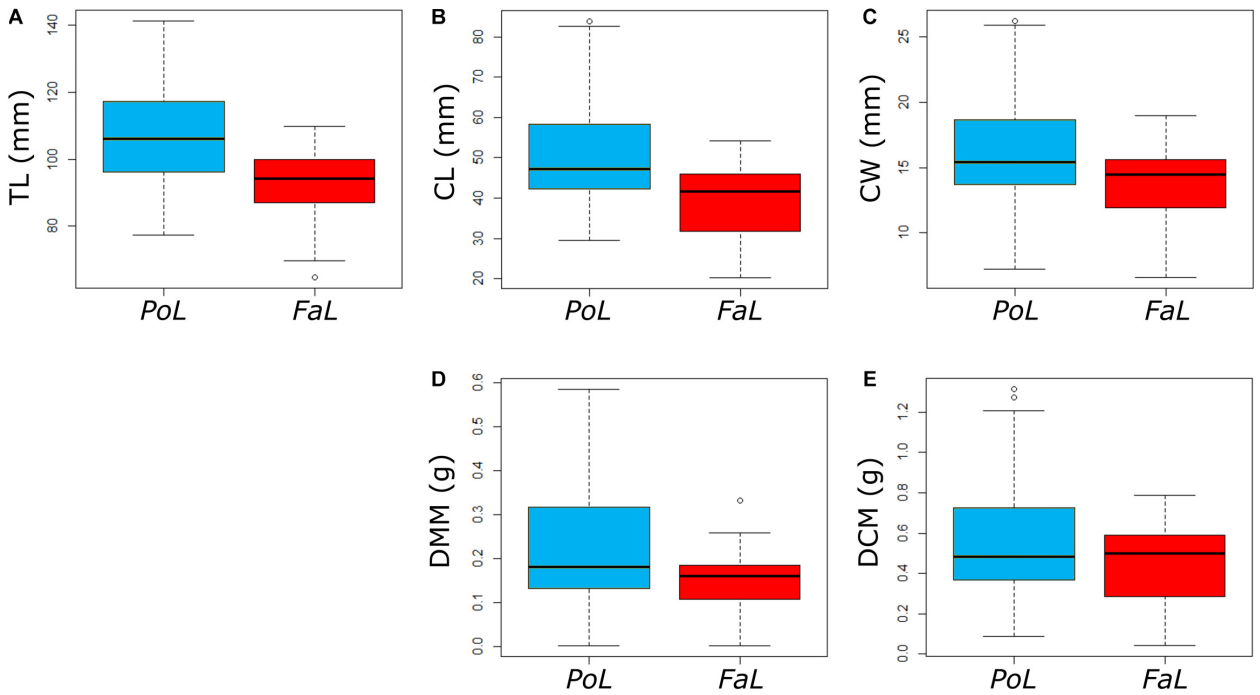

FIGURE 2 | Boxplots showing the difference between *P. leptodactylus* and *F. limosus* for the statistically relevant variables: **(A)** total length (TL), **(B)** chela length (CL), **(C)** chela width (CW), **(D)** dry chela muscle mass (DMM), and **(E)** dry chela cuticle mass (DCM). Values represent means ± SD.

TABLE 3 | Parameter estimates for logistic regression relationship models between crayfish total length (TL) and the binary successful attempts to escape (*sa* variable) to assess boldness behaviour.

	Estimate	Std. Error	*z*-value	*P*-value
Intercept	−1.902	3.978	−0.478	0.633
TL	−0.0017	0.037	−0.049	0.961

(**Figure 4C**): 19.33 ± 4.8 vs. 34.6 ± 3.9 pmol.s^{-1}.mL^{-1} ($P < 0.05$). Addition of FCCP, a protonophore, induces the inhibition of oxidative phosphorylation due to dissipation of the membrane potential, leading to the increase in respiration (i.e., phosphorylation and respiration become uncoupled) (Harris and Das, 1991). In mitochondria energised with glutamate + malate, the Uncoupled State (**Figure 4E**) was similar in *F. limosus* vs. *P. leptodactylus* groups: 58.1 ± 10.9 vs. 60.15 ± 6.2 pmol.s^{-1}.mL^{-1} (*P* NS).

In the presence of CII-dependent substrate, we observed the same trend of mitochondrial respiratory rates in *F. limosus* vs. *P. leptodactylus* groups, albeit in a lesser (not significant) degree as compared to CI-supported respiration: STATE 2 (**Figure 3B**) 19 ± 7.1 vs. 10.5 ± 3.8 pmol.s^{-1}.mL^{-1} (*P* NS), OXPHOS (**Figure 4D**) 23.9 ± 9.7 vs. 27.8 ± 9.1 pmol.s^{-1}.mL^{-1} (*P* NS) and the Uncoupled State (**Figure 4F**) 51.7 ± 30.6 vs. 43.7 ± 16.9 pmol.s^{-1}.mL^{-1} (*P* NS).

We further calculated the Respiratory Control Ratio (RCR) as the OXPHOS/State 2 ratio. Under both experimental conditions, RCR was significantly decreased ($P < 0.05$) in the *F. limosus* group (**Figures 4G,H**) as a direct reflection of the STATE 2 increase and the inhibition of ADP phosphorylation.

DISCUSSION

The Competitors' Bio-Mechanical Performance

The invasive crayfish *F. limosus* was more confident than the native *P. leptodactylus*, taking more risks as demonstrated by our laboratory experiments. The native *P. leptodactylus* appeared to be more shy, probably explaining other findings such as its limited access to shelter and food resources (Lele and Pârvulescu, 2017; Pacioglu et al., 2020). The species *F. limosus* exhibited superior values of every parameter except those related to body size, emphasising the differences also observed from a biomechanical point of view. The average of maximum forces applied by *F. limosus* revealed pinching forces of 2.3 times higher than for *P. leptodactylus*. This advantage becomes even more relevant in the context of the total body length of individuals, which did not influence the mechanism of pinching force development. However, it may contribute to the body stability condition of the larger species during a fight (Zeil and Hemmi, 2006; Bywater et al., 2008; Malavé et al., 2018; Kuo et al., 2019).

Force production is a physiological attribute of the adductor muscle, which in turn is proportional to the chelae volume. On the other hand, force transmission depends on the length of the mobile dactylus of the chelae, and therefore, both aspects should be taken into account. The reaction force measured at the tip of

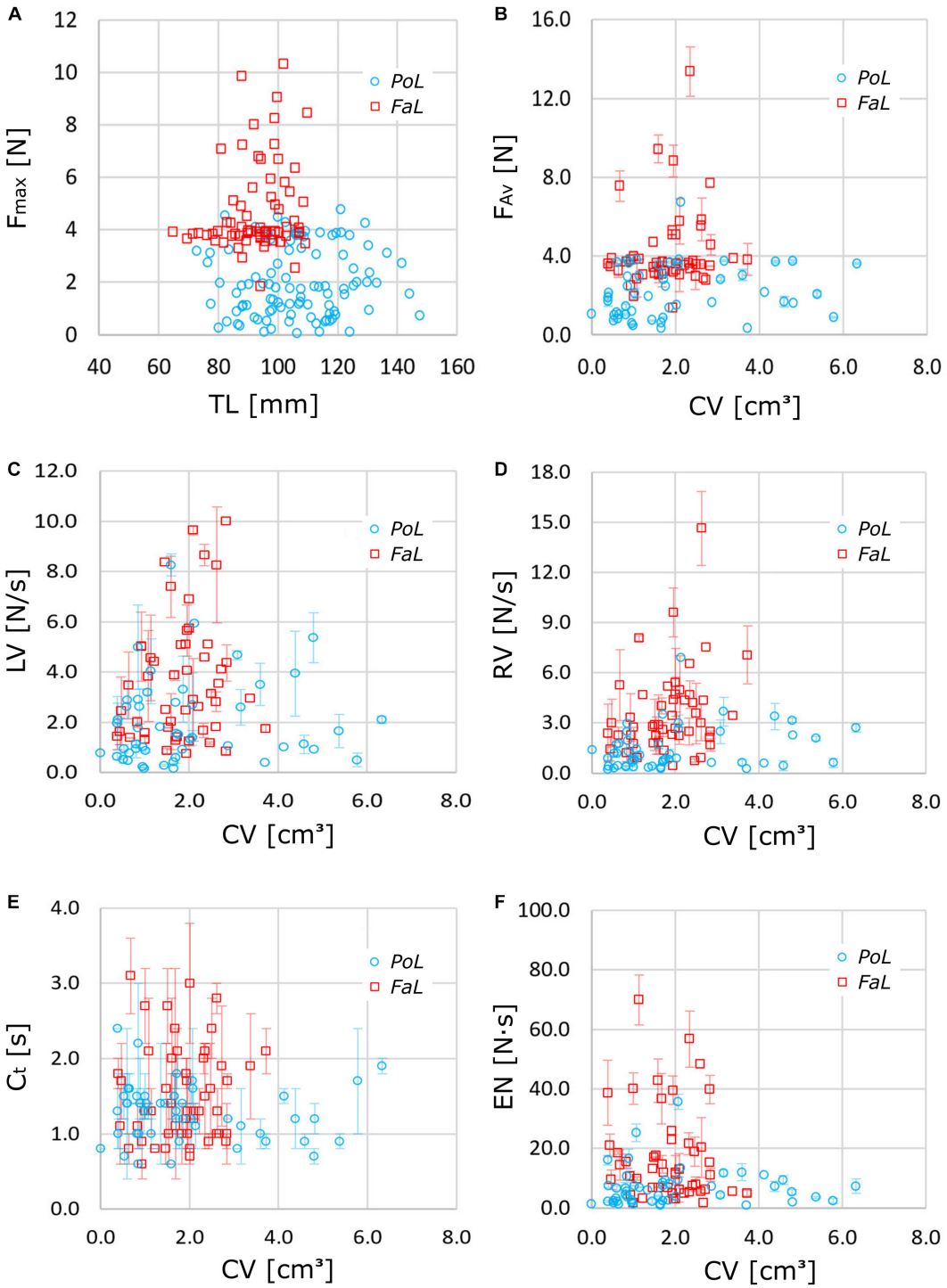

FIGURE 3 | Raw data of force and biometrical parameters: **(A)** maximum pinching force (F_{max}) vs. total length (TL), **(B)** average of maximum pinching forces (F_{Av}) vs. chela volume (CV), **(C)** average loading velocity (LV) vs. CV, **(D)** average releasing velocity (RV) vs. CV, **(E)** contraction time (C_t) vs. CV, and **(F)** energy of the pinching cycle (EN) vs. CV.

the claw can be explained by the lever mechanism developed by the muscle force through the physiological distances, as depicted in **Figure 5**. The value of the reaction force at the tip of the claw is directly influenced by the muscular force value and its insertion point, while being negatively influenced by the length of the claw. This is caused by the effect or the moment of torque developed by

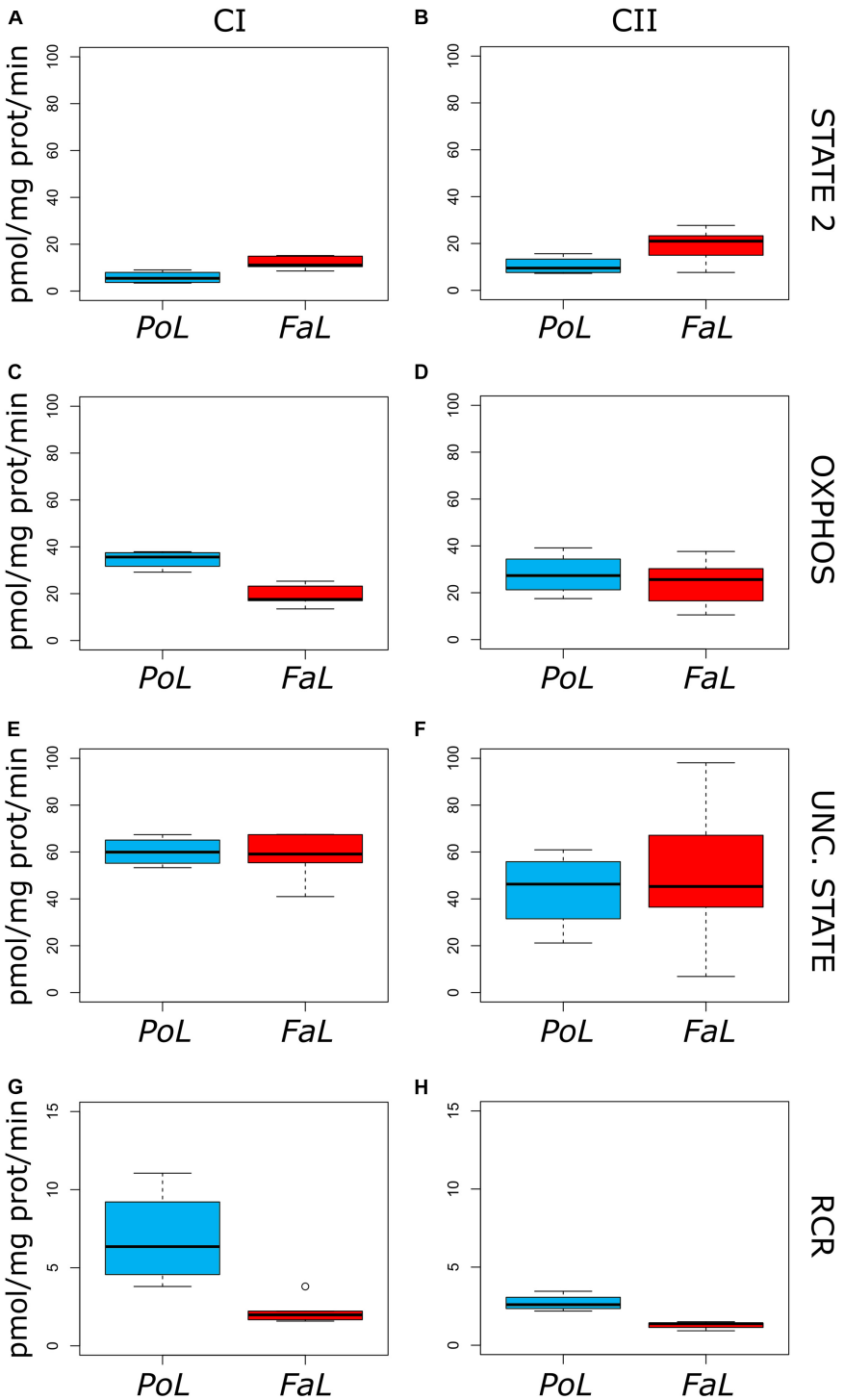

FIGURE 4 | High-resolution respirometry data for CI-supported **(A,C,E,G)** and CII-supported **(B,D,F,H)** respiration in *Pontastacus leptodactylus* and *Faxonius limosus*. Values represent means ± SD.

the forces and physiological distances. Therefore, for a constant muscle force, as the claw became longer the reaction force will be smaller. This biomechanical behaviour may explain the smaller pinching forces of *P. leptodactylus* species, which possesses longer claws compared to *F. limosus*. This disadvantage is apparently neither compensated by the observed better muscular energetic efficiency in *P. leptodactylus* nor by the increased physiological distance between the fulcrum and the adductor insertion point.

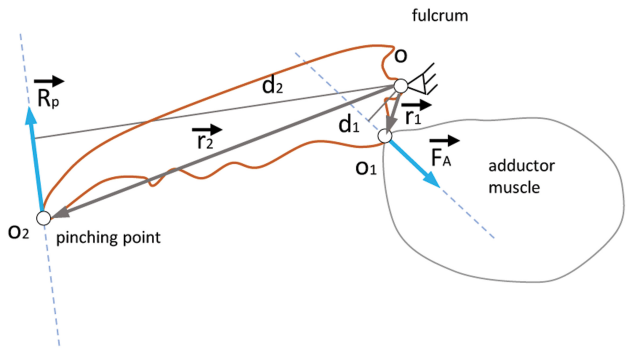

FIGURE 5 | Mechanism of force production and transmission. The equation that governs the biomechanical equilibrium is $R_p = \frac{d_1}{d_2} \cdot F_A$, where R_p is the reaction force at the tip of the claw, F_A is the adductor muscle force and d_1 and d_2 are the lever arms.

Investigations on pinching cycle energy revealed higher values for *F. limosus*, which leads us to infer that this crayfish species not only produces higher forces but is also capable of sustaining this force for an extended period of time compared to the native *P. leptodactylus*. The energy of the pinching cycle will create a significant advantage for the invasive in clash with the native species, since the application of a force for a longer time allows crack growth and propagation up to the fracture point in any biological (Egan et al., 2015) or non-living structures (Linul et al., 2020). The biomechanical parameters (biometrics and forces) make the invader prone to win most of the fights, as revealed in other studies regarding confrontations between these two species for food and shelter (Lele and Pârvulescu, 2017). The agonistic behaviour strategy of the invasive crayfish species appears to be based on physical superiority, whereas the native crayfish works on intimidation display.

The Competitors' Chelae Muscular Performance

There is evidence that metabolic capacity changes with thermal acclimation in crayfish (Seebacher and Wilson, 2006), and this might counteract threats from invasive species. We found that RCR, an indicator of the efficacy of oxidative ATP production (Montaigne et al., 2011), was significantly higher in native crayfish, and mitochondrial respiration rates, using the physiologically relevant substrate mixtures (i.e., glutamate + malate), significantly differed between *F. limosus* and *P. leptodactylus* when compared to using succinate (+rotenone) as the substrate. Thereby, an important finding of our study is that mitochondrial respiration in crayfish chelae is substrate-dependent, with a preference for CI substrates.

Another finding in our experiments was that the STATE 2 respiration rate was increased in the *F. limosus* group under both experimental conditions (with a significant difference in CI-supported respiration, see **Figure 4**), indicating partial mitochondrial uncoupling; yet, mitochondrial uncoupling was not complete since mitochondrial respiration further increased after the addition of ADP (**Figure 4**). Mild uncoupling of mitochondrial respiration, leading to the decrease in membrane potential, could yet be beneficial since it prevents the excessive generation of reactive oxygen species (Skulachev, 1998).

Finally, a major indicator of mitochondrial function is RCR, which represents a measure of the efficiency of oxidative phosphorylation. Accordingly, another important finding of our study was that RCR in the *F. limosus* group was significantly lower in mitochondria energised with both substrate types. This result, together with the finding of OXPHOS inhibition, indicates that the chelae muscle mitochondria of this species use oxygen less efficiently compared to *P. leptodactylus*. Literature data suggest that dominance can modulate behavioural dynamics through a better synchronisation of biomechanical activities (Alcala et al., 2019).

Further Coexistence Scenario

Boldness is often related to intense activity and exploration and less neophobic behaviour (Chapple et al., 2012), with bolder individuals being more likely to be found in crowded environments (Evans et al., 2010), and often associated with invasive species ready to assume more risks (Hazlett et al., 2003; Pintor et al., 2008; Reisinger et al., 2017). Invasive crayfish encounter, along their paths, many different habitats, with changing environments and biological associations. Our data detected a bolder behaviour of invasives in novel environments over the native crayfish, supporting the findings that animals in prolonged highly changeable environments display less or no neophobia (Modlinska and Stryjek, 2016; Meuthen et al., 2019). Moreover, these results confirm a similar behaviour pattern of invasive species, such as cane toads, in introduced habitats (Candler and Bernal, 2014).

The fighting strategies (both inter- and intraspecific) of different species vary (Fořt et al., 2019), and although body size and chelae display are important in the initial assessment of the competitor, these are no longer good indicators of the outcome once the fight is initiated, as there is evidence that these are unreliable signals of actual chelae strength (Walter et al., 2011; Angilletta and Wilson, 2012). Consequently, species aggressivity (Fořt et al., 2019) or even sheer force, as measured for *F. limosus* in our study, influences the individual's rank. Equally important, prior research substantiated the idea that social rank position and boldness level in animals are at least connected by the same influencing factors (Boogert et al., 2006; Mettler and Shivik, 2007). In our case, superior chela force seems determinant in dominance and, therefore, in bolder behaviour, both being advantageous for the invasive species.

The behavioural expression of boldness is influenced by various factors such as resource availability, predation-risk cues or even temperature (Hazlett et al., 2003; Seebacher and Wilson, 2006; Juette et al., 2014; Zhao and Feng, 2015; Reisinger et al., 2017). Studies primarily investigating the trophic position of these two crayfish species, in the same habitat of the Lower Danube, revealed that the invasive *F. limosus* outcompetes the native *P. leptodactylus*, pushing the latter to a lower position (Pacioglu et al., 2019, 2020). Still, in this context, recovering

pockets (i.e., sub-populations) have been documented, where males of *P. leptodactylus* have a larger chelae size compared to those populations that did not encounter the pressure of invasive species competition (Pacioglu et al., 2020). The reduced genetic structure of these recovering populations (Pacioglu et al., 2020) led to the assumption of a kind of resettlement of both crayfish species' competition over time, after invasion. Apparently, the native species selectively increased its weapon in size in the detriment of force (Seebacher and Wilson, 2006), whereas the invasive species may already have passed through the basic establishment period of a self-sustaining population in the invasion dynamics (Song et al., 2006; Blackburn et al., 2011; Hudina et al., 2015), documented as resulting in the increase of its biological (i.e., foraging and reproductive) conditions (Pârvulescu et al., 2015; Pacioglu et al., 2020). This would be an encouraging scenario for these two species to further reach a reasonable equilibrium of their coexistence.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon request, without undue reservation.

ETHICS STATEMENT

All applicable International, National, and Institutional Guidelines for the care and use of animals were followed. No protected or rare species were involved in this study. After the specimens were analysed, they were euthanised by freezing to −20°C (Oidtmann et al., 2002) to avoid any environmental contamination with the widespread crayfish plague pathogen *Aphanomyces astaci* (Ungureanu et al., 2020).

AUTHOR CONTRIBUTIONS

LP conceived the experiments. AEP conducted the laboratory investigations. IM provided assistance for measurement device. MCI, MS, and MV conducted the biometric measurements. DIS processed and interpreted the biomechanical data. MDM and OMA conducted and interpreted the muscle energetic investigations. KM statistically analysed the results. LP, DIS, MDM, OMA, and MCI wrote the first version of the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was partially supported by a grant of the Romanian National Authority for Scientific Research and Innovation (UEFISCDI), project code PN-III-P4-ID-PCE-2020-1187. MCI was supported by the Institute of Biology Bucharest, Romanian Academy, project no. RO1567-IBB04/2020.

ACKNOWLEDGMENTS

Andrei Năstase was acknowledged for providing crayfish specimens for this study, Alexandra Almăjan for supporting laboratory processes, and Coşa Alexandru for data processing and computation of derived biomechanical parameters. We thank the two reviewers whose valuable suggestions significantly improved the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.581247/full#supplementary-material

REFERENCES

Alcala, R. S., Caliva, J. M., Flesia, A. G., Marin, R. H., and Kembro, J. M. (2019). Aggressive dominance can decrease behavioral complexity on subordinates through synchronization of locomotor activities. *Commun. Biol.* 2:467. doi: 10.1038/s42003-019-0710-1

Angilletta, M. J. J., and Wilson, R. S. (2012). Cryptic asymmetry: unreliable signals mask asymmetric performance of crayfish weapons. *Biol. Lett.* 8, 551–553. doi: 10.1098/rsbl.2012.0029

Blackburn, T. M., Pyšek, P., Bacher, S., Carlton, J. T., Duncan, R. P., Jarošík, V., et al. (2011). A proposed unified framework for biological invasions. *Trends Ecol. Evol.* 26, 333–339. doi: 10.1016/j.tree.2011.03.023

Boogert, N. J., Reader, S. M., and Laland, K. N. (2006). The relation between social rank, neophobia and individual learning in starlings. *Anim. Behav.* 72, 1229–1239. doi: 10.1016/J.ANBEHAV.2006.02.021

Bywater, C. L. Jr., Angilletta, M. J., and Wilson, R. S. (2008). Weapon size is a reliable indicator of strength and social dominance in female slender crayfish (*Cherax dispar*). *Funct. Ecol.* 22, 311–316. doi: 10.2307/20142807

Candler, S., and Bernal, X. E. (2014). Differences in neophobia between cane toads from introduced and native populations. *Behav. Ecol.* 26, 97–104. doi: 10.1093/beheco/aru162

Carere, C., and Gherardi, F. (2013). Animal personalities matter for biological invasions. *Trends Ecol. Evol.* 28, 5–6. doi: 10.1016/j.tree.2012.10.006

Chapple, D. G., Simmonds, S. M., and Wong, B. B. M. (2012). Can behavioral and personality traits influence the success of unintentional species introductions? *Trends Ecol. Evol.* 27, 57–64. doi: 10.1016/J.TREE.2011.09.010

De Meyer, J., Irschick, D. J., Vanhooydonck, B., Losos, J. B., Adriaens, D., and Herrel, A. (2019). The role of bite force in the evolution of head shape and head shape dimorphism in Anolis lizards. *Funct. Ecol.* 33, 2191–2202. doi: 10.1111/1365-2435.13438

Duicu, O. M., Mirica, S. N., Gheorgheosu, D. E., Privistirescu, A. I., Fira-Mladinescu, O., and Muntean, D. M. (2013). Ageing-induced decrease in cardiac mitochondrial function in healthy rats. *Can. J. Physiol. Pharmacol.* 91, 593–600. doi: 10.1139/cjpp-2012-0422

Egan, P., Sinko, R., Leduc, P. R., and Keten, S. (2015). The role of mechanics in biological and bio-inspired systems. *Nat. Commun.* 6:7418. doi: 10.1038/ncomms8418

Essl, F., Bacher, S., Blackburn, T. M., Booy, O., Brundu, G., Brunel, S., et al. (2015). Crossing frontiers in tackling pathways of biological invasions. *Bioscience* 65, 769–782. doi: 10.1093/biosci/biv082

Evans, J., Boudreau, K., and Hyman, J. (2010). Behavioural syndromes in Urban and rural populations of song sparrows. *Ethology* 116, 588–595. doi: 10.1111/j.1439-0310.2010.01771.x

Ferrari, M. C. O., Brown, G. E., and Chivers, D. P. (2018). Understanding the effect of uncertainty on the development of neophobic antipredator phenotypes. *Anim. Behav.* 136, 101–106. doi: 10.1016/j.anbehav.2017.11.024

Flood, P. J., Duran, A., Barton, M., Mercado-Molina, A. E., and Trexler, J. C. (2020). Invasion impacts on functions and services of aquatic ecosystems. *Hydrobiologia* 847, 1571–1586. doi: 10.1007/s10750-020-04211-3

Fořt, M., Hossain, M. S., Kouba, A., Buřič, M., and Kozák, P. (2019). Agonistic interactions and dominance establishment in three crayfish species non-native to Europe. *Limnologica* 74, 73–79. doi: 10.1016/J.LIMNO.2018.11.003

Gnaiger, E. (2014). *Mitochondrial Pathways and Respiratory Control. An Introduction to OXPHOS Analysis*, ed. E. Gnaiger (Innsbruck: MiPNet Publications).

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* 177, 751–766.

Graham, Z. A., and Angilletta, M. J. (2020). Claw size predicts dominance within and between invasive species of crayfish. *Anim. Behav.* 166, 153–161. doi: 10.1016/j.anbehav.2020.06.021

Guderley, H., and Couture, P. (2005). Stickleback fights: Why do winners win? Influence of metabolic and morphometric parameters. *Physiol. Biochem. Zool.* 78, 173–181. doi: 10.1086/425204

Harris, D. A., and Das, A. M. (1991). Control of mitochondrial ATP synthesis in the heart. *Biochem. J.* 280, 561–573. doi: 10.1042/bj2800561

Haubrock, P. J., Balzani, P., Azzini, M., Inghilesi, A. F., Veselý, L., Guo, W., et al. (2019). Shared histories of co-evolution may affect trophic interactions in a freshwater community dominated by alien species. *Front. Ecol. Evol.* 7:355. doi: 10.3389/fevo.2019.00355

Hazlett, B. A., Burba, A., Gherardi, F., and Acquistapace, P. (2003). Invasive species of crayfish use a broader range of predation-risk cues than native species. *Biol. Invasions* 5, 223–228. doi: 10.1023/A:1026114623612

Holdich, D. M., Reynolds, J. D., Souty-Grosset, C., and Sibley, P. J. (2009). A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. *Knowl. Manag. Aquat. Ecosyst.* 394-395:11. doi: 10.1051/kmae/2009025

Hudina, S., Žganec, K., and Hock, K. (2015). Differences in aggressive behaviour along the expanding range of an invasive crayfish: an important component of invasion dynamics. *Biol. Invasions* 17, 3101–3112. doi: 10.1007/s10530-015-0936-x

Ion, M. C., Puha, A. E., Suciu, T., and Pârvulescu, L. (2020). Get a grip: unusual disturbances drive crayfish to improvise. *Behaviour* 157, 101–120. doi: 10.1163/1568539x-00003583

Juette, T., Cucherousset, J., and Cote, J. (2014). Animal personality and the ecological impacts of freshwater non-native species. *Curr. Zool.* 60, 417–427. doi: 10.1093/czoolo/60.3.417

Kuo, J. H., Chang, Y. T., Chen, Y. J., and Hsu, Y. (2019). Influence of previous agonistic interactions with conspecifics on contest decisions. *Ethology* 125, 660–668. doi: 10.1111/eth.12919

Lele, S.-F., and Pârvulescu, L. (2017). Experimental evidence of the successful invader *Orconectes limosus* outcompeting the native *Astacus leptodactylus* in acquiring shelter and food. *Biologia* 72, 877–885. doi: 10.1515/biolog-2017-0094

Lele, S.-F., and Pârvulescu, L. (2019). Crayfish chelae usage suggests predominantly ambidextrous habitude. *Crustaceana* 92, 257–267. doi: 10.1163/15685403-00003886

Light, T. (2003). Success and failure in a lotic crayfish invasion: the roles of hydrologic variability and habitat alteration. *Freshw. Biol.* 48, 1886–1897. doi: 10.1046/j.1365-2427.2003.01122.x

Linul, E., Marsavina, L., and Stoia, D. I. (2020). Mode I and II fracture toughness investigation of Laser-Sintered Polyamide. *Theor. Appl. Fract. Mech.* 106, 102497. doi: 10.1016/j.tafmec.2020.102497

Liu, Z.-M., Wang, G.-Z., Wu, L.-S., Zeng, Z.-S., and Chen, X.-L. (2013). Seasonal change in mitochondrial function and metabolic enzyme activity of different populations of the mud crab, *Scylla paramamosain*, from China. *Aquaculture* 376-379, 68–75. doi: 10.1016/J.AQUACULTURE.2012.11.007

Lodge, D. M. (1993). Biological invasions: lessons for ecology. *Trends Ecol. Evol.* 8, 133–137. doi: 10.1016/0169-5347(93)90025-K

Malavé, B. M., Styga, J. M., and Clotfelter, E. D. (2018). Size, shape, and sex-dependent variation in force production by crayfish chelae. *J. Morphol.* 279, 312–318. doi: 10.1002/jmor.20773

Mettler, A. E., and Shivik, J. A. (2007). Dominance and neophobia in coyote (*Canis latrans*) breeding pairs. *Appl. Anim. Behav. Sci.* 102, 85–94. doi: 10.1016/J.APPLANIM.2006.03.012

Meuthen, D., Ferrari, M., Lane, T., and Chivers, D. (2019). High background risk induces risk allocation rather than generalized neophobia in the fathead minnow. *Behav. Ecol.* 30, 1416–1424. doi: 10.1093/beheco/arz094

Modlinska, K., and Stryjek, R. (2016). Food neophobia in wild rats (*Rattus norvegicus*) inhabiting a changeable environment—a field study. *PLoS One* 11:e0156741. doi: 10.1371/journal.pone.0156741

Montaigne, D., Marechal, X., Preau, S., Baccouch, R., Modine, T., Fayad, G., et al. (2011). Doxorubicin induces mitochondrial permeability transition and contractile dysfunction in the human myocardium. *Mitochondrion* 11, 22–26. doi: 10.1016/j.mito.2010.06.001

Nentwig, W., Bacher, S., Kumschick, S., Pyšek, P., and Vilà, M. (2018). More than "100 worst" alien species in Europe. *Biol. Invasions* 20, 1611–1621. doi: 10.1007/s10530-017-1651-6

Oidtmann, B., Heitz, E., Rogers, D., and Hoffmann, R. (2002). Transmission of crayfish plague. *Dis. Aquat. Organ.* 52, 159–167. doi: 10.3354/dao052159

Pacioglu, O., Theissinger, K., Alexa, A., Samoilă, C., Sîrbu, O. I., Schrimpf, A., et al. (2020). Multifaceted implications of the competition between native and invasive crayfish: a glimmer of hope for the native's long-term survival. *Biol. Invasions* 22, 827–842. doi: 10.1007/s10530-019-02136-0

Pacioglu, O., Zubrod, J. P., Schulz, R., Jones, J. I., and Pârvulescu, L. (2019). Two is better than one: combining gut content and stable isotope analyses to infer trophic interactions between native and invasive species. *Hydrobiologia* 839, 25–35. doi: 10.1007/s10750-019-03990-8

Pârvulescu, L., Pîrvu, M., Moroşan, L.-G., and Zaharia, C. (2015). Plasticity in fecundity highlights the females' importance in the spiny-cheek crayfish invasion mechanism. *Zoology* 118, 424–432. doi: 10.1016/J.ZOOL.2015.08.003

Perrings, C., Williamson, M., Barbier, E. B., Delfino, D., Dalmazzone, S., Shogren, J., et al. (2002). Biological invasion risks and the public good: an economic perspective. *Conserv. Ecol.* 6:1. doi: 10.5751/ES-00396-060101

Pintor, L. M., Sih, A., and Bauer, M. L. (2008). Differences in aggression, activity and boldness between native and introduced populations of an invasive crayfish. *Oikos* 117, 1629–1636. doi: 10.1111/j.1600-0706.2008.16578.x

Reisinger, L. S., Elgin, A. K., Towle, K. M., Chan, D. J., and Lodge, D. M. (2017). The influence of evolution and plasticity on the behavior of an invasive crayfish. *Biol. Invasions* 19, 815–830. doi: 10.1007/s10530-016-1346-4

Richardson, A. (2019). Body size in freshwater crayfish: an intercontinental comparison. *Freshw. Crayfish* 24, 43–47. doi: 10.5869/fc.2019.v24-1.43

Seebacher, F., and Wilson, R. S. (2006). Fighting fit: thermal plasticity of metabolic function and fighting success in the crayfish *Cherax destructor*. *Funct. Ecol.* 20, 1045–1053. doi: 10.1111/j.1365-2435.2006.01194.x

Skulachev, V. P. (1998). Uncoupling: new approaches to an old problem of bioenergetics. *Biochim. Biophys. Acta Bioenerg.* 1363, 100–124. doi: 10.1016/S0005-2728(97)00091-1

Song, C. K., Herberholz, J., and Edwards, D. H. (2006). The effects of social experience on the behavioral response to unexpected touch in crayfish. *J. Exp. Biol.* 209, 1355–1363. doi: 10.1242/jeb.02126

South, J., Madzivanzira, T. C., Tshali, N., Measey, J., and Weyl, O. L. F. (2020). In a pinch: mechanisms behind potential biotic resistance toward two invasive crayfish by native African freshwater crabs. *Front. Ecol. Evol.* 8:72. doi: 10.3389/fevo.2020.00072

Strauss, A., White, A., and Boots, M. (2012). Invading with biological weapons: the importance of disease-mediated invasions. *Funct. Ecol.* 26, 1249–1261. doi: 10.1111/1365-2435.12011

Sullivan, L. L., Li, B., Miller, T. E. X., Neubert, M. G., and Shaw, A. K. (2017). Density dependence in demography and dispersal generates fluctuating invasion speeds. *Proc. Natl. Acad. Sci. U.S.A.* 114, 5053–5058. doi: 10.1073/pnas.1618744114

Ungureanu, E., Mojžišová, M., Tangerman, M., Ion, M. C., Parvulescu, L., and Petrusek, A. (2020). The spatial distribution of Aphanomyces astaci genotypes across Europe: introducing the first data from Ukraine. *Freshw. Crayfish* 25, 77–87. doi: 10.5869/fc.2020.v25-1.077

Usio, N., Konishi, M., and Nakano, S. (2001). Species displacement between an introduced and A 'vulnerable' crayfish: the role of aggressive interactions and shelter competition. *Biol. Invasions* 3, 179–185. doi: 10.1023/A:1014573915464

van der Meijden, A., Herrel, A., and Summers, A. (2010). Comparison of chela size and pincer force in scorpions; getting a first grip. *J. Zool.* 280, 319–325. doi: 10.1111/j.1469-7998.2009.00628.x

Walter, G. M., van Uitregt, V. O., and Wilson, R. S. (2011). Social control of unreliable signals of strength in male but not female crayfish, *Cherax destructor*. *J. Exp. Biol.* 214, 3294–3299. doi: 10.1242/jeb.056754

White, J. R., Meekan, M. G., McCormick, M. I., and Ferrari, M. C. O. (2013). A comparison of measures of boldness and their relationships to survival in young fish. *PLoS One* 8:e68900. doi: 10.1371/journal.pone.0068900

Zeil, J., and Hemmi, J. M. (2006). The visual ecology of fiddler crabs. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 192, 1–25. doi: 10.1007/s00359-005-0048-7

Zhao, D., and Feng, P. (2015). Temperature increase impacts personality traits in aquatic non-native species: implications for biological invasion under climate change. *Curr. Zool.* 61, 966–971. doi: 10.1093/czoolo/61.6.966

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 04 March 2021
doi: 10.3389/fevo.2021.607100

Effects of Different Diet Types on Growth and Survival of White-Clawed Crayfish *Austropotamobius pallipes* in Hatcheries

Jen Nightingale[1,2]*, *Gareth Jones*[1], *Gráinne McCabe*[2] and *Paul Stebbing*[3,4]

[1] School of Biological Sciences, University of Bristol, Bristol, United Kingdom, [2] Bristol Zoological Society, Bristol, United Kingdom, [3] Cefas, Weymouth, United Kingdom, [4] APEM Ltd., Stockport, United Kingdom

Developing an optimal diet for rearing endangered white-clawed crayfish *Austropotamobius pallipes* is important for captive breeding success prior to wild release. Four *ex situ*, 40-day experiments assessed survival and growth of crayfish fed different treatment diets. Two experiments (A and B) were undertaken with hatchlings, to determine if live food was an essential dietary component during the first few weeks after hatching. The second set of experiments (C and D) were undertaken with juvenile (60-day-old) *A. pallipes,* to determine an optimal diet after the initial critical feeding stage. In experiment A, we fed hatchlings: i) live *Artemia* nauplii + plankton (Live + P); ii) decapsulated *Artemia* cysts + plankton (Cyst + P) or iii) decapsulated *Artemia* cysts + plankton encapsulated in agar gel (Gel + CP). Survival and growth was significantly greater with Live + P than with the other two diets. In experiment B we compared Live + P with commercially available feeds by feeding hatchlings: i) live *Artemia* nauplii + Australian pellet (Live + Aus); ii) live *Artemia* nauplii plus New Zealand pellet (Live + NZ); iii) live *Artemia* nauplii + plankton (Live + P); or (iv) practical Spanish crayfish pellet diet (Spain). Under these experimental conditions crayfish survival was significantly higher with Live + P diet than with Live + Aus or Spain. Growth was also significantly greater with Live + P than with the Live + NZ or Spanish treatment diets. In experiment C, 60-day-old juvenile *A. pallipes* were fed: i) defrosted plankton plus vegetables (Standard) or (ii) defrosted plankton plus vegetables encapsulated in agar gel (Gel + PV). Survival was not significantly different between the diets; however, growth was significantly greater with the Standard diet rather than Gel + PV. In experiment D, juveniles were fed four different diets: i) Australian pellet (Australia); ii) New Zealand pellet (New Zealand); iii) plankton and vegetables (Standard); or iv) practical Spanish diet (Spain). Survival was significantly lower in crayfish fed the New Zealand diet. Crayfish growth was significantly greater with the Standard diet of plankton and vegetables than all three pellet diets. Our results showed that live food is optimal for high survival and growth in *A. pallipes* hatchlings and a plankton, plus vegetable, diet produces higher growth in juveniles compared to pellet diets.

Keywords: aquaculture, crayfish, conservation, nutrition, ecology

OPEN ACCESS

Edited by:
Ivana Maguire,
University of Zagreb, Croatia

Reviewed by:
Omid Safari,
Ferdowsi University of Mashhad, Iran
Julian Reynolds,
Trinity College Dublin, Ireland
Stephanie Peay,
Aecom (United Kingdom),
United Kingdom

***Correspondence:**
Jen Nightingale
jnightingale@bristolzoo.org.uk

Specialty section:
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *16 September 2020*
Accepted: *11 February 2021*
Published: *04 March 2021*

Citation:
Nightingale J, Jones G, McCabe G and Stebbing P (2021) Effects of Different Diet Types on Growth and Survival of White-Clawed Crayfish Austropotamobius pallipes in Hatcheries. Front. Ecol. Evol. 9:607100. doi: 10.3389/fevo.2021.607100

INTRODUCTION

The white-clawed crayfish *Austropotamobius pallipes* is endangered throughout its native range in the United Kingdom and mainland Europe (Sibley et al., 2011). The loss of this species is attributed to the spread of the invasive American signal crayfish *Pacifastacus leniusculus* and associated crayfish plague, caused by the pathogen/oomycete *Aphanomyces astaci*, along with habitat degradation and pollution (Sibley et al., 2011). In response to this decline, white-clawed crayfish aquaculture is increasing in the United Kingdom and mainland Europe (Nightingale et al., 2017).

A major issue with the mass-rearing of animals in captivity is the provision of a well-balanced and nutritional diet, to ensure good survival, growth and development, which is particularly important for juvenile life stages. With crayfish, a critical period of survival occurs during the first weeks, post-hatching, when high mortality rates can occur due to a lack of adequate nutrition (González et al., 2011; Celada et al., 2012). When crayfish hatch, they initially feed on their egg yolk and remain attached to the female's pleopods. When they have undergone two moults and are free-living, their exogenous mouth parts have formed and feeding begins (Reynolds, 2002). Observations from the wild can provide key information on suitable diets for captive-bred animals. Wild *A. pallipes* are opportunistic omnivores, feeding on invertebrates, carrion, vegetable matter, and organic and inorganic detritus (Gherardi et al., 2004). Scalici and Gibertini (2007) found stomach contents of wild-caught *A. pallipes* differing with age and sex. Insect larvae were found to be a key component of juvenile and adult female diets, in contrast, adult males mainly fed on vegetable matter. These findings are also supported by *in situ* analysis of the gut contents of *A. pallipes* juveniles, which were shown to be feeding predominantly on aquatic invertebrates (Paglianti and Gherardi, 2004). This ontogenetic diet shift may be a result of the digestive enzymes within the hepatopancreas altering as crayfish mature and this corresponds to changes in diets (Hammer et al., 2000; Figueiredo and Anderson, 2003).

Several studies have examined feeding and nutrition requirements of captive-bred crayfish, including *P. leniusculus* (Carral et al., 2011; González et al., 2012), red swamp crayfish *Procambarus* clarkii (Hua et al., 2015) and common yabby *Cherax destructor* (Austin et al., 1997). Commercially available fish-feed pellets were historically fed to all age-classes resulting in low survival rates of hatchlings in both *A. pallipes* (Sáez-Royuela et al., 2001) and *P. leniusculus* (Ulikowski et al., 2006; Sáez-Royuela et al., 2007). *Artemia* nauplii have also been used as feed for captive-bred juvenile crayfish, as these are a readily available and easily produced substitute for insect larvae found in the diet of wild juvenile crayfish. *Artemia* nauplii are a popular first feed within aquaculture as they are high in protein and lipids and contain proteolytic enzymes, which can aid the digestive abilities of young animals (Bengtson et al., 1991). As *Artemia* will readily feed on a wide variety of food items, they provide a useful vessel for enrichment products, such as lipids and algae, to be easily incorporated (Léger et al., 1986).

Artemia spp. is deficient in some nutrients, such as poly unsaturated fatty acids and therefore is often enriched prior to feeding juveniles (Coutteau and Mourente, 1997). When hatchling *P. leniusculus* were reared using enriched live *Artemia* nauplii, high survival rates of up to 80% were achieved (González et al., 2008). After day 20, however, there was no significant difference in survival between *P. leniusculus* fed on a pellet diet and those fed live *Artemia* nauplii, although growth was significantly greater if live *Artemia* were fed up to day 50 (González et al., 2011). Following on from this research, live *Artemia* nauplii were replaced with *Artemia* cysts and fed to free-living hatchling *P. lenisuculus*, also resulting in high survival rates (81%) (González et al., 2009). Subsequently, a practical pellet feed, incorporating decapsulated *Artemia* cysts, was developed and high survival rates (86%) were achieved with hatchling *P. leniusculus* (Carral et al., 2011).

Despite the ecological importance of *A. pallipes*, and likely due to the previous lack of aquaculture of the species, commercially available diets have not been tested or developed for the species. This study presents results from a series of experiments testing different diet formulations on the growth and survival of hatchling and juvenile *A. pallipes*.

MATERIALS AND METHODS

Study Animals and Site

The crayfish used within the experiments were captive-born juvenile *A. pallipes*, hatched from 21, wild-caught, ovigerous females (collected from a local river population in South Gloucestershire, England, under Natural England licence). The females were brought into an indoor, closed-circuit, aquaculture facility (in Somerset, England), two-months prior to the experiment commencing. They were removed once the juveniles had undergone two moults and were free-living. The experiments took place within the same aquaculture facility. This comprised 24 glass tanks (0.12 m^2 bottom area; 45 L^3 total tank volume), on a closed-circuit, recirculating system including a filtration sump, with a de-gassing chamber, filled with bio-balls and a fluidised sand bed. Water returned from the lidded tanks to the sump via 2 mm meshed tanks outlets (to prevent escape) and was fed back to the tanks from the sump via an ultraviolet filtration unit. Turnover rate was four times per hour and total system water volume was 1,200 L^3. Water temperature varied between 12 and 18°C over the course of the experiments. The temperature range was controlled with coolers, to ensure there was a maximum temperature variation of <3°C, over a 24 h period. The photoperiod was ambient and averaged 12 h light and 12 h dark. Each experimental tank had a 30 mm substrate base layer of coral sand and fine gravel (0.4–1.0 mm diameter). Polycarbonate 10 mm sheeting, held down with substrate, provided refuges (two per crayfish), together with 15 mm internal diameter pipe for the larger animals. All tanks were gravel-siphoned weekly and 20% of the water was replaced with rainwater, collected within a water reservoir adjacent to the aquaculture facility. Water quality was measured weekly using a Colombo Testlab water testing kit (Aquadistri UK Ltd., Cambridgeshire, United Kingdom). Chemical levels remained consistent throughout the experiment: ammonia <0.1 mg/L,

nitrite <0.1 mg/L, nitrate <15 mg/L, phosphate 0.2 mg/L, pH 7.8, calcium ≥35 mg/L, general hardness 10 KH, potassium hardness 8 KH and a level of dissolved oxygen ≥90%.

Experimental Procedure

Four feeding experiments (A–D) took place over two breeding seasons. Experiments A and B were with juvenile (20-day-old), hatchling *A. pallipes* with an initial mean carapace length (mm ± SD) of 5.3 ± 0.14 mm. All hatchlings had undergone two moults, having fully formed mouth parts and uropods. Experiments C and D used 60-day-old crayfish, with an initial mean carapace length of 7.9 ± 0.23 mm. For each experiment, the juvenile *A. pallipes* were randomly selected and put into different treatment tanks, with six replicates of each, at varying densities for each food treatment to be trialled (**Table 1**). All experiments ran for 40 days.

Experiment C used juvenile crayfish that had not been used in experimental trials and had been fed an enriched diet of live *Artemia fransicana* nauplii and defrosted plankton since hatching. In contrast, experiment D used the same experimental crayfish that were in B as an extension of this experiment. Therefore, in experiment D, the live food component was removed after day-40 for treatments i, ii and iii, whereas treatment iv remained the same throughout both experiments B and D.

All diets, (except the pellet-only Spanish practical diet treatment, tested in experiments B and D), were enriched with 1 mL multivitamins, 1 g *Spirulina*, 1 mL lipids, plus 1 g of the carotenoid Astaxanthin, which was added to prevent the crayfish turning blue (Menasveta et al., 1993; Lorenz and Cysewski, 2000). As these enrichments were already included in the Spanish practical diet they were excluded. Additionally, the Spanish practical diet was not presented with live *Artemia* as the pellet already contained *Artemia* cysts. The defrosted plankton used in all four experiments was gamma-irradiated, prior to freezing. The *A. fransicana* nauplii were hatched from *A. fransicana* eggs and 24 h, post-hatching, were then gut-loaded with 0.1 mL *Nannochloropsis* spp., and enriched with highly unsaturated fatty acids (HUFA), for 8 h, prior to feeding to crayfish (**Supplementary Table 2**). The gel diet (for experiments A and C) was made by mixing 1.5 g of potato dextrose agar powder and 0.8 g of locust bean gum, which was added to help binding. To this mixture, 50 mL of water was added and then boiled for 1 min. The mixture was allowed to cool to 35°C before the other food items were added, to ensure that the mixture did not solidify but was cool enough to prevent the protein within the food items denaturing (Lepock, 1997). The mixture was then put in a fridge to set. For experiment A, we added 9 g of enriched plankton and 2 g of enriched decapsulated *A. fransicana* cysts to the gel. For experiment C, we added 8 g of enriched plankton and 3 g of vegetable mix (**Supplementary Table 1**), plus 1g of *Spirulina*. The pellet diets were commercially available crayfish-specific pellets (**Supplementary Table 2**). As these are commercially available diets not all content of the formulas was available. For experiments B and D, the crayfish the standard diets were made up at a ratio of three parts plankton to one part vegetable mix.

All crayfish were fed to excess, at a rate of approximately 4% of bodyweight of food per individual, presented at 18:00 daily. For hatchlings (experiments A and B), live *A. fransicana* or decapsulated *A. fransicana* cysts were fed at a rate of 500/crayfish/day; plankton, pellet or gel diets were fed at a rate of 0.01 g per day. For juveniles (experiments C and D), crayfish were fed at a rate of 0.02 g of food per animal per day. In both hatchling experiments, plankton or pellet was offered, in addition

TABLE 1 | Four feeding experiments A–D, on *A. pallipes* including dietary treatments, density equivalent, treatment replicates, duration of experiment, and age-class of animals.

Experiment	Treatments	Date	Crayfish/tank (/m^2)	Treatment replicates	Age-class
A	(i): Live *Artemia* + plankton[1] (Live + P) (ii): *Artemia* cysts + plankton[1] (Cyst + P) (iii): *Artemia* cysts + plankton[1] in gel (Gel + CP)	Jul–Aug'16	12 (100)	6	hatchlings
B	(i): Live *Artemia* + Australian pellet (Live + Aus) (ii): Live *Artemia* + New Zealand pellet (Live + NZ) (iii): Live *Artemia* + plankton[1] (Live + P) (iv): Spanish practical pellet diet (Spain)	Jun–Jul'17	16 (150)	6	hatchlings
C	(i): Plankton[2] + vegetable[3] (Standard) (ii): Plankton[2] + vegetable[3] in gel (Gel + PV)	Aug–Sep'16	12 (100)	6	60-day
D	(i): Australian pellet (Australia) (ii): New Zealand pellet (New Zealand) (iii): Plankton[2] + vegetable[3] (Standard) (iv): Spanish practical pellet diet (Spain)	Jul–Aug'17	16 (150)	6	60-day

[1] *Experiments A and B – defrosted plankton = equal proportions cyclops, Daphnia and rotifers.*
[2] *Experiments C and D – defrosted plankton = equal proportions of bloodworm, Mysis, krill and Daphnia.*
[3] *Vegetable – equal proportions of spinach, chard, peas, carrot and kale blended and frozen.*

to the live food element, recognising that a combination diet may be important for crayfish growth and survival.

All four experiments were approved by the University of Bristol's Animal Welfare and Ethical Review Board (AWERB) and the Bristol Zoological Society's Conservation, Ethics and Sustainability Committee (CESC). Wild collection of animals was carried out under Natural England crayfish survey licence and an Environment Agency trapping licence. Crayfish were maintained in captivity under a Natural England scientific handling licence. The Crayfish Research Unit was inspected and certified as a hatchery facility by the Centre for the Environment, Fisheries and Aquaculture Science (Cefas).

Data Analysis

All crayfish were counted and individually measured on day-1 and day-40 (mm ± SD) of the experiments, to determine survival and growth. Growth was quantified by the carapace length, which was measured from the anterior edge of the rostrum to the posterior edge of the cephalothorax to the nearest 0.1 mm using Vernier callipers (Moore and Wright, Sheffield). Crayfish growth in each treatment group was calculated by subtracting the starting average carapace length from the final average carapace length.

To determine if there were any significant differences between the survival (% ± SD) with the different dietary treatments, data were examined using binomial generalized linear models (function *glm*, R package *lme4*) (Bates et al., 2015). To determine if there was any difference in growth among the treatments, data were examined with an ANOVA. Goodness-of-fit to normal distributions was checked by running the Shapiro - Wilk test on residuals (Shapiro and Wilk, 1965), prior to using an ANOVA. To determine if there were differences in growth among the treatments, data were log-transformed and examined with linear mixed models (function *lmer*, R package *lme4*) (Bates et al., 2015) or ANOVA if tested at tank level. The treatments were considered as fixed effects, and tanks were considered a random effect. The alpha level was set at $p < 0.05$. Only variables that had a significant effect were retained in models. Statistical analyses were performed using R 3.2.5 (R Development Core Team, 2006).

RESULTS

Experiment A

Survival for those fed with the Live + P diet (91.7 ± 6.4%) was significantly higher than for those fed the Cyst + P (43.7 ± 23.2%) or Gel + CP (43.7 ± 8.9%) diets ($z_{285} = 6.28$, $p < 0.001$). There was no significant difference in crayfish survival between the Gel + CP and Cyst + P diet ($z_{285} = 0.06$, $p = 0.95$) (**Figure 1**).

Crayfish growth with the Live + P was significantly greater (2.4 ± 0.1 mm), than with the Cyst + P (2.0 ± 0.3 mm), or Gel + CP (2.0 ± 0.2 mm) diets ($F_{2,169} = 14.94$, $p < 0.001$). There was no significant difference in growth between the Gel + CP and Cyst + P diet (**Figure 2**).

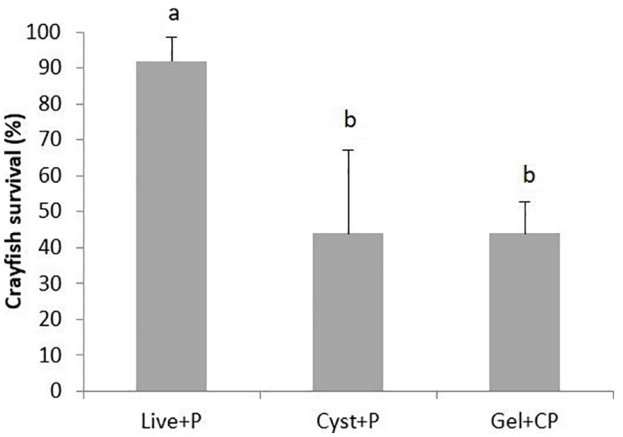

FIGURE 1 | Percentage survival of hatchling *A. palliipes*, within the different treatment diets: live *Artemia* nauplii plus plankton (Live + P); *Artemia* cysts plus plankton (Cyst + P); or *Artemia* cysts plus plankton incorporated into agar gel (Gel + CP), at day-40. A different letter denotes significance between treatments. Error bars represent standard deviations.

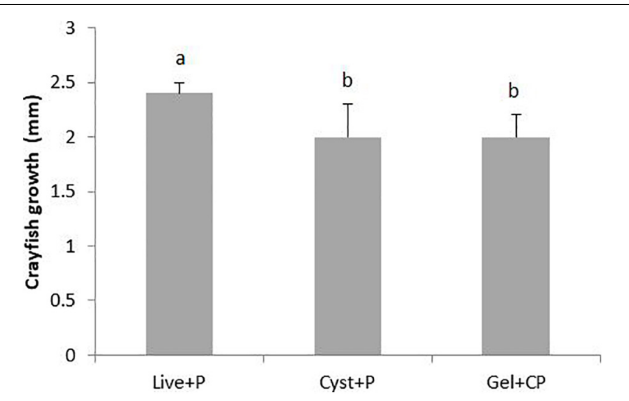

FIGURE 2 | Mean growth (final carapace length – start carapace length mm) of hatchling *A. pallipes*, within the different treatment diets: live *Artemia* nauplii plus plankton (Live + P); *Artemia* cysts plus plankton (Cyst + P); or *Artemia* cysts plus plankton incorporated into agar gel (Gel + CP), at day-40. A different letter above a treatment denotes significance. Error bars represent standard deviations.

Experiment B

Hatchling crayfish survival from day-1 to day-40 was significantly higher ($z_{383} = 2.3$, $p = 0.02$) with the Live + P diet (95.8 ± 5.1%) than with the Live + Aus (85.4 ± 12.3%) and Spain treatment diets (80.2 ± 6.1%) ($z_{383} = -3.0$, $p = 0.002$). Crayfish survival with the Live + NZ diet (91.7 ± 7.6%) was significantly higher than with the Spain diet ($z_{383} = 5.5$, $p = 0.03$) but was not significantly different from the Live + Aus diet (**Figure 3**).

From day-1 to day-40, crayfish growth was significantly greater with the Live + P treatment diet (2.5 ± 0.1 mm) than with the Live + NZ and Spain diet ($F_{3,335} = 7.1$, $p < 0.001$). There was no significant difference between growth with the other three diet treatments (mean 2.24 ± 0.06 mm) (**Figure 4**).

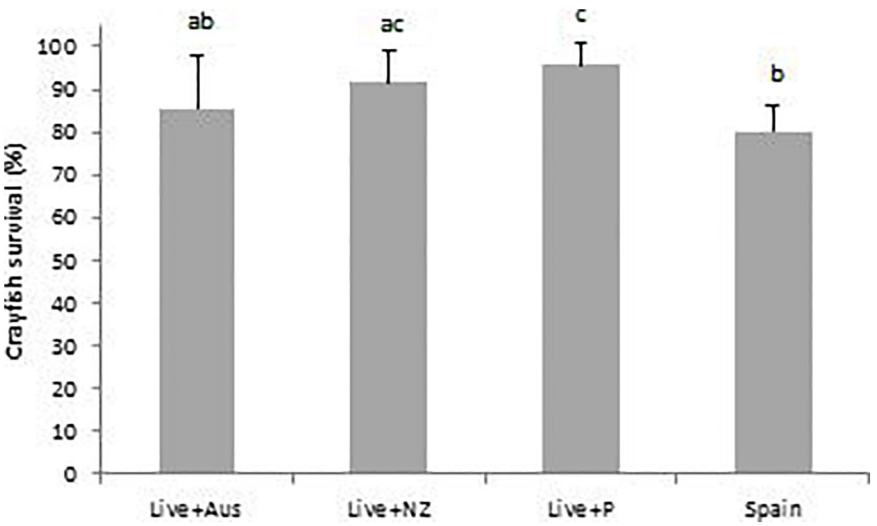

FIGURE 3 | Percentage survival of hatchling *A. pallipes* for the four different treatment diets: Live *Artemia* nauplii plus Australian pellet (Live + Aus); Live *Artemia* nauplii plus plankton (Live + P); Live *Artemia* nauplii plus New Zealand pellet (Live + NZ) and the Spanish practical crayfish pellet diet (Spain), from day-1 to day-40. A different letter above a treatment denotes significance. Error bars represent standard deviations.

Experiment C

For the juvenile *A. pallipes*, there was no significant difference in crayfish survival between Standard (97.9 ± 5.9%) and Gel + PV diet (96.9 ± 6.2%) ($t_{187} = 0.45$, $p = 0.65$) (**Figure 5**).

Crayfish growth (mm ± SD) on the Standard diet (3.1 ± 0.3 mm) was significantly greater than those on the Gel + PV diet (2.4 ± 0.2 mm), ($t_{187} = 4.38$, $p < 0.001$) (**Figure 6**).

Experiment D

For the juvenile crayfish, survival from day-40 to day-80 was significantly lower in the New Zealand treatment diet (78.4 ± 10.3%) than all the other three treatments: Australia (97.4 ± 4.1%); ($z_{383} = 3.2$, $p < 0.001$), Standard (92.5 ± 11.6%); ($z_{383} = 2.2$, $p = 0.03$) and Spain (96.3 ± 4.0%); ($z_{383} = 3.2$, $p < 0.001$). Crayfish survival was not significantly different between the other three treatment diets (**Figure 7**).

From day-40 to day-80, crayfish growth was significantly greater within the Standard treatment diet (2.5 ± 0.4 mm) than with all three other treatment diets: Australian (1.4 ± 0.3 mm, $p < 0.001$); New Zealand (2.0 ± 0.2 mm, $p = 0.01$); and Spanish diets (1.1 ± 0.3 mm, $F_{3,20} = 28.52$, $p < 0.001$). Crayfish growth with the New Zealand pellet was significantly greater than with the Australian pellet ($p = 0.01$) and Spanish practical diet ($F_{3,20} = 28.52$, $p < 0.001$) (**Figure 8**).

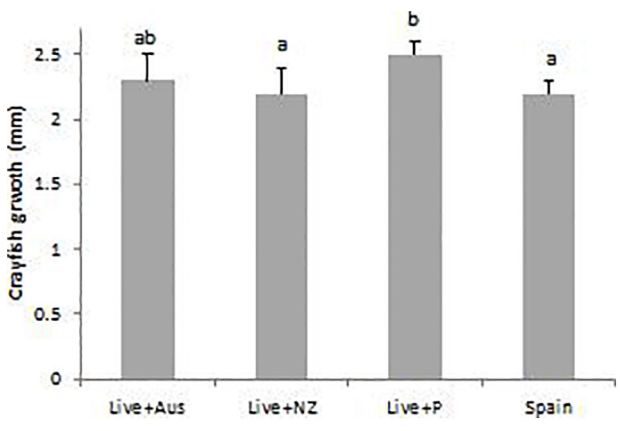

FIGURE 4 | Mean growth (final CL – start CL mm) of hatchling *A. pallipes* for the four different treatment diets: Live *Artemia* nauplii plus Australian pellet (Live + Aus); Live *Artemia* nauplii plus plankton (Live + P); Live *Artemia* nauplii plus New Zealand pellet (Live + NZ) and the Spanish practical crayfish pellet diet (Spain), from day-1 to day-40. A different letter above a treatment denotes significance. Error bars represent standard deviations.

DISCUSSION

Hatchling Survival

In both experiments with hatchling *A. pallipes* (A and B), consistently high survival rates (>85%) were achieved with crayfish fed with enriched live *Artemia* (with additional food sources). This is thought to be due to its nutritional components, having a high protein content (>50%) and levels of lipids and fatty acids (Treece, 2000). González et al. (2012), demonstrated that a level of 55% protein was optimal for survival and growth of hatchling *P. leniusculus*; with levels of over 33% required, to not compromise survival. However, the essential nutritional elements that hatchling crayfish require still remain unknown (González et al., 2011). During the first 4–6 weeks of life, the levels of digestive enzymes steadily increase as the hepatopancreas matures (Hammer et al., 2000). The proteolytic enzymes that *Artemia* nauplii contain may make digestion of dietary items easier for crayfish hatchlings and contribute to the

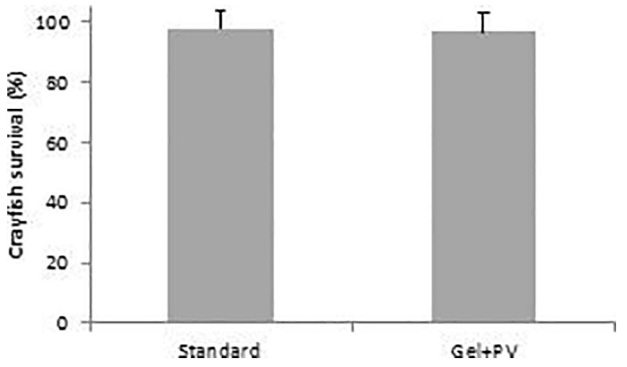

FIGURE 5 | Percentage survival of juvenile *A. pallipes*, within the different treatment diet, plankton plus vegetable (Standard) or plankton plus vegetables incorporated into agar gel (Gel + PV), between day-1 to day-40. Error bars represent standard deviations.

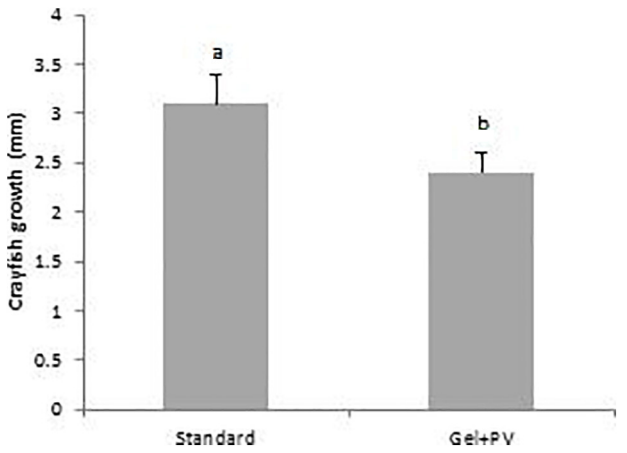

FIGURE 6 | Mean growth (final CL – start CL mm) of juvenile *A. pallipes*, within the different treatment diets, plankton plus vegetable (Standard) or plankton plus vegetable incorporated into agar gel (Gel + PV), between day-1 to day-40. A different letter above a treatment denotes significance. Error bars represent standard deviations.

maturation process of the hepatopancreas (Léger et al., 1986; Bengtson et al., 1991).

Lower survival rates occurred with *Artemia* cysts plus plankton (experiment A), than with live *Artemia*. This suggests that the nutritional content was not optimal and live *Artemia* nauplii are a more suitable first food item than *Artemia* cysts. This is potentially because prey motility is important for *A. pallipes* hatchlings with the movement of the nauplii stimulating them to feed. This is in contrast to a study on hatchling *P. leniusculus*, where no significant difference in survival between *Artemia* cysts and live *Artemia* nauplii was observed (González et al., 2009). When four age classes of yabby *Cherax destructor* were tested with live plankton versus pellet food, they spent 85% of their time feeding on live food and 15% feeding on the inert pellets (Meakin et al., 2008), demonstrating a preference for live food items. This was also observed in a study where juvenile hairy marron *Cherax tenuimanus*, when presented with both live *Daphnia* and pellet, showed a significant preference for feeding on the live food (Meakin et al., 2009). While observations on feeding preferences were not made as part of this study, a preference for live food items may be a result of their greater nutritional value. A stronger feeding response might have also been triggered by the presence of the live *A. fransicana* and therefore more food was ingested in total by the hatchlings in comparison to alternative diets.

The practical Spanish diet (experiment B), which contained *Artemia* cysts, within the pellet mix, achieved higher survival rates in hatchling crayfish than the rehydrated, decapsulated *Artemia* cysts, with or without agar gel. The higher survival rate in trials, which contained live elements, suggests there were some nutritional elements lacking from the non-live treatment diets, which could be due to the quality of the original food elements (such as the cysts) or the preparation. For example, in a study by Kouba et al. (2011), industrially decapsulated *Artemia* cysts produced lower growth and survival rates than freshly decapsulated cysts fed to six-month-old noble crayfish *Astacus astacus*.

Artemia cysts like *Artemia* nauplii also contain proteolytic enzymes. In a study by Garcı́a-Ortega et al. (1998) the composition of both *Artemia* cysts and newly hatched *Artemia* nauplii was investigated. There was little difference in protease enzyme activity at the time of hatching; however, the enzyme levels increased as the nauplii developed. In our experiments, we fed the *Artemia* nauplii at 30 + hours post-hatching. When newly hatched, the *Artemia* nauplii lack exogenous mouth parts (Sorgeloos et al., 2001). We therefore grew on the nauplii until they had sufficiently developed mouth parts, to enable them to feed on the food items offered. From Garcı́a-Ortega et al. (1998), protease activity was elevated and increasing up until the end of their study (7-hours post-hatching), suggesting that this proteolytic enzyme levels would increase with older nauplii, which could help to explain the increase in growth and survival that we experienced in our experiments.

Hatchling survival was lowest in the Spanish diet compared to the other treatment diets and survival was highest in the Live + P diet (experiment B). Due to the potential for increased food consumption in the presence of live feed, the exclusion of live *Artemia* when animals were fed the Spanish practical diet may have limited consumption in comparison to other pellet diets tested.

Hatchling Growth

Growth of the hatchlings (experiment A) was significantly greater when fed live *Artemia* nauplii plus plankton diet (Live + P) in comparison to decapsulated *Artemia* cysts plus plankton (Cyst + P). This was despite significantly lower numbers of crayfish within the cyst treatment groups during this time period due to mortalities. Previous studies have shown there is an increase in growth with a reduction in crayfish density (Nightingale et al., 2018), which was not observed in this case, and therefore suggests that diet was a limiting factor. Growth of the hatchlings (experiment B) was also significantly greater when fed live *Artemia* nauplii plus plankton diet (Live + P) than when they were fed the Live + NZ or Spanish diets.

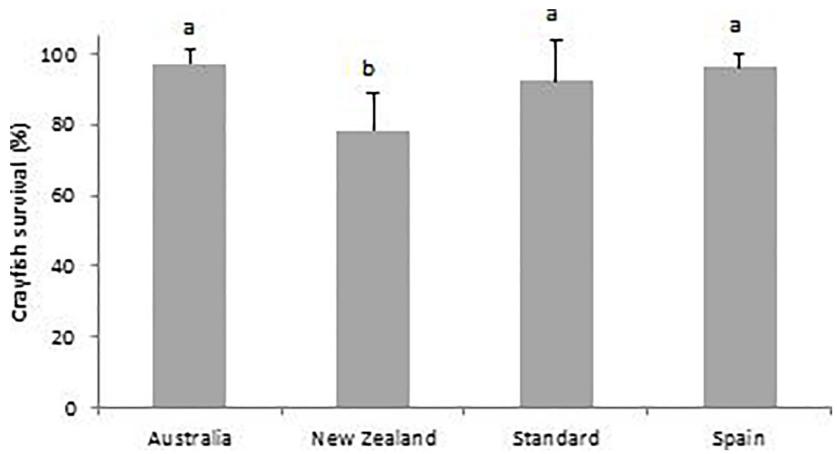

FIGURE 7 | Percentage survival of juvenile *A. pallipes* for the four different treatment diets: Australian pellet (Australia); New Zealand pellet (New Zealand); plankton plus vegetable (Standard); and the Spanish practical crayfish diet (Spain); from day-40 to day-80. A different letter above a treatment denotes significance. Error bars represent standard deviations.

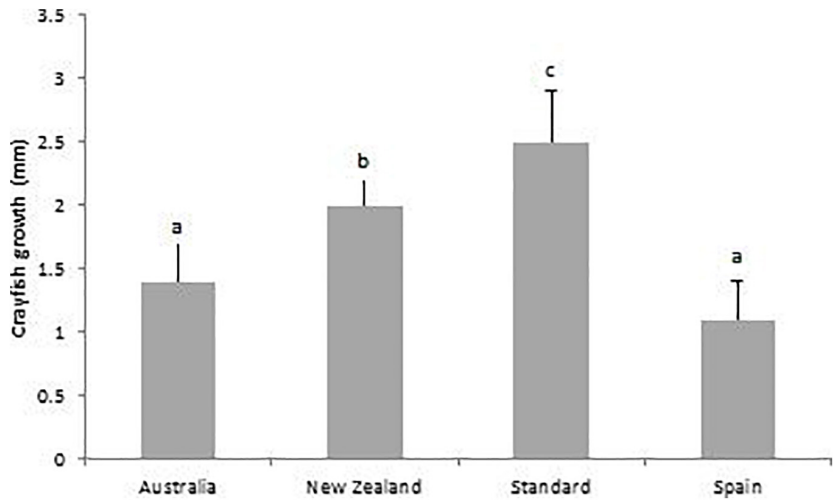

FIGURE 8 | Mean growth (final CL – start CL mm) of juvenile *A. pallipes* for the four different treatment diets: Australian pellet (Australia); New Zealand pellet (New Zealand); plankton plus vegetables (Standard); and the Spanish practical crayfish diet (Spain), from day-40 to day-80. A different letter above a treatment denotes significance. Error bars represent standard deviations.

The survival and growth of hatchling *A. pallipes* fed live food is consistent with previous studies on *P. leniusculus* (Sáez-Royuela et al., 2007) and *C. destructor* (Austin et al., 1997) where growth and survival of hatchling crayfish was significantly higher when fed live food rather than other treatment diets. The fact that hatchling survival and growth was improved with live food and plankton is supported by studies of *A. pallipes* analysing gut contents, which found that juvenile crayfish ingest a high proportion of invertebrates in their diet (Gherardi et al., 2004; Reynolds and O'Keeffe, 2005).

Juvenile Survival

There was no difference in survival between the treatments in experiment B, indicating that the critical life phase was over. However, in experiment D, crayfish fed the New Zealand pellet diet had significantly lower survival rates than the three other treatment diets, suggesting there may be some nutritional element lacking within this particular diet for *A. pallipes* juveniles. There are lower levels of protein in the New Zealand diet, compared to the other pellet feeds, which may result in lower survival rates. Mortalities were often observed during the moulting stage, where a lack of adequate nutrition can cause crayfish to die whilst moulting due to a lack of sufficient energy (Bowser and Rosemark, 1981).

Juvenile Growth

Crayfish growth was significantly higher in the Standard diet than in the Standard diet encapsulated in agar diet (experiment

C), suggesting that the crayfish were not ingesting the same food items in the same quantities. In experiment D, crayfish growth in the Standard diet was significantly greater than in the all three other pellet treatment diets, suggesting that the crayfish were either eating more or better quality food items. In contrast to juvenile survival rates, the New Zealand pellet diet elicited better growth when compared to the Australian and Spanish diets; although this may have been due to cannibalism in the New Zealand pellet trial tanks, as this corresponded to a reduction in crayfish numbers within the tanks and not all the mortalities were accounted for. The crayfish may have been attacking moulting crayfish due to a nutritional deficiency within the diet (synchronised moulting was not occurring and therefore some crayfish were susceptible to cannibalism at this time). Alternatively, the reduced numbers of crayfish in the treatment tanks, due to the lower survival rate, caused an increase in growth of the remaining individuals (Savolainen et al., 2004; Nightingale et al., 2018).

The reduction in *A. pallipes* growth experienced with several of the treatment diets from day 40-80 is typical for this species. In a recent paper by Nightingale et al. (2018), a 240-day study of *A. pallipes* hatchlings, found growth was highest in the first 60-days (47.1% ± 6.6%). Growth then dropped to an average 14.1% ± 5.8% increase with every 60-day increment. This corresponds to other crayfish studies, which found that smaller crayfish grow faster than larger crayfish (Evans and Jussila, 1997; Jones and Ruscoe, 2000).

Agar Treatments

Crayfish graze periodically; therefore, if their food source is encapsulated within a gel, it should not degrade as quickly in water and should retain both its palatability and nutritional value for longer. This is supported by a study investigating survival and growth in juvenile (10 g) white yabby *Cherax albidus*. The crayfish were either fed fish and potatoes or this fresh food was encapsulated within pectin, alginate, agar or chitosan. There was a significant increase in growth when using the gel diets in comparison to a fresh food diet without gel (Coccia et al., 2010). However, this was not the case in our experiments (A and C). The agar may not be suitable as an early food item for *A. pallipes* hatchlings and juveniles, performing poorly in contrast to the plankton diets in both experiments A and C. Crayfish may be consuming too much agar and not enough of the other dietary, protein-based items at a life-stage where protein is an important dietary component.

Palatability

Studies on the palatability of diets have shown crustaceans will increase their feeding when particular stimulants are added to their diet (Harpaz et al., 1987; Hua et al., 2015). Crustacean species within aquaculture have preferences for specific textures and softness of food items (Cox and Johnston, 2003). In the case of both the agar-encapsulated (experiments A and C) and the pellet diets (experiments B and D), versus the plankton diets (experiments A–D), the plankton may be more palatable to the crayfish, in terms of both taste and texture, and therefore more is ingested, which increases growth.

Ontogenetic Dietary Changes

It has been suggested that as crayfish species mature they become predominantly detritivores (Paglianti and Gherardi, 2004); however, isotopic analysis of tissue suggests that aquatic invertebrates form a significant part of the diet throughout all age classes as found in koura *Paranephrops zealandicus* (Hollows et al., 2002) and *P. leniusculus* (Stenroth et al., 2006). A recent study investigated reproductive ability and growth in *P. clarkii* showed there was a significant increase in both fecundity and specific growth rate when zooplankton diets were offered rather than a commercial pellet feed. It indicates that the importance of zooplankton within crayfish diets at all age classes should not be underestimated (Sonsupharp and Dahms, 2017). This could explain why even in the older crayfish growth was still improved with the Standard diet, which was still predominantly consisting of plankton.

CONCLUSION

Enriched live diets are important for high survival and growth of *A. pallipes* from when the hatchlings are free-living, up until day-60, after which time the hepatopancreas and associated proteolytic enzymes are more mature. The hatchling may be using the proteolytic enzymes from the *A. fransicanca* to aid digestion at this early life stage. The enriched plankton diet consistently produced high rates of survival and growth in *A. pallipes* in both the hatchling and juvenile experiments (A–D), in comparison to all other diets offered, which suggests that nutritional quality and palatability is optimal. This is supported by studies of wild-caught *A. pallipes* juveniles, which consume a diet of predominantly aquatic invertebrates (Scalici and Gibertini, 2007). Therefore, it is possible that pellets do not give optimal nutrition in comparison to a more natural diet and the *A. pallipes* juveniles are showing a natural tendency to consume plankton rather than the artificial feeds offered. Evidence suggests that zooplankton may be important within crayfish diets at all age classes Hollows et al., 2002; Reynolds and O'Keeffe, 2005; Stenroth et al., 2006; Sonsupharp and Dahms, 2017). Therefore further research is required, to assess the long-term effects on growth, survival and fecundity of *A. pallipes* offered a solely pellet-based diet, rather than more natural food items.

The pellet diets used in this study were commercially available crayfish-specific pellets. As these are commercially produced diets, not all content and quantities of the formulas was available from the suppliers. However, the percentage values for proteins and lipids were provided for all diets; these are the components of the diets that have been discussed.

Pellet diets are a cheaper and more convenient option than producing bio-secure live food or gamma-irradiated frozen plankton. Where time and financial constraints are not an issue, feeding live *Artemia* plus enriched plankton for the first 40-days and then moving on to an enriched plankton diet is optimal for high survival and growth in *A. pallipes*. However, if *A. pallipes* are to be produced on a larger-scale, it may be more efficient to offer the practical crayfish pellet diet for all life-stages, but growth, survival and potentially fecundity may be compromised.

All the diets trialled in these experiments have been specifically designed for particular species of freshwater crayfish, which may have different nutritional requirements, and therefore there may not be a specific diet that will work for all the species that are bred and reared. Therefore, for juvenile *A. pallipes* it is suggested that an enriched plankton and vegetable diet, with a live food element during the first weeks, will produce the best survival and growth.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Bristol's Animal Welfare and Ethical Review Board (AWERB) and the Bristol Zoological Society's Conservation, Ethics and Sustainability Committee (CESC).

AUTHOR CONTRIBUTIONS

JN designed and orchestrated the research and wrote up the findings. GM, PS, and GJ provided advice regarding the experimental design and analysis and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the Mohamed Bin Zayed Species Conservation Fund and Bristol Zoological Society.

ACKNOWLEDGMENTS

Thanks to Jose Carral, John Hollows, and Damian Rigg for allowing your crayfish diets to be trialled in the experiments. Special thanks to Jonathan Williams and Timothy Clements for assisting with husbandry care, during the course of the four experiments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.607100/full#supplementary-material

REFERENCES

Austin, C. M., Jones, P. L., Stagnitti, F., and Mitchell, B. D. (1997). Response of the yabby, cherax destructor clark, to natural and artificial diets: phenotypic variation in juvenile growth. *Aquaculture* 149, 39–46. doi: 10.1016/s0044-8486(96)01429-9

Bates, D., Maechler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models using Lme4. *J. Stat. Softw.* 67, 1–48.

Bengtson, D. A., Léger, P., and Sorgeloos, P. (1991). Use of Artemia as a food source for aquaculture. in *Artemia Biology*, eds R. A. Browne, P. Sorgeloos, C. N. A. Trotman. (Boca Raton, FL: CRC Press)11, 255–285. doi: 10.1201/9781351069892-11

Bowser, P. R., and Rosemark, R. (1981). Mortalities of cultured lobsters, *Homarus*, associated with a moult death syndrome. *Aquaculture* 23, 11–18. doi: 10.1016/0044-8486(81)90003-x

Carral, J. M., Gonzalez, A., Celada, J. D., Saez-Royuela, M., Garcia, V., and Gonazalez, R. (2011). Proposal of a practical diet for juvenile astacid crayfish studies from the onset of exogenous feeding under controlled conditions. *Knowl. Manag. Aquat. Ecosyst.* 401, 20–28. doi: 10.1051/kmae/2011034

Celada, J. D., Fuertes, J. B., Carral, J. M., Sáez-Royuela, M., González, Á, and González-Rodríguez, Á (2012). Effects of vitamin C inclusion in practical diets on survival and growth of juvenile crayfish (*Pacifastacus leniusculus* Dana, Astacidae) from the onset of exogenous feeding. *Aquac. Nutr.* 19, 110-116.

Coccia, E., Santagata, G., Malinconico, M., Volpe, M. G., Di Stasio, M., and Paolucci, M. (2010). *Cherax albidus* juveniles fed polysaccharide-based pellets: rheological behaviour effect on growth. *Freshw. Crayfish* 17, 13–18.

Coutteau, P., and Mourente, G. (1997). Lipid classes and their content of n-3 highly unsaturated fatty acids (HUFA) in *Artemia franciscana* after hatching, HUFA-enrichment and subsequent starvation. *Mar. Biol.* 130, 81–91. doi: 10.1007/s002270050227

Cox, S., and Johnston, D. (2003). Feeding biology of spiny lobster larvae and implications for culture. *Rev. Fish. Sci.* 11, 89–106. doi: 10.1080/713610924

Evans, L., and Jussila, J. (1997). Freshwater crayfish growth under culture conditions: proposition for a standard reporting approach. *J. World Aquac. Soc.* 28, 11–19. doi: 10.1111/j.1749-7345.1997.tb00956.x

Figueiredo, M., and Anderson, A. (2003). Ontogenetic changes in digestive proteases and carbohydrases from the Australian freshwater crayfish, redclaw *Cherax quadricarinatus* (Crustacea, Decapoda, Parastacidae). *Aquac. Res.* 34, 1235–1239. doi: 10.1046/j.1365-2109.2003.00929.x

Garcıa-Ortega, A., Verreth, J. A. J., Coutteau, P., Segner, H., Huisman, E. A., and Sorgeloos, P. (1998). Biochemical and enzymatic characterization of decapsulated cysts and nauplii of the brine shrimp *Artemia* at different developmental stages. *Aquaculture* 161, 501–514. doi: 10.1016/s0044-8486(97)00297-4

Gherardi, F., Acquistapace, P., and Santini, G. (2004). Food selection in freshwater omnivores: a case study of crayfish Austropotamobius pallipes. *Arch. Hydrobiol.* 159, 357–376. doi: 10.1127/0003-9136/2004/0159-0357

González, A., Celada, J. D., González, R., García, V., Carral, J. M., and Sáez-Royuela, M. (2008). *Artemia* nauplii and two commercial replacements as dietary supplement for juvenile signal crayfish, *Pacifastacus leniusculus* (Astacidae), from the onset of exogenous feeding under controlled conditions. *Aquaculture* 281, 83–86. doi: 10.1016/j.aquaculture.2008.06.015

González, Á, Celada, J. D., Sáez-Royuela, M., González, R., Carral, J. M., and García, V. (2012). Response of juvenile astacid crayfish (*Pacifastacus leniusculus*) to three commercial dry diets with different protein levels during the first 6 months of intensive rearing. *Aquac. Res.* 43, 99–105. doi: 10.1111/j.1365-2109.2011.02808.x

González, R., Celada, J., Carral, J., García, V., Sáez-Royuela, M., and González, A. (2011). Intensive rearing of juvenile crayfish (*Pacifastacus leniusculus*, Astacidae) during the first 6 months: effects of size grading. *Aquac. Res.* 42, 1385–1392. doi: 10.1111/j.1365-2109.2010.02732.x

González, R., Celada, J., Carral, J., González, A., Sáez-Royuela, M., and García, V. (2009). Decapsulated *Artemia* cysts as dietary supplement for juvenile crayfish (*Pacifastacus leniusculus,* Astacidae) at different food supply frequencies from the onset of exogenous feeding under controlled conditions. *Aquaculture* 295, 200–204. doi: 10.1016/j.aquaculture.2009.07.009

Hammer, H. S., Bishop, C. D., and Watts, S. A. (2000). Activities of three digestive enzymes during development in the crayfish *Procambarus clarkii* (Decapoda). *J. Crustac. Biol.* 20, 614–620. doi: 10.1651/0278-0372(2000)020[0614:aotded]2.0.co;2

Harpaz, S., Kahan, D., Galun, R., and Moore, I. (1987). Response of freshwater prawn *Macrobrachuim rosenbergii* to chemical attractants. *J. Chem. Ecol.* 13, 1957–1965. doi: 10.1007/bf01014678

Hollows, J., Townsend, C. R., and Collier, K. (2002). Diet of the crayfish *Paranephrops zealandicus* in bush and pasture streams: insights from stable isotopes and stomach analysis. *N. Z. J. Mar. Freshwater Res.* 36, 129–142. doi: 10.1080/00288330.2002.9517076

Hua, X. M., Shui, C., He, Y. D., Xing, S. H., Yu, N., Zhu, Z. Y., et al. (2015). Effects of different feed stimulants on freshwater crayfish (*Procambarus clarkii*), fed diets with or without partial replacement of fish meal by biofeed. *Aquac. Nutr.* 21, 113–120. doi: 10.1111/anu.12148

Jones, C., and Ruscoe, I. (2000). Assessment of stocking size and density in the production of redclaw crayfish, *Cherax quadricarinatus* (von Martens) (Decapoda: Parastacidae), cultured under earthen pond conditions. *Aquaculture* 189, 63–71. doi: 10.1016/s0044-8486(00)00359-8

Kouba, A., Hamáčkov, J., Buřič, M., Policar, T., and Kozák, P. (2011). Use of three forms of decapsulated *Artemia* cysts as food for juvenile noble crayfish (*Astacus astacus*). *Czech J. Anim. Sci.* 3, 114–118. doi: 10.17221/1301-cjas

Léger, P. H., Bengtson, D., Simpson, K., and Sorgeloos, P. (1986). The use and nutritional value of *Artemia* as a food source. *Oceanogr. Mar. Biol. Annu Rev.* 24, 521–623.

Lepock, J. R. (1997). Protein denaturation during heat shock. *Adv. Mol. Cell Biol.* 19, 223–259. doi: 10.1016/s1569-2558(08)60079-x

Lorenz, T., and Cysewski, G. (2000). Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. *Trends Biotechnol.* 4, 160–167. doi: 10.1016/s0167-7799(00)01433-5

Meakin, C., Qin, J., and Mair, G. (2008). Feeding behaviour, efficiency and food preference in yabbies *Cherax destructor*. *Hydrobiologia* 605, 29–35. doi: 10.1007/s10750-008-9297-0

Meakin, C., Qin, J., and Mair, G. (2009). Zooplankton predation and consumption by freshwater crayfish, *Cherax tenuimanus*, at different sizes and light conditions. *J. Freshwater Ecol.* 24, 159–167. doi: 10.1080/02705060.2009.9664276

Menasveta, P., Worawattanamateekul, W., Latscha, T., and Clark, J. (1993). Correction of black tiger prawn (*Penaeus monodon* Fabricius) colouration by Astaxanthin. *Aquac. Eng.* 12, 203–213. doi: 10.1016/0144-8609(93)90012-z

Nightingale, J., Stebbing, P., McCabe, G., and Jones, G. (2018). Determining an effective density regime for rearing juvenile *Austropotamobius pallipes* in a small-scale closed system hatchery. *Aquac. Res.* 49, 3055–3062. doi: 10.1111/are.13766

Nightingale, J., Stebbing, P., Sibley, P., Brown, O., Rushbrook, B., and Jones, G. (2017). The use of ark sites and associated conservation measures to secure the long-term survival of white-clawed crayfish in the UK. *Int. Zoo Year J.* 53, 50–68. doi: 10.1111/izy.12161

Paglianti, A., and Gherardi, F. (2004). Combined effects of temperature and diet on growth and survival of young of year crayfish: a comparison between indigenous and invasive species. *J. Crustac. Biol.* 24, 140–148. doi: 10.1651/c-2374

R Development Core Team (2006). *R: A language And Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. ISBN 3-900051-07-0, URL http://www.R-project.org

Reynolds, J. D. (2002). "Growth and reproduction," in *In Biology and Freshwater Crayfish*, ed. D. M. Holdich (Oxford: Blackwell Science), 152–191.

Reynolds, J. D., and O'Keeffe, C. (2005). Dietary patterns in stream-and lake-dwelling populations of *Austropotamobius pallipes*. *Bull. Fr. Pêche Piscic.* 376-377, 715–730. doi: 10.1051/kmae:2005028

Sáez-Royuela, M., Carral, J., Celada, J., and Pérez, J. (2001). Effects of shelter type and food supply frequency on survival and growth of stage-2 juvenile white-clawed crayfish (*Austropotamobius pallipes* Lereboullet) under laboratory conditions. *Aquac. Int.* 9, 489–497.

Sáez-Royuela, M., Carral, J., Celada, J., Pérez, J., and González, A. (2007). Live feed as a supplement from the onset of external feeding of juvenile signal crayfish (*Pacifastacus leniusculus* Dana Astacidae) under controlled conditions. *Aquaculture* 269, 321–327. doi: 10.1016/j.aquaculture.2007.04.053

Savolainen, R., Ruohonen, K., and Tulonen, J. (2004). Effect of stocking density on growth, survival and cheliped injuries of stage 2 juvenile signal crayfish *Pacifastacus leniusculus* Dana. *Aquac. Res.* 231, 237–248. doi: 10.1016/j.aquaculture.2003.09.045

Scalici, M., and Gibertini, G. (2007). Feeding habits of the crayfish *Austropotamobius pallipes* (Decapoda, Astacidae) in a brook in Latium (central Italy). *Ital. J. Zool.* 74, 157–168. doi: 10.1080/11250000701248688

Shapiro, S., and Wilk, M. (1965). An analysis of variance test for normality (complete samples). *Biometrika* 52, 591–611. doi: 10.1093/biomet/52.3-4.591

Sibley, P. J., Holdich, D. M., and Richman, N. (2011). "Monitoring the global status of crayfish, with particular reference to the white-clawed crayfish," in *Proceedings of the Species Survival: Securing White-Clawed Crayfish in a Changing Environment, Conference Held on 16th and 17th November 2010*, (Bristol), 45–54.

Sonsupharp, S., and Dahms, H. U. (2017). Effect of frozen zooplankton feed on growth and reproductive performance of crayfish (*Procambarus clarkii*). *Int. J. Agric. Technol.* 13, 2317–2324.

Sorgeloos, P., Dhert, P., and Candreva, P. (2001). Use of the brine shrimp, *Artemia* spp., in marine fish larviculture. *Aquaculture* 200, 147–159. doi: 10.1016/s0044-8486(01)00698-6

Stenroth, P., Holmqvist, N., Nyström, P., Berglund, O., Larsson, P., and Granéli, W. (2006). Stable isotopes as an indicator of diet in omnivorous crayfish (*Pacifastacus leniusculus*): the influence of tissue, sample treatment, and season. *Can. J. Fish. Aquat. Sci.* 63, 821–831. doi: 10.1139/f05-265

Treece, G. (2000). Artemia production for marine larval fish culture. *South. Reg. Aquac. Centre* 702, 1–8. doi: 10.1007/978-1-4615-4911-6_1

Ulikowski, D., Krzywosz, T., and Śmietana, P. (2006). A comparison of survival and growth in juvenile *Astacus leptodactylus* (Eschscholtz) and *Pacifastacus leniusculus* (Dana) under controlled conditions. *Bull. Fr. Pêche Piscic.* 38, 1245–1253. doi: 10.1051/kmae:2006023

Conflict of Interest: PS was employed by the company APEM Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 04 March 2021
doi: 10.3389/fevo.2021.621613

Not Only Environmental Conditions but Also Human Awareness Matters: A Successful Post-Crayfish Plague Reintroduction of the White-Clawed Crayfish (*Austropotamobius pallipes*) in Northern Italy

Raoul Manenti[1,2], Benedetta Barzaghi[1], Alessandro Nessi[1]*, Sara Cioccarelli[1], Mauro Villa[3] and Gentile Francesco Ficetola[1,4]*

[1] Department of Environmental Science and Policy, Faculty of Science and Technology, Università degli Studi di Milano, Milan, Italy, [2] Laboratory of Subterranean Biology "Enrico Pezzoli," Parco Regionale del Monte Barro, Galbiate, Italy, [3] Parco Regionale del Monte Barro, Galbiate, Italy, [4] University Grenoble Alpes, CNRS, Université Savoie Mont Blanc, LECA, Laboratoire d'Ecologie Alpine, Grenoble, France

OPEN ACCESS

Edited by:
Japo Jussila,
University of Eastern Finland, Finland

Reviewed by:
Adam Petrusek,
Charles University, Czechia
William Perry,
Illinois State University, United States

***Correspondence:**
Raoul Manenti
raoulmanenti@gmail.com;
raoul.manenti@unimi.it
Alessandro Nessi
alessandro.nessi93@gmail.com

Specialty section:
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *26 October 2020*
Accepted: *26 January 2021*
Published: *04 March 2021*

Citation:
Manenti R, Barzaghi B, Nessi A, Cioccarelli S, Villa M and Ficetola GF (2021) Not Only Environmental Conditions but Also Human Awareness Matters: A Successful Post-Crayfish Plague Reintroduction of the White-Clawed Crayfish (Austropotamobius pallipes) in Northern Italy. Front. Ecol. Evol. 9:621613. doi: 10.3389/fevo.2021.621613

In Europe, invasive freshwater crayfish are not only changing freshwater ecosystems, but they are also leading to local extinctions of native freshwater crayfish. This is particularly evident for the populations of red swamp crayfish and spiny-cheek crayfish in northern Italy, which are threatening the last and isolated populations of the white-clawed crayfish. Here, we describe the steps that accompanied a successful reintroduction of the white-clawed crayfish in an Italian stream (Park Monte Barro) that, although isolated from other freshwater sites, suffered from an illegal introduction of the spiny-cheek crayfish in 2013. After the removal of presumably all the introduced spiny-cheek crayfish individuals, we started periodical surveys (twice a year) of the stream to confirm the absence of further introductions and to monitor environmental conditions. Prior to the reintroduction of the white-clawed crayfish that started in autumn 2018, we developed an intense dissemination activity to raise awareness of white-clawed crayfish features and importance among landowners surrounding the stream, including those suspected of the introduction of the spiny-cheek crayfish: we organized public meetings and we performed seven direct visits, house to house, to the local people providing information on good practices for white-clawed crayfish conservation. From 2018 to 2020, every autumn, we reintroduced a batch of 3-month-old white-clawed crayfish juveniles, and we developed a program for the monitoring of crayfish growth and density, water quality, and direct landowners' disturbance of the site. We detected a significant increase of the white-clawed crayfish total length (TL) from the first reintroduction (October 2018) to June 2020. In 2020, crayfish were consistently larger than in the 2019 surveys; some of them were able to breed less than 2 years after the first reintroduction. In 2020, the estimated density of large crayfish reached 0.57 individuals/m^2, which is lower than the

density observed prior to extinction. We did not detect any case of human disturbance of the site. Our results underline that the reintroduction actions could be more effective when the stakeholders having the greatest potential impact on the species are identified, informed, and involved as primary caretakers of the activities.

Keywords: invertebrate, restoration, conservation, freshwater, *Faxonius limosus*

INTRODUCTION

Crayfish species are important components of freshwaters' biodiversity, which play key roles in the food web and can provide important services (e.g., nutrient recycling and structural diversification) for aquatic ecosystems (Gherardi et al., 2003; Manenti et al., 2019b; Unger et al., 2020). Moreover, freshwater crayfish have a relevant economic and cultural value; their management, thus, has an impact on the preservation of food resources and cultural heritages (Gherardi and Souty-Grosset, 2006; Manenti et al., 2019b). This relevance for humans has heavily shaped the geographical distribution of some European species because of human activities and human-mediated translocations (Souty-Grosset et al., 1997, 2006). Along with an increasingly globalized trade, crayfish introductions outside their natural range have increased dramatically, with the growing spread of multiple species of invasive crayfish, that are threatening native biodiversity worldwide (Nishijima et al., 2017; Manenti et al., 2020).

Multiple American crayfish species are invading European freshwaters, causing an impact on freshwater ecosystems and causing several local extinctions of native freshwater crayfish species (Kouba et al., 2014; Strand et al., 2019). North American crayfish are chronic carriers of the oomycete *Aphanomyces astaci*, the crayfish plague pathogen, which causes this severe disease in susceptible taxa. Crayfish plague is responsible for extensive mass deaths of native European crayfish that often have a 100% rate of mortality (Svoboda et al., 2017; Caprioli et al., 2018; Martín-Torrijos et al., 2019). Extinctions of native crayfish populations caused by the crayfish plague have been reported since the mid-19th century (Alderman, 1996). The first mass mortalities occurred in northern Italy (Lombardy region) in 1859. Since then, numerous crayfish plague outbreaks have been reported throughout Europe and are still continuing today (Bland, 2017). The spread of crayfish plague initially followed the crayfish trade and the location of rearing facilities established across Europe (Souty Grosset et al., 2006). Nowadays, at least nine species of North American crayfish are well established in Europe (Kouba et al., 2014; Weiperth et al., 2017); among them, the most widespread are an astacid, the signal crayfish (*Pacifastacus leniusculus*), and three cambarids, the red swamp crayfish (*Procambarus clarkii*), the spiny-cheek crayfish (*Faxonius limosus*), and the marbled crayfish (*Procambarus virginalis*) (Kouba et al., 2014; Lo Parrino et al., 2020). With the recent observation of the cambarid *Cambarellus patzcuarensis* in Hungary, the Central and Western European indigenous crayfish species have been strongly outnumbered by non-indigenous species (Weiperth et al., 2017, 2020).

The patterns of crayfish invasion are complex and have tremendous effects on the spread of crayfish plague. For instance, in central Italy, multiple genotype groups of *A. astaci* have been identified, suggesting the existence of multiple infection sources associated with alien crayfish host species even when they are not widespread in the area (Caprioli et al., 2018). In northern Italy, populations of invasive crayfish (red swamp and spiny-cheek crayfish) are threatening the last and isolated populations of the native white-clawed crayfish (*Austropotamobius pallipes*) (Manenti et al., 2014). On the one hand, invasive crayfish are able to naturally disperse and colonize nearby suitable sites (Siesa et al., 2011). However, sites naturally colonized by these two alien crayfish are ecologically different from those occupied by white-clawed crayfish populations (Gil-Sanchez and Alba-Tercedor, 2006; Manenti et al., 2014; Chucholl, 2016). Both alien species, in fact, mostly select small lakes or downstream streams and rivers that are not used by the native crayfish, or from where the native crayfish disappeared long time ago because of pollution and the crayfish plague infections that occurred during the 19th century (Gherardi and Barbaresi, 2000; Ghia et al., 2013; Manenti et al., 2014). Even in catchment basins in which alien crayfish appear, the presence of barriers, such as waterfalls, can prevent the spread of invasive crayfish and crayfish plague outbreaks (Manenti et al., 2019b). On the other hand, local citizens may become, intentionally or accidentally, the main vectors of both alien crayfish and *A. astaci* in areas where the native crayfish still exist. This is due to the long tradition of crayfish consumption as food in Europe (Gherardi, 2011). Furthermore, in northern Italy, the white-clawed crayfish still have value as a cultural heritage, and numerous people pay attention to existing populations or sites in which they observed crayfish when they were younger (Manenti, 2006), even though this attention can be detrimental, and occasional poaching occurs (Manenti, 2006). Unfortunately, local people are often unaware of the occurrence of alien crayfish species and the disease they carry, or are unable to distinguish the invasive from native species. Unaware stakeholders can thus bring alien crayfish in sites where the native white-clawed crayfish still survive, causing their extinction due to *A. astaci* outbreaks (Bonelli et al., 2017). After infection, the spread of *A. astaci* in an astacid population cannot be stopped as *A. astaci* sporulation is particularly high in dying crayfish (Makkonen et al., 2013), and affected crayfish can disperse further the disease (Souty Grosset et al., 2006), even if the occurrence of unaffected refuges in the same catchment basin can allow species persistence and recovery (Kozubíková-Balcarová et al., 2014).

Nevertheless, once a native crayfish population is extinct, restoration actions are feasible if alien crayfish species are not present or, in the rarer cases, if they can be totally eradicated

(Jourdan et al., 2019). *A. astaci* has indeed a limited life span (from a few hours to weeks) in the absence of crayfish or other suitable alternative hosts like freshwater crabs (Souty Grosset et al., 2006; Svoboda et al., 2014, 2020; Jussila et al., 2020). In sites where alien crayfish or freshwater crabs are void, the pathogen can thus disappear (Souty Grosset et al., 2006). Despite Europe-wide newscasts and internet reporting of many ongoing reintroduction actions of the white-clawed crayfish, the number of peer-reviewed studies reporting information on failure or success of reintroduction actions is surprisingly low. Published papers (**Table 1**) and reintroduction plans (Diéguez-Uribeondo et al., 1997; Kemp et al., 2003; Marquis, 2006) highlight water quality and the presence or absence of further crayfish plague outbreaks as key determinants of reintroduction success. Habitat preferences of the white-clawed crayfish are well known and include good water quality and high diversity of microhabitats which provide shelters for both adults and juveniles (Holdich et al., 1999). Assessing the presence of ephemeropteran communities is a simple way to identify brooks for white-clawed crayfish restocking, as mayflies are good indicators of their requirements (Grandjean et al., 2011; Jandry et al., 2014). To avoid further outbreaks of the crayfish plague, reintroductions are generally performed some years after the extinction and in places where no alien crayfish species occur (Spink and Frayling, 2000; Durlet et al., 2009). Conversely, we found no published studies on the role played by the stakeholders (fishermen, landowners) of the reintroduction sites in allowing successful crayfish recovery in Italy, even though it is increasingly evident that local stakeholders play a key role in the success of all conservation actions (Perino et al., 2019). With this paper, we describe a recent case of white-clawed crayfish reintroduction, in which a key aspect was the involvement of local stakeholders. The reintroduction action was performed in a stream of a protected area in northern Italy, that, although isolated from other freshwater sites, was affected in 2013 by the introduction of adults of the spiny-cheek crayfish (Bonelli et al., 2017); after the introduction, the whole white-clawed crayfish population that lived in the stream went extinct in less than 15 days, likely because of crayfish plague outbreak (Bonelli et al., 2017). With successive investigations, we understood that those responsible for the introduction were the local landowners and inhabitants of the nearby village, and one old landowner partially acknowledged of having introduced, with the approval of other local people, the spiny-cheek crayfish with the intention to provide "bigger individuals" to the population of white-clawed crayfish inhabiting the stream (RM pers. obs.). We thus performed an intense action of information and involvement to increase stakeholders' awareness of white-clawed crayfish importance that we aim to describe in this paper together with all the steps that allowed the successful reintroduction of white-clawed crayfish.

The aims of this paper are to (a) describe the steps of the restoration action initiated in 2018, (b) compare freshwater characteristics before the extinction and after the reintroduction of the white-clawed crayfish, (c) assess white-clawed crayfish breeding success after the reintroduction, and (d) provide insights for successful reintroduction projects.

MATERIALS AND METHODS

Study Site

The study site is the San Michele creek within the Monte Barro Regional Park (45.84 N, 9.39 E; **Figure 1**), in the Lecco district (NW Italy). The creek is in a site of community importance (SCI), as defined by the European Commission Habitats Directive (92/43/EEC). The creek arises from a spring with a water flow of approximately 8 L per minute. The creek offers various microhabitats with repeated successions of small falls, riffles, and pools and the presence of two larger pools laterally connected with the creek that were built in 2009 to increase the habitat available for freshwater crayfish (**Figure 1B**). After 200 m, the creek continues by a 60-m-high waterfall; downstream to it, the creek becomes ephemeral and is connected with the nearby Garlate Lake and the Adda River only after a heavy rainfall. The waterfall and the irregular hydroperiod act as efficient barriers for the spread of the spiny-cheek crayfish that inhabits Garlate Lake (Bonelli et al., 2017; Manenti et al., 2019b). At 85 m upstream to the waterfall, the creek is currently difficult to access and sample because of dense vegetation. Until 2013, the creek hosted a well-structured population of white-clawed crayfish that had been periodically surveyed since 2003 (Manenti, 2006). The population belonged to the *carinthiacus* clade of the *A. pallipes* complex, which is typical for Western Lombardy (Bernini et al., 2016).

On July 26, 2013, a mass mortality occurred among white-clawed crayfish with typical features of crayfish plague outbreaks, and many adult spiny-cheek crayfish were recorded right in the same stretch of San Michele creek (Bonelli et al., 2017). In the successive weeks, no further individuals of white-clawed crayfish were observed, but, thanks to the limited extension of the creek, it was possible to perform efficient removals of the spiny-cheek crayfish individuals (Bonelli et al., 2017). A zebra mussel (*Dreissena polimorpha*), which only inhabits standing waters, recorded on a spiny-cheek crayfish male (Bonelli et al., 2017) allowed us to hypothesize that spiny-cheek crayfish came from nearby localities, particularly the Lario Lake where zebra mussels are rather common. As stated above, successive investigations confirmed the intentional introduction of the alien crayfish by local landowners and inhabitants of San Michele village (RM pers. obs.).

Reintroduction Strategy and Methods

The reintroduction of white-clawed crayfish was decided by Monte Barro Park authorities and carried out as part of a larger project co-financed by the Cariplo Foundation. The reintroduction strategy has been developed in the context of the project LIFE14 IPE/IT/000018 project "LIFE IP GESTIRE 2020 – Nature Integrated Management to 2020," activated in the Lombardy region in 2016 and involving different actions for the conservation of the white-clawed crayfish. These include the rearing of juveniles for stocking purposes, monitoring of the sites inhabited by the white-clawed crayfish, providing educational activities, and creating local task forces for interventions in case of emergencies regarding freshwater crayfish. The reintroduction performed in the park of Monte Barro has been

TABLE 1 | Sites of white-clawed crayfish reintroductions described in peer-reviewed studies.

Locality	Nation	Extinction causes	Year reintroduction	Success/ failure	Causes of success/failure	Source
River Lathkill	United Kingdom	Mass mortality (likely crayfish plague)	2000	Not clear; partial success	Shelter creation reduced mortality	Rogers and Watson, 2007
Sherston Easton Grey	United Kingdom	Crayfish plague	1986	Success	No more outbreaks of crayfish plague	Spink and Frayling, 2000
Sherston Fosse Mill	United Kingdom	Crayfish plague	1982	Success	No more outbreaks of crayfish plague	Spink and Frayling, 2000
Sherston Hyam Farm	United Kingdom	Crayfish plague	1994	Success	No more outbreaks of crayfish plague	Spink and Frayling, 2000
Tetbury Shipton Mill	United Kingdom	Crayfish plague	1987	Success	No more outbreaks of crayfish plague	Spink and Frayling, 2000
Tetbury Merchants Farm	United Kingdom	Crayfish plague	1987	Success	No more outbreaks of crayfish plague	Spink and Frayling, 2000
Tetbury Boakley Farm	United Kingdom	Crayfish plague	1987	Success	No more outbreaks of crayfish plague	Spink and Frayling, 2000
Lhau	France	Crayfish plague	2006	Success	Not specified	Durlet et al., 2009
Vurpillières	France	Crayfish plague	2006	Success	Not specified	Durlet et al., 2009
White Lake	Ireland	Crayfish plague	1999	Success	Release in enclosures	Reynolds et al., 2000

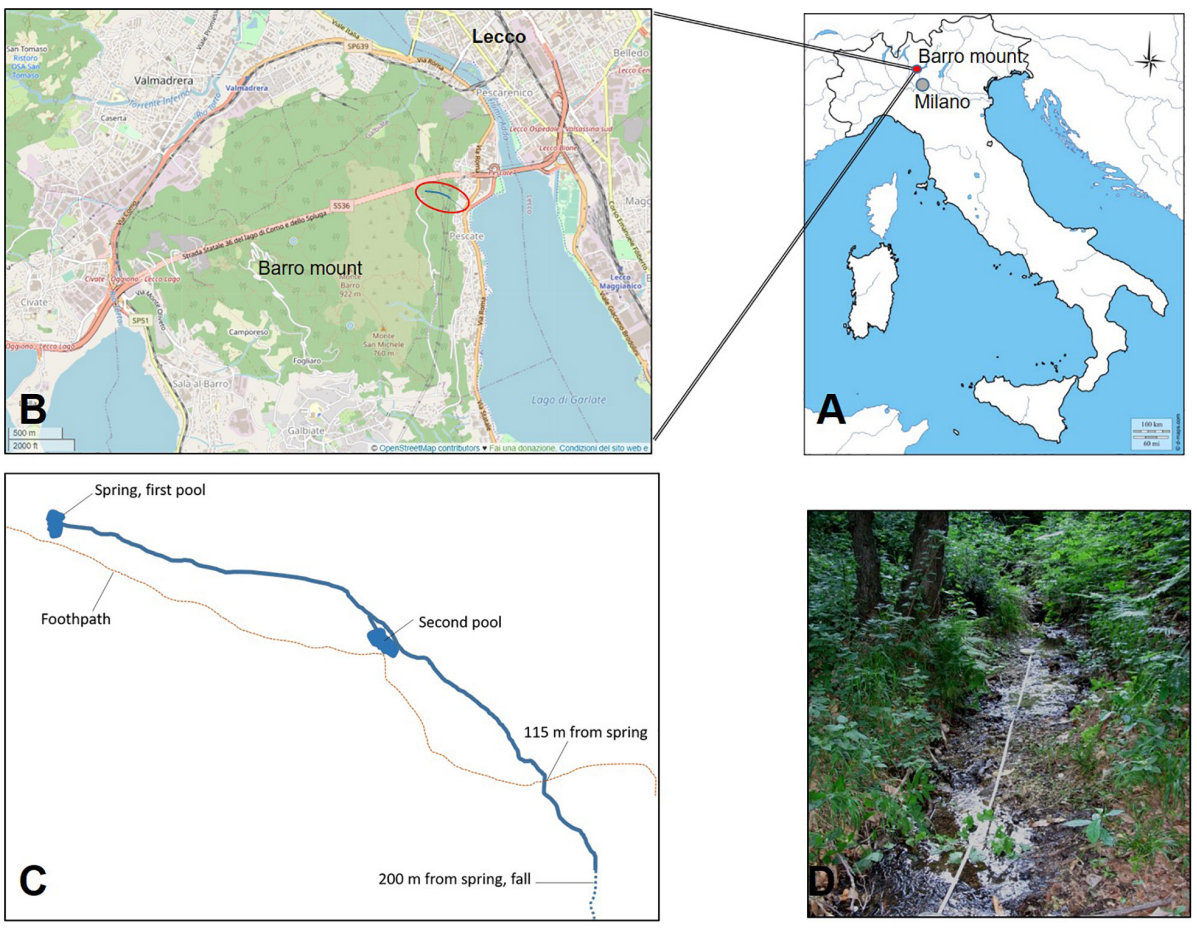

FIGURE 1 | Location **(A,B)** of the San Michele stream **(C,D)** where white-clawed crayfish reintroduction took place. In **(B)**, the red circle represents the location of the San Michele stream in the context of Mount Barro. **(C)** A schema of the San Michele stream where the main sites mentioned in the paper are indicated; the stretch between the stream spring and a waterfall with intermittent flow that makes a barrier for invasive crayfish species is 200 m long. Reintroduced white-clawed crayfish have been released in the two pools, built in 2009, and along the stream stretch 115 m long downstream from the spring. **(C)** Not drawn to scale.

developed in different steps with specific methodologies that are described here below.

Site Monitoring and Alien Crayfish Removal Before Reintroduction

The spiny-cheek crayfish introduced by local stakeholders were removed in summer 2013 (Bonelli et al., 2017). From autumn 2013 to autumn 2018, each year, we performed two surveys of the creek (at least one survey in each spring and at least one survey in each autumn) to assess spiny-cheek crayfish occurrence. Surveys were performed during the night (from 21:00 to 01:00). Moreover, during 2017, we assessed water quality using the extended biotic index (EBI) modified for the Italian streams (Woodiwiss, 1978; Ghetti, 1997). We compared it to EBI scores of 2005 and 2013 to verify that water quality did not change after the white-clawed crayfish extinction. EBI is a standard method used in Europe to assess stream quality; on the basis of the number and identity of taxa found, each stream has a score ranging from 1 (lowest quality: poor communities including very tolerant species) to 13 (maximum quality: the richest communities, including stenoecious species).

Stakeholders' Control and Communication Campaign

White-clawed crayfish reintroduction was planned for autumn 2018. At the beginning of 2018, we started multiple actions of public communication to raise awareness of white-clawed crayfish features and crayfish plague and to inform on how to distinguish native from invasive crayfish species. It must be pointed out that two main categories of stakeholders exist in the park. The first includes occasional visitors that live in cities near the park; they mostly use the main footpaths of the park that do not cross the San Michele locality. The second category includes people living in the San Michele village, which are mostly represented by old persons with apparent limited educational levels and often a hostile view against wildlife management actions proposed by outsiders. During the investigations performed after the freshwater crayfish's extinction, we observed that the old inhabitants believed themselves to be the first managers of the natural environments surrounding the village and the only ones with valid management techniques. The local practices of stream management included riverbed and stream bank cleanings that can alter shelter availability, translocations of frog clutches from unknown localities that can favor pathogen transmission, and water organic enrichment through bread and organic waste placed in the pools to feed frog tadpoles (RM pers. obs.).

To counteract this situation, first we held in February and March 2018 two meetings in the presence of biologists and forestry officers. The meetings were directed toward both park visitors and San Michele local people. The participation of the local people was limited, but park managers convinced at least the main suspect of alien crayfish introduction to participate. Second, from March to September 2018, we performed seven direct visits, house to house, to the local people. At each visit, we provided information on freshwater crayfish and explained with practical examples the risks of continuing the usual management practices performed along the stream for white-clawed crayfish once reintroduced. Each inhabitant was visited at least twice and the main suspect five times; each visit lasted at least 30 min. We also listened to their opinion and asked their collaboration in the surveillance of both territory and white-clawed crayfish health. When we held the February and March public meetings, only occasional visitors of the park and local inhabitants with negative attitude toward questionnaires participated; we thus avoided pre- and post-outreach assessments to evaluate changes in attitude of local landowners. Instead, we evaluated if the reintroduction site suffered human disturbance.

To evaluate disturbance, we placed in one of the pools connected with the creek four cylindrical stainless steel cages with a diameter of 50 cm and a mesh of 3 × 3 mm; the chosen pool was easily accessible, along the main path, but sheltered from distant sighting points. Therefore, it was in principle possible in this pool to intentionally damage objects presumably placed there by park and external authorities and alter stream bed by traditional management without being noticed from the surrounding area. Each cage circumscribed a column of water from the substrate of the pool's bottom to the surface and was grounded in the substrate for 20 cm and at a distance of 30 cm from the pool border. The cages let water and small invertebrates flow through them but did not allow people to step on the pool's bottom without moving them. The cages allowed us to detect possible disturbances and unplanned interventions (e.g., substrate cleaning by local people) by evaluating whether they were moved and by comparing their substrate with the substrate of the pool. Moreover, at the pool access point, we placed a small informative sign reporting that there was an ongoing action (not detailed) by an external institution (the University of Milan), which could have induced hostility and suspicion in the local people. We checked the sign position at each survey after reintroduction.

Crayfish Reintroduction

In October 2018, September 2019, and September 2020, we reintroduced in the creek altogether 568 3-month-old juveniles of white-clawed crayfish (for numbers in each year, see **Table 2**). These were raised in a breeding facility of the Regional Agency for the Agricultural and Forestry Services (ERSAF) and originated from 90 breeding white-clawed crayfish from different streams of the Como district whose genotype corresponds to the white-clawed crayfish clade found in Western Lombardy. For their first 3 months, juveniles were reared in two outdoor ponds with seminatural conditions and shelters. We recorded rostrum–telson total length (TL) and sex. Juveniles were reintroduced during the night in different pools of the creek over 115 m away from the springs; we avoided the 85-m-long inaccessible area directly upstream of the waterfall.

Post-reintroduction Monitoring

From October 2018 to September 2020, we performed periodical visual encounter surveys of the reintroduced white-clawed crayfish. We performed the surveys during nighttime at 9:00 p.m., going upstream from the final stretch before the waterfall to the

spring for 115 m. We included in each survey the two pools built in 2009. Each survey was performed by one to three observers along the same stretch and lasted 40–60 min (**Table 2**). During the four surveys, we also captured all the detected white-clawed crayfish, measured their TL, weighed them, recorded their sex, and released them at the site of capture.

Features of the Reintroduced Population

We assessed variation of morphological features of the reintroduced white-clawed crayfish in October 2018 comparing their TL and weight during successive surveys in June 2019, September 2019, May 2020, and June 2020; we did not record individual weight at reintroduction, but only during the successive surveys.

We also compared white-clawed crayfish morphological parameters with data from a survey performed in the same site prior to 2013. In particular, we compared crayfish TL and weight recorded in May 2020 for 19 individuals with the data collected in May 2007 for 84 individuals. Both datasets were collected in a single survey date and were limited to individuals > 40 mm, which have higher detectability compared with juveniles (Arrignon, 1981).

To assess current differences in density before extinction and after the first year of reintroduction, we used the estimation obtained in May 2007 through removal samplings; in that year, we performed two successive samplings during the same night over a stream stretch with length 195 m (the 5 m of stretch before the waterfall was dry) and average width 0.47 m. During the first survey, we removed all the white-clawed crayfish with TL > 40 mm seen outside of shelters, and we placed them in two tanks outside the creek; we waited for 30 min and repeated the sampling by placing the collected individuals in two other tanks. We then measured all the individuals and released them in the creek. Subsequently, we calculated white-clawed crayfish population abundance using the method proposed by Chao and Chang (1999) to analyze removal samplings. In 2019–2020, we did not perform removal sampling to avoid excessive disturbance of the individuals, and we performed three multiple counting surveys in successive nights in order to estimate population abundance using N-mixture models (see below). The stretch surveyed in 2019–2020 was 115 m long and the average width was 0.6 m; we avoided the inaccessible stretch before the waterfall, but we included the two new pools located laterally.

TABLE 2 | Number of white-clawed crayfish released and observed in the successive surveys during the period October 2018–September 2020.

Date	Total crayfish released	Total crayfish observed	Duration of survey (min)	Number of observers
18/10/2018	231	–	60	2
25/10/2018	–	7	40	1
28/10/2018	–	1	40	2
30/10/2018	–	2	60	1
13/11/2018	–	0	60	2
18/11/2018	–	1	45	1
12/12/2018	–	0	60	1
18/01/2019	–	0	40	1
06/06/2019[a]	–	23	45	2
14/06/2019[a,b]	–	25	45	2
03/09/2019	157	–	40	1
23/09/2019[b]	–	29	45	3
20/05/2020[a]	–	22	50	2
25/05/2020[a,b]	–	27	50	2
24/06/2020	–	19	45	2
14/09/2020[b]	–	20	45	2
17/09/2020	180	–	40	1

[a]*Surveys used to estimate the density of white-clawed crayfish longer than 40 mm through N-mixture models. The surveyed stretch was always 115 m long and included two pools built in 2009 to increase habitat availability for white-clawed crayfish.*

[b]*Post-introduction dates in which white-clawed crayfish were collected, weighted, and measured.*

Statistical Analyses

To assess variation of morphological features (TL and weight) of the white-clawed crayfish, we built linear models (LMs) using the lm function in R. We considered TL and weight as dependent variables, sampling period as a fixed factor, and sex of individuals as a covariate. We also tested the occurrence of significant interactions between period and sex. We assessed the significance of the variables using a Wald *F* test (Bolker et al., 2008). Subsequently, we performed Tukey's *post hoc* tests to assess differences between the different periods, using the function glht of the package multcomp (Hothorn et al., 2008).

To compare crayfish TL and weight between May 2007 and May 2020, we built LMs with TL and weight as dependent variables. We considered as fixed factors the period of sampling (May 2007 or May 2020) and sex. We assessed the significance of the variables using a Wald *F* test (Bolker et al., 2008).

We used N-mixture models to estimate population abundance after the reintroduction. Previous studies showed that N-mixture models provide reliable estimates of abundance, with results comparable to those obtained with removal samplings (Ficetola et al., 2018). To estimate the density of white-clawed crayfish in 2019 and 2020, we used N-mixture models for closed populations. We assumed that the population was closed during two successive surveys performed during the same month (June 2019 and May 2020; **Table 2**); we used Akaike's information criterion (AIC) to select the most appropriate error distribution (Poisson). The analysis was performed using the R package unmarked (Fiske and Chandler, 2011). The surveys considered for N-mixture models were performed by the same operators and had similar length (45–50 min); thus, the duration of the survey was not included as a covariate in the model. We considered for this analysis the individuals with TL > 40 mm. Density was calculated by dividing the estimated number of individuals by the area surveyed in each year. All the analyses were performed in R 3.6.3 environment.

RESULTS

Environmental conditions remained relatively stable from 2013 to 2017. Since October 2013, no spiny-cheek crayfish were

observed in the creek. The macrobenthos community both before and after the extinction showed a good degree of diversification with different bioindicator taxa typical of unpolluted small and slow-flowing streams like plecopterans of the genus *Amphinemura* and ephemeropterans of the genus *Ecdyonurus* and *Paraleptophlebia*. The EBI score was high and indicated a site with the highest class of water quality (**Table 3**). Both before and after extinction, benthic communities were dominated by amphipod crustaceans of the genus *Echinogammarus*.

The increasing understanding and knowledge of white-clawed crayfish features and importance by local stakeholders and landowners positively influenced their attitude toward the reintroduction action. After the reintroduction, no further introductions of alien species occurred, and no disturbance actions were detected (e.g., no bread and organic waste were thrown to feed tadpoles and no cleaning of riverbed and banks occurred). The cages and the sign placed in the most accessible pool remained untouched. Moreover, the substrate showed the same layer of small organic particles and decomposed leaves in both pool and cages.

In October 2018, we reintroduced 231 3-month-old white-clawed crayfish (122 males and 109 females) ranging from 32 to 43 mm of TL. The subsequent surveys confirmed the survival of the juveniles (**Table 3**), even though the number of detected individuals was limited. In early summer 2019, the number of young white-clawed crayfish detected increased. LMs detected significant changes of white-clawed crayfish TL from the first reintroduction (October 2018) to June 2020 ($F_{4,290} = 184.68$, $P < 0.001$; **Figure 2**). In all the surveys, white-clawed crayfish were significantly larger than at reintroduction, and in 2020, crayfish were consistently larger than in the 2019 surveys (Tukey's *post hoc* tests; **Supplementary Table 1**). Considering all the surveys, we detected no differences in TL between the collected males and females ($F_{1,290} = 0.73$, $P = 0.39$), but there was a significant interaction between period and sex with males larger than females in May 2020 ($F_{4,290} = 4.79$, $P < 0.01$). Weight showed significant differences between sexes, with males being significantly heavier than females ($F_{1,68} = 8.44$, $P < 0.01$). Furthermore, there was a significant difference of weight across periods ($F_{3,68} = 3.25$, $P = 0.02$), but Tukey's *post hoc* tests only showed that in May 2020 white-clawed crayfish were significantly heavier than in June 2019 ($t = 3.17$; $P = 0.01$).

We then compared the individuals sampled in May 2020 with the population measured before the extinction (May 2007). In May 2020, white-clawed crayfish were longer ($F_{1,100} = 11.85$, $P < 0.001$), but not heavier ($F_{1,100} = 2.80$, $P = 0.10$) than in 2007; in both periods, males were larger ($F_{1,100} = 16.01$, $P < 0.001$) and heavier ($F_{1,100} = 22.21$, $P < 0.001$) than females. Moreover, in October 2019, we detected one very large male (114 mm TL, 30 g); this individual was larger than any other white-clawed crayfish found in the stream on the same date.

Removal sampling of the population, performed in May 2007, estimated a total population of 106 individuals larger than 40 mm, which represents a density of 1.15 individuals/m^2. In 2019, N-mixture models estimated a total population size of 39 individuals larger than 40 mm, i.e., a density of 0.56 individuals/m^2 (95% CI 0.49–0.66). In 2020, we estimated a total population size of 40 individuals, i.e., a density of 0.57 individuals/m^2 (95% CI 0.50–0.68).

The reintroduction of young white-clawed crayfish was repeated in September 2019 and in September 2020. In September 2019, we reintroduced 157 3-month-old white-clawed crayfish (73 males and 84 females) with TL ranging from 20 to 33 mm. In September 2020, we reintroduced 180 3-month-old white-clawed crayfish (76 males and 104 females) with TL ranging from 18 to 31 mm. White-clawed crayfish breeding was already recorded during the first year of reintroduction. In October 2019, we detected three females with spermatophores on their ventral side, and in May 2020, we observed a female with newly hatched crayfish still attached at the pleopods (**Figure 3**).

TABLE 3 | Results of the extended biotic index (EBI) assessment in different years along the same transect of the stream where the reintroduction was conducted.

Year	Date	Before/ after extinction	EBI score	Quality class	Total S.U.	Ephemeropteran genera found
2005	December 2005	Before	10.5	I	21	*Ecdyonurus*, *Palaeptophlebia*
2013	July 2013	Before	10	I	16	*Ecdyonurus*, *Ephemera*
2017	July 2017	After	10	I	18	*Ecdyonurs*, *Ephemera*, *Paraleptophlebia*

S.U. refers to the number of systematic units (species, genera of families depending on the EBI requirements for the different taxa) recorded.

DISCUSSION

Post-reintroduction surveys showed that white-clawed crayfish reintroduction in San Michele creek was successful; the intense dissemination activity was crucial in avoiding further introduction of alien crayfish and prevented disturbance by local stakeholders. The careful attention paid to environmental conditions prior to reintroduction enhanced the possibility to perform an effective action.

The high quality of environmental conditions of the site both before and after the reintroduction was likely a factor determining reintroduction success. EBI score remained substantially unvaried over 12 years, confirming that the stream was and remained unpolluted, providing appropriate environmental conditions for the white-clawed crayfish; even the potential organic enrichment caused by local stakeholders when feeding tadpoles did not seem to have altered water quality. In reintroduction projects dealing with freshwater fauna, insufficient water quality is a major potential cause of failure (Jourdan et al., 2019). In fact, considering all white-clawed crayfish reintroductions described in peer-reviewed literature (**Table 1**), unexpected pollution events affected white-clawed crayfish abundance in at least two sites where they were reintroduced after *A. astaci* propagation (Spink and Frayling, 2000). Our stream hosted a diversified mayfly

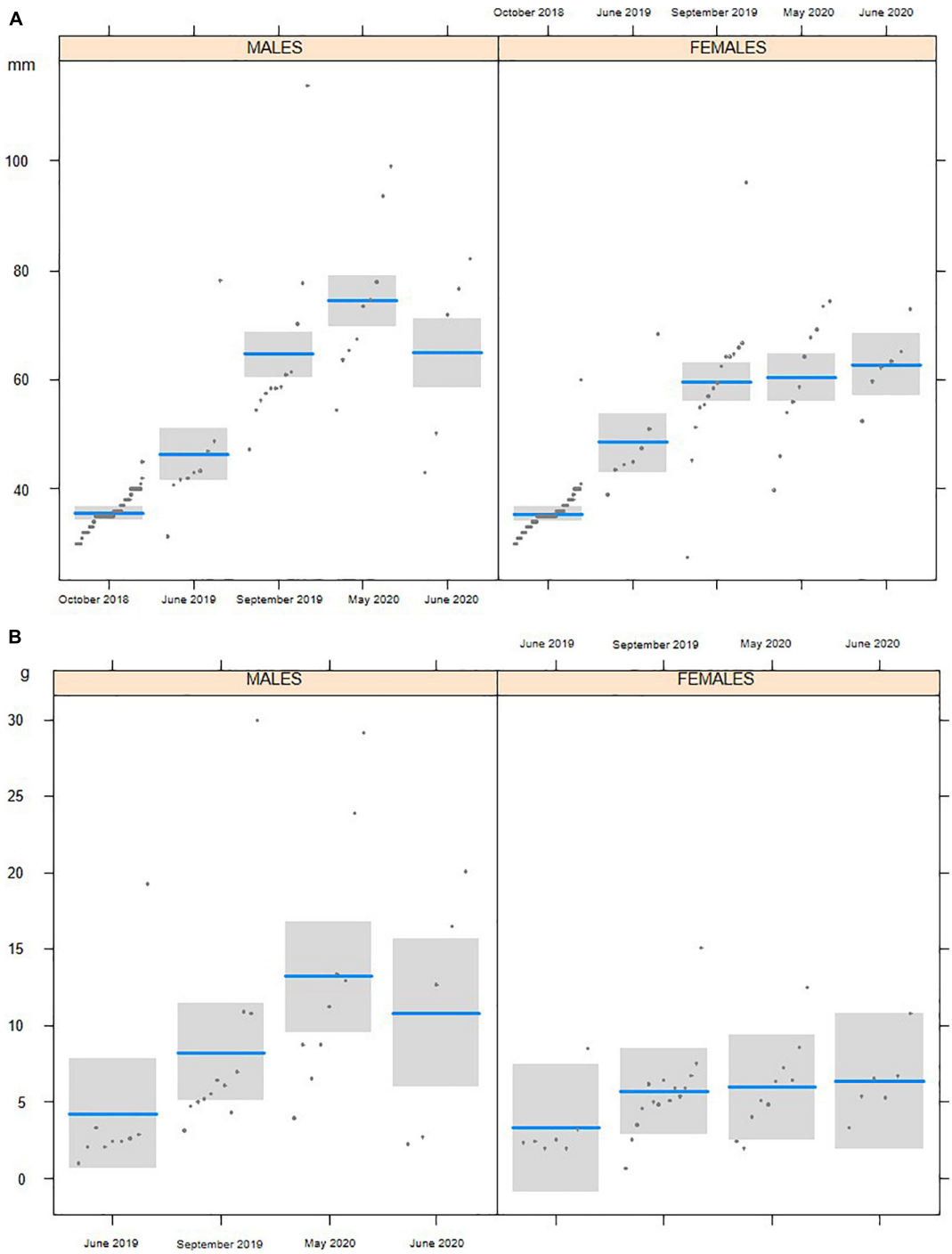

FIGURE 2 | Total length **(A)** and weight **(B)** of the individuals of *Austropotamobius pallipes* collected during the surveys performed from the first reintroduction in October 2018 to June 2020.

community, with occurrence of the genus *Paraleptophlebia*, which is considered an indicator of favorable conditions for the white-clawed crayfish (Grandjean et al., 2011). White-clawed crayfish are good indicators of freshwater ecosystem quality and functioning (Nardi et al., 2005) and also play a fundamental role in maintaining the structure of benthic communities (Manenti et al., 2019b) especially considering their large biomass (Richman et al., 2015). Assessing how crayfish extinctions and reintroductions impact the communities and the functionality of streams and creeks could be extremely important to understand the community-wide impacts of the extinction of these keystone species

FIGURE 3 | Different steps and structures of the white-clawed crayfish reintroduction action. **(A)** The breeding facility of the ERSAF organization. **(B)** 3-month-old white-clawed crayfish ready for the release. **(C)** Measurement of the 3-month-old white-clawed crayfish. **(D)** Introduction of the white-clawed crayfish in September 2019. **(E)** Grown reintroduced white-clawed crayfish female in June 2019. **(F)** The finding of a reintroduced female in May 2020 with a newly hatched white-clawed crayfish still attached to the pleopods. Written informed consent was obtained from the person depicted in picture **(D)** for the publication of any potentially identifiable images or data included in this article.

(Ripple et al., 2014) and to quantify the amplitude of services provided by them.

The activities of the study, the control, and the dissemination directed toward local stakeholders are other factors that prompted reintroduction success. The geography of the reintroduction site ensures that the crayfish population is naturally isolated from surrounding populations of alien crayfish by a high natural fall and a temporary stream stretch. However, the introduction of alien crayfish carrying the crayfish plague pathogen determined the local extinction and revealed the interest in the site by local inhabitants of the nearby village (Bonelli et al., 2017). Prior to the start of reintroduction

activities, we thus performed multiple dissemination actions at a local scale, targeting the inhabitants of the surrounding area. The success of this effort is confirmed by the fact that no further introductions of alien crayfish occurred, nor did we detect disturbing activities by landowners and inhabitants of the area. The cages and sign placed prominently in one of the most accessible points of the stream remained untouched, and substrate features of the pool were not different inside and outside of the cages. So far, analyses of reintroduction actions performed with white-clawed crayfish paid limited attention to the involvement of local stakeholders and the impact of outreach campaigns. Local landowners were involved in the reintroduction of the noble crayfish (*Astacus astacus*) in different sites of Fennoscandia, but only very limited details are available on how this involvement took place (Taugbøl, 2004; Jussila et al., 2008; Edsman and Schröder, 2009); in particular, it is reported that local landowners collaborated with local authorities in reducing the motivation for illegal stocking of alien crayfish species and performing a fast reintroduction of native crayfish, but it is not described what local stakeholders did. Our case study suggests that local practices of self-made stream management can be a threat to native crayfish. Local stakeholders of isolated hilly and mountainous areas can include people with no awareness of current threats to biodiversity and native species (Lindemann-Matthies and Bose, 2008) which act based on personal beliefs rather than proper management practices. Convincing the inhabitants to avoid activities such as inappropriate cleaning of the stream and translocations has been a hard task. In our case study, it was impossible to perform a formal assessment of the success of the outreach campaign, yet the available indicators and the success of the reintroduction action suggest they have been helpful, with potential positive effects on the long-term persistence of this isolated white-clawed crayfish population.

A certain number of white-clawed crayfish survived and at least some individuals were quickly able to successfully breed. This suggests that 3-month-old juveniles are a good choice for reintroduction actions, as they can easily adapt to new habitats and conditions (Rogers and Watson, 2007). Without intraspecific competitors, the first reintroduced juveniles quickly reached larger sizes compared with individuals comprising the extinct population. The few published studies of white-clawed crayfish reintroductions highlight that, when juveniles are introduced, shelter availability is pivotal for survival (Rogers and Watson, 2007). Most of the available studies, however, dealt with translocated adults (Reynolds et al., 2000; Durlet et al., 2009) or did not provide information on the size or age of the reintroduced white-clawed crayfish (Spink and Frayling, 2000).

In our case study, the first breeding activity was detected just 12 months after the first reintroduction, when the reintroduced individuals were in their second year of life. In two French streams where 50- to 70-mm-long white-clawed crayfish were reintroduced, breeding was recorded for the first time after 3 years (Durlet et al., 2009). The sexual maturity of the white-clawed crayfish is strongly correlated to body length (Rhodes and Holdich, 1979; Grandjean et al., 1997), and both temperature and diet strongly affect their growth, molting, and survival (Paglianti and Gherardi, 2004). The size at which the white-clawed crayfish reaches sexual maturity is usually 22–25 mm of carapace length and 50–60 mm of TL (Mason, 1975; Arrignon, 1981; Ghia et al., 2015). Usually, sexual maturity is achieved around the third or fourth year of life, and even if alleged, breeding in 2-year-old individuals has not been recorded yet (Brewis and Bowler, 1982; Mancini, 1986; Ghia et al., 2015). The very fast growth rate observed suggests that, in suitable streams without intraspecific competition, juveniles can reach maturity very quickly. Even if the species is K-selected, with a slow growth rate and long life (Ghia et al., 2015), our results underline the fact that, in the absence of further disturbances, reintroductions of white-clawed crayfish can allow the species to recover in relatively short times. Nevertheless, the present-day apparent density remains 50% lower than the one reached by the extinct population. Further surveys should allow to assess whether in the next years the population will reach the abundance observed before the extinction.

The observation of a large male in 2019 is particularly noticeable, as this individual showed the size typical of old individuals. The life span of the white-clawed crayfish may last even over 12–13 years (Mancini, 1986; Ghia et al., 2015); it is thus possible that this individual could be a survivor of the former population. Isolated cases of survival after the crayfish plague have been recorded in different species of European freshwater crayfish (Kozubíková-Balcarová et al., 2014; Jussila et al., 2016; Strand et al., 2019), but this record might also represent unauthorized attempts of reintroduction performed by the local people as we recorded for at least in one stream in the same hydrographic catchment (Manenti, 2006). In San Michele creek, our monitoring suggests that illegal introductions could happen but are unlikely, and this might indeed represent a rare case of survival from the crayfish plague outbreak.

Our results suggest that the communication campaigns addressed to stakeholders and the natural separation of the stream from environments inhabited by alien crayfish species should allow the success of the reintroduction action, with a complete recovery of the species. Even though the study site can be considered a typical ark site, i.e., a refuge site safe from non-native crayfish and crayfish plague (Kozák et al., 2011; Haddaway et al., 2012; Rosewarne et al., 2017), major concerns remain for the long-term persistence of this population. For instance, long-term isolation could expose the population to the risk of extinction because of stochastic or genetic factors. Future efforts should thus prevent extinctions in the few nearby streams where native white-clawed crayfish still survive and try to re-establish additional populations that can enable long-term persistence, for instance, by forming a metapopulation network isolated from crayfish plague outbreaks.

Successful reintroductions require accurate planning and the execution of multiple steps. Each step, from habitat assessment to dissemination activities and continued monitoring, has had a key role in the reintroduction of white-clawed crayfish. In our case study, as for most conservation programs and policies (Chazdon et al., 2017; Manenti et al., 2019a), the aim was to reverse the impacts of human actions. For this reason, a substantial part of both preliminary actions and monitoring activities were directed

toward preventing the detrimental actions of stakeholders living near the reintroduction site. Reintroduction actions could be more effective when the stakeholders having the greatest potential impact on the species are identified and involved in the activities. This is especially important when reintroductions focus on animals having major cultural, gastronomic, and commercial interest.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Written informed consent was obtained from the individuals for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

RM and GFF conceived the manuscript. RM and MV planned the reintroduction. AN, BB, SC, and RM performed the surveys. All authors contributed to the writing of the manuscript.

FUNDING

Monitoring from 2016 to 2018 was made possible by The Mohamed bin Zayed Species Conservation Fund, project n. 162512949. Reintroduction actions were funded by the Cariplo Foundation through the project "Capitale naturale nel Monte di Brianza" of the Monte Barro Regional Park and by LIFE14 IPE/IT/000018 project "LIFE IP GESTIRE 2020 – Nature Integrated Management to 2020," website: https://naturachevale.it/. Publication APC was covered by the University of Milan. Young crayfish for reintroductions were provided by the facility of the ERSAF organization which has two breeding capacities in Canzo (CO) and in Tignale (BS).

ACKNOWLEDGMENTS

We are grateful to all from the LIFE IP Gestire2020 Action C6 team and to D. Ghia, G. Fea, G. Fracassi, A. Casoni, and P. Colombo. We thank Robin Whalley for proofreading the text.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.621613/full#supplementary-material

REFERENCES

Alderman, D. J. (1996). Geographical spread of bacterial and fungal diseases of crustacean. *Rev. Sci. Tech. Off. Int. Épizoot.* 15, 603–632. doi: 10.20506/rst.15.2.943

Arrignon, J. (1981). *L'écrevisse et Son Élevage*. Paris: Gauthier-Villars.

Bernini, G., Bellati, A., Pellegrino, I., Negri, A., Ghia, D., Fea, G., et al. (2016). Complexity of biogeographic pattern in the endangered crayfish *Austropotamobius italicus* in northern Italy: molecular insights of conservation concern. *Conserv. Genet.* 17, 141–154. doi: 10.1007/s10592-015-0767-4

Bland, L. M. (2017). Global correlates of extinction risk in freshwater crayfish. *Anim. Conserv.* 20, 532–542. doi: 10.1111/acv.12350

Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H. H., et al. (2008). Generalized linear mixed models: a practical guide for ecology and evolution. *Trends Ecol. Evol.* 24, 127–135. doi: 10.1016/j.tree.2008.10.008

Bonelli, M., Manenti, R., and Scaccini, D. (2017). Mountain protected areas as refuges for threatened freshwater species: the detrimental effect of the direct introduction of alien species. *Ecol. Mont.* 9, 23–29. doi: 10.1553/eco.mont-9-2s23

Brewis, J. M., and Bowler, K. (1982). The growth of the freshwater crayfish *Austropotamobius pallipes* in Northumbria. *Freshw. Biol.* 12, 187–200. doi: 10.1111/j.1365-2427.1982.tb00613.x

Caprioli, R., Mrugała, A., Di Domenico, M., Curini, V., Giansante, C., Camma, C., et al. (2018). *Aphanomyces astaci* genotypes involved in recent crayfish plague outbreaks in central Italy. *Dis. Aquat. Org.* 130, 209–219. doi: 10.3354/dao03275

Chao, A., and Chang, S. H. (1999). An estimating function approach to the inference of catch-effort models. *Environ. Ecol. Stat.* 6, 313–334. doi: 10.1023/A:1009687514770

Chazdon, R. L., Brancalion, P. H. S., Lamb, D., Laestadius, L., Calmon, L., and Kumar, C. (2017). A policy-driven knowledge agenda for global forest and landscape restoration. *Conserv. Lett.* 10, 125–132. doi: 10.1111/conl.12220

Chucholl, C. (2016). The bad and the super-bad: prioritising the threat of six invasive alien to three imperilled native crayfishes. *Biol. Invas.* 18, 1967–1988. doi: 10.1007/s10530-016-1141-2

Diéguez-Uribeondo, J., Rueda, A., Castien, E., and Bascones, J. C. (1997). A plan of restoration in Navarra for the native freshwater crayfish species of Spain, *Austropotamobius pallipes*. *Bull. Fr. Pêche Piscic.* 347, 625–637. doi: 10.1051/kmae/1997056

Durlet, P., Tissot, B., Pesme, E., Marquis, A., and Besson, S. (2009). Expérience de réintroduction de deux populations d'écrevisses à pattes blanches *Austropotamobius pallipes* (Lereboulet, 1858). *Rev. Sci. Bourgogne Nat.* 10, 243–249.

Edsman, L., and Schröder, S. (2009). *Action Plan for the Noble Crayfish (Astacus astacus), 2008–2013*. Stockholm: Swedish Environmental Protection Agency, 5955. Report.

Ficetola, G. F., Barzaghi, B., Melotto, A., Muraro, M., Lunghi, E., Canedoli, C., et al. (2018). N-mixture models reliably estimate the abundance of small vertebrates. *Sci. Rep.* 8:10357. doi: 10.1038/s41598-018-28432-8

Fiske, I., and Chandler, R. B. (2011). unmarked: an R package for fitting hierarchical models of wildlife occurrence and abundance. *J. Stat. Softw.* 43, 1–23. doi: 10.18637/jss.v043.i10

Gherardi, F. (2011). Towards a sustainable human use of freshwater crayfish (Crustacea, Decapoda, Astacidea). *Knowl. Manag. Aquat. Ecosyst.* 2:22. doi: 10.1051/Kmae/2011038

Gherardi, F., Acquistapace, P., Aquiloni, L., Barbaresi, S., Cioni, A., Paglianti, A., et al. (2003). The red swamp crayfish in Europe: lessons for invasion biology. *Integr. Comp. Biol.* 43, 1005–1005.

Gherardi, F., and Barbaresi, S. (2000). Invasive crayfish: activity patterns of *Procambarus clarkii* in the rice fields of the Lower Guadalquivir (Spain). *Arch. Hydrobiol.* 150, 153–168. doi: 10.1127/archiv-hydrobiol/150/2000/153

Gherardi, F., and Souty-Grosset, C. (2006). European crayfish as heritage species-linking research and management strategies to conservation and socio-economic development-Preface. *Bull. Fr. Pêche Piscic.* 38, 851–852.

Ghetti, P. F. (1997). *Indice Biotico Esteso (I.B.E.): Manuale di Applicazione*. Trento: Provincia Autonoma di Trento.

Ghia, D., Fea, G., Conti, A., Sacchi, R., and Nardi, P. A. (2015). Estimating age composition in Alpine native populations of *Austropotamobius pallipes* complex. *J. Limnol.* 74, 501–511. doi: 10.4081/jlimnol.2015.1139

Ghia, D., Fea, G., Sacchi, R., Di Renzo, G., Garozzo, P., Marrone, M., et al. (2013). Modelling environmental niche for the endangered crayfish *Austropotamobius pallipes* complex in Northern and Central Italy. *Freshw.Crayfish.* 19, 189–195. doi: 10.5869/fc.2013.v19-2.189

Gil-Sanchez, J., and Alba-Tercedor, J. (2006). The decline of the endangered populations of the native freshwater crayfish (*Austropotamobius pallipes*) in southern Spain: it is possible to avoid extinction? *Hydrobiologia* 559, 113–122. doi: 10.1007/s10750-005-1024-5

Grandjean, F., Jandry, J., Bardon, E., Coignet, A., Trouilhe, M. C., Parinet, B., et al. (2011). Use of Ephemeroptera as bioindicators of the occurrence of white-clawed crayfish (*Austropotamobius pallipes*). *Hydrobiologia* 671, 253–258. doi: 10.1007/s10750-011-0717-1

Grandjean, F., Romain, D., AvilaZarza, C., Bramard, M., SoutyGrosset, C., and Mocquard, J. P. (1997). Morphometry, sexual dimorphism and size at maturity of the white-clawed crayfish *Austropotamobius pallipes pallipes* (Lereboullet) from a wild French population at Deux-Sèvres (Decapoda, Astacidea). *Crustaceana* 70, 31–44. doi: 10.1163/156854097x00320

Haddaway, N. R., Mortimer, R. J. G., Christmas, M., Grahame, J. W., and Dunn, A. M. (2012). Morphological diversity and phenotypic plasticity in the threatened British white-clawed crayfish (*Austropotamobius pallipes*). *Aquat. Conserv.* 22, 220–231. doi: 10.1002/aqc.2225

Holdich, D. M., Rogers, W., and Reynolds, J. (1999). Native and alien crayfish in the British Isles. *Crustac* 11, 221–236. doi: 10.1201/9781315140469-14

Hothorn, T., Bretz, F., and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biomed. J.* 50, 346–363. doi: 10.1002/bimj.200810425

Jandry, J., Brulin, M., Parinet, B., and Grandjean, F. (2014). Ephemeroptera communities as bioindicators of the suitability of headwater streams for restocking with white-clawed crayfish, *Austropotamobius pallipes*. *Ecol. Indic.* 46, 560–565. doi: 10.1016/j.ecolind.2014.07.005

Jourdan, J., Plath, M., Tonkin, J. D., Ceylan, M., Dumeier, A. C., Gellert, G., et al. (2019). Reintroduction of freshwater macroinvertebrates: challenges and opportunities. *Biol. Rev.* 94, 368–387. doi: 10.1111/brv.12458

Jussila, J., Maguire, I., Kokko, H., Tiitinen, V., and Makkonen, J. (2020). Narrow-clawed crayfish in Finland: *Aphanomyces astaci* resistance and genetic relationship to other selected European and Asian populations. *Knowl. Manag. Aquat. Ecosyst.* 421:30. doi: 10.1051/kmae/2020022

Jussila, J., Ojala, K., and Mannonen, A. (2008). Noble crayfish (*Astacus astacus*) reintroduction project in the river Pyhäjoki, western Finland: a case study. *Freshw. Crayfish.* 16, 51–56. doi: 10.5869/fc.2008.v16.51

Jussila, J., Tiitinen, V., Edsman, L., Kokko, H., and Fotedar, R. (2016). Signal crayfish in Lake Saimaa could be maladapted to the local conditions due to *Aphanomyces astaci* infection: a seven-year study. *Freshw. Crayfish.* 22, 53–60. doi: 10.5869/fc.2016.v22-1.53

Kemp, E., Birkinshaw, N., Peay, S., and Hiley, P. D. (2003). *Reintroducing the White-Clawed Crayfish Austropotamobius pallipes*. Peterborough: English Nature.

Kouba, A., Petrusek, A., and Kozák, P. (2014). Continental-wide distribution of crayfish species in Europe: update and maps. *Knowl. Manag. Aquat. Ecosyst.* 5:7. doi: 10.1051/kmae/2014007

Kozák, P., Füreder, L., Kouba, A., Reynolds, J., and Souty-Grosset, C. (2011). Current conservation strategies for European crayfish. *Knowl. Manag. Aquat. Ecosyst.* 1:18. doi: 10.1051/kmae/2011018

Kozubíková-Balcarová, E., Beran, L., Ďuriš, Z., Fischer, D., Horká, I., Svobodová, J., et al. (2014). Status and recovery of indigenous crayfish populations after recent crayfish plague outbreaks in the Czech Republic. *Ethol. Ecol. Evol.* 26, 299–319. doi: 10.1080/03949370.2014.897652

Lindemann-Matthies, P., and Bose, E. (2008). How Many Species Are There? Public Understanding and Awareness of Biodiversity in Switzerland. *Hum. Ecol.* 36, 731–742. doi: 10.1007/s10745-008-9194-1

Lo Parrino, E., Ficetola, G. F., Manenti, R., and Falaschi, M. (2020). Thirty years of invasion: the distribution of the invasive crayfish *Procambarus clarkii* in Italy. *Biogeographia* 35, 43–50. doi: 10.21426/B635047157

Makkonen, J., Strand, D. A., Kokko, H., Vrålstad, T., and Jussila, J. (2013). Timing and quantifying Aphanomyces astaci sporulation from the noble crayfish suffering from the crayfish plague. *Vet. Microbiol.* 162, 750–755. doi: 10.1016/j.vetmic.2012.09.027

Mancini, A. (1986). *Astacicoltura: Allevamento e Pesca dei Gamberi d'acqua Dolce*. Bologna: Edagricole.

Manenti, R. (2006). *Rilievi sul Patrimonio Astacicolo Della Provincia di Lecco*. Thesis, Università degli Studi di Milano, Milan.

Manenti, R., Barzaghi, B., Tonni, G., Ficetola, G. F., and Melotto, A. (2019a). Even worms matter: cave habitat restoration for a planarian species has increased prey availability but not population density. *Oryx* 53, 216–221. doi: 10.1017/S0030605318000741

Manenti, R., Ghia, D., Fea, G., Ficetola, G. F., Padoa-Schioppa, E., and Canedoli, C. (2019b). Causes and consequences of crayfish extinction: stream connectivity, habitat changes, alien species and ecosystem services. *Freshw. Biol.* 64, 284–293. doi: 10.1111/fwb.13215

Manenti, R., Bonelli, M., Scaccini, D., Binda, A., and Zugnoni, A. (2014). *Austropotamobius pallipes* reduction vs. *Procambarus clarkii* spreading: management implications. *J. Nat. Conserv.* 22, 586–591. doi: 10.1016/j.jnc.2014.09.001

Manenti, R., Falaschi, M., Delle Monache, D., Marta, S., and Ficetola, G. F. (2020). Network-scale effects of invasive species on spatially-structured amphibian populations. *Ecography* 43, 119–127. doi: 10.1111/ecog.04571

Marquis, A. (2006). *Protocole de Reintroduction de l'ecrevisse a Pieds Blancs (Austropotamobius pallipes)*. Besançon: Universite de Franche-Comte.

Martín-Torrijos, L., Kokko, H., Makkonen, J., Jussila, J., and Diéguez-Uribeondo, J. (2019). Mapping 15 years of crayfish plague in the Iberian Peninsula: the impact of two invasive species on the endangered native crayfish. *PLoS One* 14:e0219223. doi: 10.1371/journal.pone.0219223

Mason, J. C. (1975). Crayfish production in a small woodland stream. *Freshw. Crayfish.* 2, 449–479.

Nardi, P. A., Bernini, F., Bo, T., Bonardi, A., Fea, G., Ghia, D., et al. (2005). Status of *Austropotamobius pallipes* complex in the watercourses of the Alessandria province (N-W Italy). *Bull. Fr. Pêche Piscic.* 37, 585–598. doi: 10.1051/Kmae:2005017

Nishijima, S., Nishikawa, C., and Miyashita, T. (2017). Habitat modification by invasive crayfish can facilitate its growth through enhanced food accessibility. *BMC Ecol.* 17:37. doi: 10.1186/s12898-017-0147-7

Paglianti, A., and Gherardi, F. (2004). Combined effects of temperature and diet on growth and survival of young-of-year crayfish: a comparison between indigenous and invasive species. *J. Crust. Biol.* 24, 140–148. doi: 10.1651/C-2374

Perino, A., Pereira, H. M., Navarro, L. M., Fernandez, N., Bullock, J. M., Ceausu, S., et al. (2019). Rewilding complex ecosystems. *Science* 364:eaav5570. doi: 10.1126/science.aav5570

Reynolds, J., Souty-Grosset, C., Gouin, N., Devaney, S., and Grandjean, F. (2000). "Experimental restocking of native crayfish in White lake, Co. Westmeath, Ireland," in *Proceedings of the Crayfish Conference Leeds*, eds D. Rogers and H. Brickland (London: Environment Agency), 123–130.

Rhodes, C. P., and Holdich, D. M. (1979). On size and sexual dimorphism in *Austropotamobius pallipes* (Lereboullet). *Aquaculture* 17, 345–358. doi: 10.1016/0044-8486(79)90089-9

Richman, N. I., Boehm, M., Adams, S. B., Alvarez, F., Bergey, E. A., Bunn, J. J. S., et al. (2015). Multiple drivers of decline in the global status of freshwater crayfish (Decapoda: Astacidea). *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370, 20140060. doi: 10.1098/Rstb.2014.0060

Ripple, W. J., Estes, J. A., Beschta, R. L., Wilmers, C. C., Ritchie, E. G., Hebblewhite, M., et al. (2014). Status and ecological effects of the World's largest carnivores. *Science* 343:151. doi: 10.1126/science.1241484

Rogers, D., and Watson, E. (2007). Increasing the chances of successful reintroduction of white-clawed crayfish (*Austropotamobius pallipes*) in the Peak District National Park, UK. *Int. J. Biodivers. Sci. Ecosyst. Serv. Manag.* 3, 209–216. doi: 10.1080/17451590709618174

Rosewarne, P. J., Mortimer, R. J. G., and Dunn, A. M. (2017). Habitat use by the endangered white-clawed crayfish *Austropotamobius* species complex: a systematic review. *Knowl. Manag. Aquat. Ecosyst.* 4:9.

Siesa, M. E., Manenti, R., Padoa-Schioppa, E., De Bernardi, F., and Ficetola, G. F. (2011). Spatial autocorrelation and the analysis of invasion processes from

distribution data: a study with the crayfish *Procambarus clarkii*. *Biol. Invas.* 13, 2147–2160. doi: 10.1007/s10530-011-0032-9

Souty Grosset, C., Holdich, D. M., Noël, P., Reynolds, J. D., and Haffner, P. (2006). *Atlas of Crayfish in Europe*. Paris: Muséum National d'Histoire Naturelle.

Souty-Grosset, C., Grandjean, F., Raimond, R., Frelon, M., Debenest, C., and Bramard, M. (1997). Conservation genetics of the white-clawed crayfish *Austropotamobius pallipes*: the usefulness of the mitochondrial DNA marker. *Bull. Fr. Pêche Piscic.* 347, 677–692. doi: 10.1051/kmae/1997055

Souty-Grosset, C., Reynolds, J., Gherardi, F., Schulz, R., Edsman, L., Füreder, L., et al. (2006). Craynet-Achievements in scientific management of European crayfish, the way forward and future challenges. *Bull. Fr. Pêche Piscic.* 38, 1395–1399. doi: 10.1051/Kmae:2006043

Spink, J., and Frayling, M. (2000). An assessment of post-plague reintroduced native white-clawed crayfish *Austropotamobius pallipes* in the Sherston Avon and Tetbury Avon. *Wiltshire. Freshw. Forum* 14, 59–69.

Strand, D. A., Johnsen, S. I., Rusch, J. C., Agersnap, S., Larsen, W. B., Knudsen, S. W., et al. (2019). Monitoring a Norwegian freshwater crayfish tragedy: eDNA snapshots of invasion, infection and extinction. *J. Appl. Ecol.* 56, 1661–1673. doi: 10.1111/1365-2664.13404

Svoboda, J., Fischer, D., Kozubíková-Balcarová, E., Šťástková, A., Brůčková, M., Kouba, A., et al. (2020). Experimental evaluation of the potential for crayfish plague transmission through the digestive system of warm-blooded predators. *J. Fish Dis.* 43, 129–138. doi: 10.1111/jfd.13109

Svoboda, J., Mrugała, A., Kozubiková-Balcarová, E., and Petrusek, A. (2017). Hosts and transmission of the crayfish plague pathogen *Aphanomyces astaci*: a review. *J. Fish Dis.* 40, 127–140. doi: 10.1111/jfd.12472

Svoboda, J., Strand, D. A., Vralstad, T., Grandjean, F., Edsman, L., Kozák, P., et al. (2014). The crayfish plague pathogen can infect freshwater-inhabiting crabs. *Freshw. Biol.* 59, 918–929. doi: 10.1111/fwb.12315

Taugbøl, T. (2004). Reintroduction of noble crayfish *Astacus astacus* after crayfish plague in Norway. *Bull. Fr. Pêche Piscic.* 372–373, 315–328. doi: 10.1051/Kmae:2004006

Unger, S., Bodinof-Jachowski, C., Diaz, L., and Williams, L. A. (2020). Observations on habitat preference of juvenile eastern hellbender salamanders (*Cryptobranchus alleganiensis*). *Acta Ethol.* 23, 119–124. doi: 10.1007/s10211-020-00344-9

Weiperth, A., Blaha, M., Szajbert, B., Sepros, R., Banyai, Z., Patoka, J., et al. (2020). Hungary: a European hotspot of non-native crayfish biodiversity. *Knowl. Manag. Aquat. Ecosyst.* 421:43. doi: 10.1051/kmae/2020035

Weiperth, A., Gal, B., Kurikova, P., Blaha, M., Kouba, A., and Patoka, J. (2017). *Cambarellus patzcuarensis* in Hungary: the first dwarf crayfish established outside of North America. *Biologia* 72, 1529–1532. doi: 10.1515/biolog-2017-0159

Woodiwiss, F. S. (1978). *Comparative Study of Biological-Ecological Water Quality Assessment Methods*. Summary Report. Commission of the European Communities. Coventry: Severn Trent Water Authority.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Environmental Science

ORIGINAL RESEARCH
published: 11 March 2021
doi: 10.3389/fenvs.2021.639380

eDNA Detection of Native and Invasive Crayfish Species Allows for Year-Round Monitoring and Large-Scale Screening of Lotic Systems

Franziska Chucholl[1,2], Franziska Fiolka[1], Gernot Segelbacher[2] and Laura Saskia Epp[1]**

[1]*Laboratory of Environmental Genomics, Limnological Institute, University of Konstanz, Konstanz, Germany,* [2]*Laboratory of Conservation Genetics, Wildlife Ecology and Management, University of Freiburg, Freiburg im Breisgau, Germany*

Effective management of both endangered native and invasive alien crayfishes requires knowledge about distribution, monitoring of existing and early detection of newly established populations. Complementary to traditional survey methods, eDNA sampling has recently emerged as a highly sensitive non-invasive detection method to monitor crayfish populations. To advance the use of eDNA as detection tool for crayfish we used a twofold approach: 1) we designed a novel set of specific eDNA-assays for all native (*Austropotamobius torrentium, Austropotamobius pallipes, Astacus astacus*) and the most relevant invasive crayfish species (*Pacifastacus leniusculus, Faxonius limosus, Faxonius immunis*) in Central Europe. To ensure specificity each primer pair was tested *in silico*, *in vitro,* and *in situ*; 2) we assessed the influence of spatio-temporal variables (distance to upstream population, season, stream size) on eDNA detection in seven streams using two different detection methods (qualitative endpoint PCR and quantitative droplet digital PCR, ddPCR). The newly developed eDNA assays successfully detected all crayfish species across different lotic and lentic habitats. eDNA detection rate (endpoint PCR) and eDNA-concentration (ddPCR) were significantly influenced by distance and season. eDNA detection was successful up to 7 km downstream of the source population and across all seasons, although detectability was lowest in winter. eDNA detection rate further decreased with increasing stream size. Finally, eDNA-concentration correlated positively with estimated upstream population size. Overall, we provide near operational eDNA assays for six crayfish species, enabling year-round detection, which represents a clear benefit over conventional methods. Due to its high sensitivity, eDNA detection is also suitable for the targeted search of as-yet unrecorded or newly emerging populations. Using quantitative ddPCR might further allow for a rough estimation of population size, provided that the identified spatio-temporal factors are accounted for. We therefore recommend implementing eDNA-detection as a complementary survey tool, particularly for a large-scale screening of data-deficient catchments or a year-round monitoring.

Keywords: environmental DNA, species detection, crayfish, freshwater systems, monitoring

OPEN ACCESS

Edited by:
Ivana Maguire,
University of Zagreb, Croatia

Reviewed by:
Chloe Victoria Robinson,
University of Guelph, Canada
David A. Strand,
Norwegian Veterinary Institute, Norway

****Correspondence:***
Franziska Chucholl
franziska.chucholl@uni-konstanz.de
Laura Saskia Epp
laura.saskia.epp@gmail.com

Specialty section:
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Environmental Science

Received: *08 December 2020*
Accepted: *20 January 2021*
Published: *11 March 2021*

Citation:
Chucholl F, Fiolka F, Segelbacher G and Epp LS (2021) eDNA Detection of Native and Invasive Crayfish Species Allows for Year-Round Monitoring and Large-Scale Screening of Lotic Systems. Front. Environ. Sci. 9:639380. doi: 10.3389/fenvs.2021.639380

INTRODUCTION

Freshwater crayfish (Crustacea, Decapoda, Astacidae) are among the most threatened animal taxa in Central European fresh waters (Souty-Grosset et al., 2006; Chucholl and Schrimpf, 2016). All of the three indigenous crayfish species (ICS; *Astacus astacus, Austropotamobius pallipes, Austropotamobius torrentium*) are listed under the Habitats Directive and their conservation status is "unfavourable-inadequate" to "unfavourable-bad" in all biogeographical regions of the European Union with deteriorating population trends (Habitats Directive 92/43/EEC). The alarming population declines of native crayfish are driven by several threats, of which the most imminent is the ongoing spread of invasive non-indigenous crayfish species (NICS) of North American origin (Holdich et al., 2009). These act as a reservoir host for the causative agent of crayfish plague (*Aphanomyces astaci*), a lethal disease for native crayfish (Almeida et al., 2014; Chucholl and Schrimpf, 2016). In addition to displacement by NICS and crayfish plague, ICS suffer from pollution and degradation of habitats, and the increasing frequency of summer droughts due to climate change (Chucholl and Schrimpf, 2016).

Effective management of both ICS and NICS requires knowledge about distribution, early detection of newly emerging and monitoring of existing populations (Chucholl and Dehus, 2011; Kouba et al., 2014; Agersnap et al., 2017). However, conventional detection methods, such as manual search and trapping, are often hampered by the nocturnal behavior and elusive nature of crayfish (Peay, 2004). Specifically, crayfish populations are very hard to detect at low population densities, which are typical for early invasion stages of NICS and deteriorating populations of ICS (Cowart et al., 2018; Rice et al., 2018). What is more, conventional methods are mostly successful during summer when animals are active, and are normally associated with a disturbance of the habitat (Peay, 2004; Olarte et al., 2019).

In recent years, environmental DNA (eDNA) has emerged as new monitoring tool to survey aquatic environments (Ficetola et al., 2008; Goldberg et al., 2015; Yates et al., 2019), including crayfish (Dougherty et al., 2016; Mauvisseau et al., 2017; Rusch et al., 2020). The advantages of eDNA as monitoring tool are the high sensitivity of the method, the possibility to screen large sections of running waters with only a few sampling sites and the non-invasive nature of the sampling (Larson et al., 2017; Geerts et al., 2018). Detection by means of eDNA therefore shows considerable promise for both the early detection of new or spreading biological invasions (Jerde et al., 2011; Cowart et al., 2018), as well as the monitoring of rare or endangered species that also often occur at low abundances (Atkinson et al., 2019; Ikeda et al., 2019). Moreover, the simultaneous detection of both ICS and NICS is possible (Robinson et al., 2018). Studies on eDNA detection of crayfish show general applicability of this novel method and confirm its high sensitivity (Agersnap et al., 2017; Mauvisseau et al., 2017; Rusch et al., 2020). For instance, in small headwater streams in Japan, crayfish detection by means of eDNA was more sensitive than conventional methods, *i.e.* eDNA was found in sampling sites where a manual capture failed to detect crayfish (Ikeda et al., 2016).

However, it is still largely unknown how environmental factors and population properties affect detectability of crayfish populations. For instance, eDNA transport distances in lotic systems, that are often taxon-specific (Deiner and Altermatt, 2014; Wacker et al., 2019), are unknown for freshwater crayfish, although persistence of eDNA was assessed in laboratory and mesocosm experiments (Dunn et al., 2017; Harper et al., 2018). Moreover, unlike in other taxa (Buxton et al., 2018; Wacker et al., 2019; Curtis et al., 2020), seasonal variation of crayfish eDNA detectability has never been consistently assessed; existing studies either sampled only during one season or included preliminary field experiments (Ikeda et al., 2016; Harper et al., 2018; Rusch et al., 2020). Finally, water volume, e.g. during flood conditions, has been found to influence eDNA detectability (Curtis et al., 2020). This might imply an influence of stream size on crayfish eDNA detectability, which, however, has not been investigated yet. Overall, this lack of information is unfortunate as the routine application of eDNA detection, for instance in applied conservation projects, generally requires knowledge about these relationships.

To advance eDNA as detection tool for Central European crayfish we used a twofold approach: 1) we designed a novel set of specific endpoint eDNA-assays for all native (*Austropotamobius torrentium, Austropotamobius pallipes, Astacus astacus*) and the most relevant invasive crayfish species (*Pacifastacus leniusculus, Faxonius limosus, Faxonius immunis*) using a consistent multiple step approach, including an *in silico*, *in vitro* and *in situ* evaluation; 2) we assessed the influence of spatio-temporal variables (distance to upstream population, season, stream size) on eDNA detection. For this, we took water samples from 40 sampling sites in seven streams using two different detection methods (qualitative endpoint PCR and quantitative droplet digital PCR, ddPCR). All samples were subjected to endpoint PCR, whereas only a subset of samples (30 sampling sites in four streams) was analyzed with ddPCR. Finally, ddPCR was used to assess the relationship between estimated population size and eDNA concentration in the water.

We expected that eDNA detection probability is highest within or directly downstream of the population (Rice et al., 2018) and during the main activity time from spring to autumn (Harper et al., 2018). For small streams we expected a higher eDNA detection probability than for large rivers because the eDNA signal is probably diluted in a larger volume of water. Furthermore, we hypothesized a correlation between eDNA concentration in the water and estimated upstream population size, as the eDNA signal probably integrates with the populated stretch through downstream flow (Rice et al., 2018).

MATERIALS AND METHODS

Primer Design and Evaluation

For each crayfish species a specific eDNA-assay with species-specific primers was developed. Primers were initially designed by

TABLE 1 | Specific primer pairs for all crayfish species investigated in this study and ddPCR probes for *A. torrentium* and *A. pallipes* (species name and status, primer/probe name, primer sequence, length in basepairs, annealing temperature (Ta) used in the PCR reaction).

Target species	Species status	Primer/probe	Primer name	Sequence (5′-3′)	Length	Ta
A. torrentium	native	forward	Torr_COI_333F	GGGTACCGGTTGAACTGTCTAC	22	58
A. torrentium	native	probe	Torr_COI_381P	HEX/CTCACGCAG/ZEN/GAGCCTCTGTAGAT/3IABkFQ	23	64
A. torrentium	native	reverse	Torr_COI_514R	CGATCTAAAGTTATTCCCACACCC	24	58
A. pallipes	native	forward	Pall_COI_299F	GAGGGTTAGTGGAGAGAGGG	20	60
A. pallipes	native	probe	Pall_COI_353P	FAM/CATCACT/ZEN/TTGCCCACGCAGG/3IABkFQ	22	66
A. pallipes	native	reverse	Pall_COI_401R	AAATCCCCAGATCCACAGACG	21	60
A. astacus	native	forward	Ast_COI_324F	GATTAGAGGAATAGTAGAGAGAGG	24	54
A. astacus	native	reverse	Ast_COI_434R	TGCCAAGTGTAATGAAAAAATCC	23	54
P. leniusculus	invasive	forward	Len_COI_320F	AAGAGGAGTGGGTACTGGAT	20	60
P. leniusculus	invasive	reverse	Len_COI_428R	AACACCCGCTAAATGAAGTG	20	60
F. limosus	invasive	forward	Lim_COI_380F	GAACAGTGTATCCTCCTCTC	20	54
F. limosus	invasive	reverse	Lim_COI_522R	GGCCCGTATATTAATAGCCG	20	54
F. immunis	invasive	forward	Imm_COI_76F	GAATAGTTGGGACTTCGTTAAGAT	24	54
F. immunis	invasive	reverse	Imm_COI_516R	CTGCACGTATATTAATAGCCGT	22	54

visually screening alignments using the software Geneious Prime 2020.0.5 and evaluated for specificity using the program ecoPCR (Ficetola et al., 2010). Species-specific primers, amplifying fragments of 58–295 bp, were designed using alignments of the standard animal barcoding marker Cytochrome c Oxidase subunit I (COI). Alignments were built using published sequences of the respective target species and sequences of a range of non-target species. Accession numbers of the sequences used are listed in **Supplementary Table S1**.

For *Astacus astacus, Faxonius limosus* and *Faxonius immunis* all European sequences were used as target sequences. For *Austropotamobius pallipes* only sequences from the West European haplotype were included in the alignment, as *A. pallipes* represents a species complex with several proposed (sub) species, of which only the western form occurs in Central and Northern Europe (Grandjean et al., 2000; Gouin et al., 2006). For *Austropotamobius torrentium*, only haplotypes from the North of the Alps were included as target sequences, as haplotypes from the South of the Alpes belong to genetically different lineages (Trontelj et al., 2005) For non-native *Pacifastacus leniusculus* only sequences that belong to the subspecies *P. l. leniusculus* were included, because the other subspecies have never been imported from North America into Europe (Larson et al., 2012). As outgroups, sequences of all native and non-native crayfish species occurring in Europe, aquatic species that are likely to occur in the same habitats (*e.g.*, *Cottus gobio, Salmo trutta,* and *Gammarus fossarum*) and species that represent common contaminants (e.g., humans and chicken) were included in the alignment.

The alignment was visually inspected for suitable primer pairs, which were subsequently tested in an *in silico* PCR for their specificity against the public NCBI database, covering approximately 160,000 taxa, using the program ecoPCR (Ficetola et al., 2010). The specified conditions for the *in silico* amplification allowed for a maximum of three mismatches for each primer, but demanded a perfect match on the last two nucleotides of the 3' end of each primer. The minimum and maximum amplified sequence lengths (excluding primers) were 5 and 1,000 bp, respectively. Output of the ecoPCR was subsequently screened to ensure that amplified non-target species do not occur in European freshwater habitats (*i.e.* they were marine species, terrestrial species or species with a distribution range outside Europe).

After the *in silico* evaluation, the most suitable primer pairs were tested *in vitro* for their efficiency and specificity against tissue and environmental samples (see below) in an endpoint PCR. Each primer pair was tested with DNA extractions from tissue samples of both the target species and of non-target crayfish species, using DNA of the following: *A. torrentium, A. pallipes, A. astacus, P. leniusculus, F. limosus, F. immunis, Procambarus virginalis*. Finally, the best performing primer pair for each crayfish species was selected (**Table 1**).

To ensure that the selected primers correctly amplified the target amplicon, also in DNA extractions of environmental samples, PCR-products of at least one environmental sample for each primer were purified with ExoSAP-IT™ PCR Product Cleanup (ThermoFisher Scientific) and DNA sequences were obtained through Sanger-Sequencing (performed by Microsynth Seqlab, Göttingen).

Study Populations

To test the efficiency and specificity of the novel set of eDNA-assays *in situ,* a total of 13 water bodies with known populations of the six target species were sampled (**Figure 1**). Detailed information on water body characteristics and eDNA sampling is summarized in **Table 2**. To prevent accidental transmission of the crayfish plague agent between water bodies, the used sampling equipment was cleaned and thoroughly dried for a minimum of 7 days upon each sampling occasion (OIE, 2019). In streams with several sampling locations, sites were visited in direction of stream flow to avoid upstream transmission of *A. astaci* spores. Furthermore, in streams with both manual capture and eDNA sampling (see *Population Abundance and Size*), the eDNA sampling was always done before the manual capture, to prevent contamination of eDNA sampling equipment with crayfish DNA.

eDNA Sample Collection and Extraction

At each sampling site and occasion, four replicates of water samples were taken using sterile standup Whirl-Pak® sampling

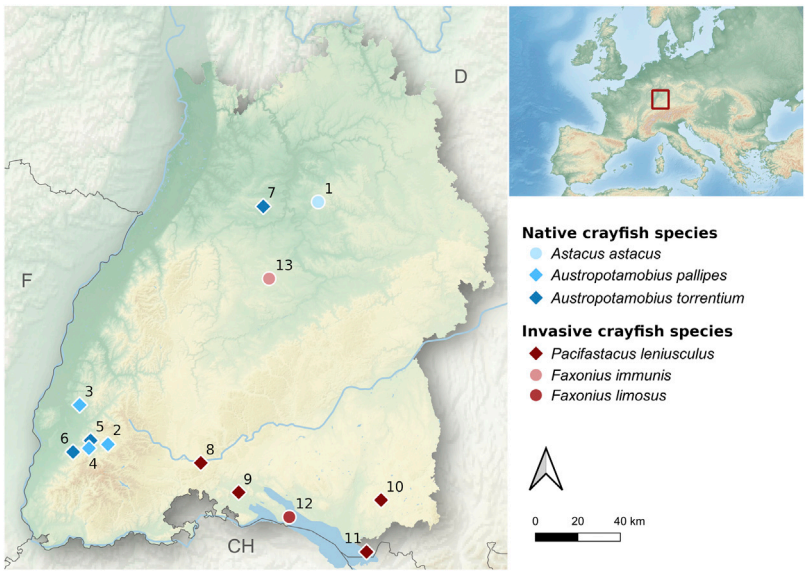

FIGURE 1 | Map of the study area (federal state of Baden-Württemberg, southwestern Germany) showing all investigated water bodies. Native and invasive crayfish populations are presented by blue- and red-colored symbols, respectively. Sampling sites with diamonds were used for spatio-temporal eDNA sampling, whereas sampling sites with circles were only used for primer evaluation. *See* **Table 2** for habitat characteristics.

TABLE 2 | Overview of the investigated water bodies with information on the target species, name and catchment of water body, habitat characteristics, sampling date and sampling method.

No.	Name	Species	Catchment	Habitat	Elevation [m]	Sampling date	Spatio-temp. Sampling
1	Bottwar	*A. astacus*	Neckar	stream	277	Aug 2020	no
2	Wagensteigbach	*A. pallipes*	Rhine	stream	531	Aug, Oct 2019 Feb, Apr 2020	yes (four sites), two sites in Oct
3	Aubächle	*A. pallipes*	Rhine	small stream	344	Aug 2019	no
4	Brugga	*A. pallipes*	Rhine	stream	352	Apr 2020	no
5	Attentalerbach	*A. torrentium*	Rhine	small stream	412	Aug, Oct 2019 Feb, Apr 2020	yes (two sites)
6	Mühlbach	*A. torrentium*	Rhine	small stream	318	Oct 2020	yes (seven sites)
7	Kirbach	*A. torrentium*	Neckar	stream	244	Aug 2019	yes (two sites)
8	Danube	*P. leniusculus*	Danube	river	663	May 2020	yes (three sites)
9	Radolfzeller Aach	*P. leniusculus*	Lake Constance	small river	437	May 2020	yes (three sites)
10	Eggenbach	*P. leniusculus*	Lake Constance	stream	652	Jun 2020	yes (five sites)
11	Lake Constance	*P. leniusculus*	Lake Constance	large lake	395	May 2020	no
12	Lake Constance	*F. limosus*	Lake Constance	large lake	395	Nov 2019	no
13	Goldbachsee	*F. limosus F. immunis*	Neckar	small lake	450	Jul 2020	no

The numbering of water bodies corresponds to the respective numbers in ***Figure 1****.*

bags (V = 2041 ml, Nasco). Upon sampling, water samples were immediately put on ice in an opaque box and transported to the laboratory. Per sample 2 L of water were filtered, but in some cases, where the filter was clogged, the filtered volume was reduced to 1.5 L. All water samples were filtered on the same day using a membrane vacuum pump (VWR vacuum pump/compressor VCP 130) connected to a filter flask attached to a sterile analytical filter funnel with a Cellulose-Nitrate filter inside (analytical filter funnels, CN, Nalgene®, pore size 0.45 µm). For each sample, a new sterile filter funnel was used. Filters were removed from the funnel with sterile forceps and the filter was loosely rolled and put into a sterile 8 ml tube (Sarstedt). Subsequently, samples were frozen at −20°C until DNA-extraction. Extractions of eDNA samples were performed in a clean laboratory that had never been exposed to crayfish DNA. All extractions were carried out under an eDNA-extraction hood in a pre-PCR lab that is physically separated from the PCR and post-PCR lab to avoid back-contamination from PCR products. Before and after every DNA-extraction the bench of the extraction hood was cleaned with DNA-exitus, wiped with ddH_2O and sterilized with UV-light for 15 min. Extractions were carried out using the DNeasy Power Water kit from Qiagen, following the protocol of the manufacturer. For every 18 samples, one extraction blank was included.

Spatio-Temporal Sampling Design

To test for spatio-temporal effects (distance to upstream population, season, and stream size) on eDNA detection a

total of 40 sampling sites in seven streams with known populations of either *A. torrentium* (N = 3), *A. pallipes* (N = 1) or *P. leniusculus* (N = 3) were sampled (**Table 2**). Contemporary population extent was either known from monitoring surveys within the last 6 months or was surveyed during this study in May and August 2020 using manual capture following Chucholl and Schrimpf (2016). To account for spatial effects on eDNA detection, each of these streams was sampled with two to seven sampling sites. The most upstream sampling site was always located within the core population and the remaining sampling sites were distributed downstream of the population in an equally spaced distance from each other. The distance to the upstream crayfish population was measured as in-stream distance between the lower distribution limit of the population and the respective sampling site and ranged between 800 and 7,000 m. The number of sampling sites downstream of the core population was determined by the distance between the lower distribution limit and the confluence of the stream with the main water course. To make sure that eDNA-concentration in the flow was not significantly diluted by other waters, only streams without major tributaries were selected. To investigate seasonal effects on eDNA detection, a year-round sampling in spring, summer, autumn and winter was done in two streams (*c.f.* **Table 2**). To assess the effect of stream size on eDNA detection all investigated streams were categorized into four size classes, according to their width (0–5 m = small stream, >5–10 m = stream, >10–20 m = small river, >20 m = river).

PCR Procedures

Endpoint PCR

Qualitative endpoint PCR was performed for all tissue and environmental samples using a Flex Cycler (Analytik Jena). All PCR assays were prepared in a prePCR lab either on a bench (tissue samples) or under a UV hood reserved for PCR setup of eDNA extractions (environmental water samples) that was cleaned as outlined above for the UV hood used for DNA extraction.

For runs that contained only tissue samples, each 21.5 µl reaction contained 10 µl myTaq mix (MyTaq™ Mix, Bioline), 8 µl DEPC treated H2O, 0.5 µl BSA (4 mg/ml), 1 µl of each primer (10 µM) and 1 µl template DNA. For amplification the following cycling protocol was used: 95°C for 1 min (initial denaturation), 35 cycles of 95°C for 20 s (denaturation), 55 °C for 30 s (annealing), 72°C for 20 s (elongation), and a final elongation step at 72°C for 2 min.

For environmental water samples, all reactions were run in triplicates including a positive control (tissue), an extraction blank and a negative template control (NTC) for every run of N = 22 samples. Prior to use, the PCR buffer, $MgSO_4$ and BSA solution were subjected to 5 min of UV irradiation at short distance from the light bulbs in a UVP crosslinker CL-1000 (see recommendations by Champlot et al. 2010). Each subsequent 25 µl reaction contained 15.05 µl water, 2.5 µl buffer (10x), 0.25 µl dNTPs (25 mM), 1 µl BSA (4 mg/ml), 1 µl $MgSO_4$ (50 mM), 1 µl of each Primer (10 µM), 0.2 µl polymerase (Platinum™ *Taq* DNA-Polymerase High Fidelity, Invitrogen™, ThermoFisher Scientific, 5 U/ul) and 3 µl template DNA. For amplification the following cycling protocol was used: Initial denaturation at 94°C for 4 min, 55 cycles of denaturation at 94°C for 30 s, primer-specific annealing at 52°C (*F. limosus*), 56°C (*F. immunis, A. astacus*), 58°C (*A. torrentium*), 60°C (*A. pallipes, P. leniusculus*) for 30 s, elongation at 72°C for 20 s, and a final elongation step at 72°C for 2 min. After amplification PCR products were visually checked for bands of the correct amplicon size using a 2% agarose gel electrophoresis stained with GelRed (Biotium, Hayward, CA). If only one technical PCR replicate was positive, at least one PCR product from this site was purified with ExoSAP-IT™ PCR Product Cleanup (ThermoFisher Scientific) and subjected to Sanger-Sequencing (performed by Microsynth Seqlab, Göttingen) to exclude the possibility of false positives. Finally, the detection rate was calculated as proportion of positive PCR replicates per sampling site by dividing the number of positive PCR replicates per site by the total number of PCR replicates per site. The lower and a upper 95% confidence interval (CI) of the detection rate was calculated using the R package epiR (R Core Team, 2019; Nunes et al., 2020).

Droplet Digital PCR

In addition to the endpoint PCR, environmental samples from the *A. pallipes* and *A. torrentium* streams (*cf.* **Table 2**) were subjected to a quantitative droplet digital PCR (ddPCR) using a BioRad QX200 system. To quantify target DNA of *A. pallipes* and *A. torrentium* a double-quenched TaqMan probe was developed according to the assay design guidelines for ddPCR (Bio-Rad). The *A. pallipes* probe had a length of 22 base pairs: Pallipes_COI_353P (/56-FAM/CAT CAG CTA/ZEN/TTG CCC ACG CAG G/3IABkFQ/) and the *A. torrentium* probe had a length of 23 base pairs: Torrentium_COI_381P (/56-HEX/CTC ACG CAG/ZEN/GAG CCT CTG TAG AT/3IABkFQ/). In the ddPCR every sample is partitioned in 20,000 droplets and target and non-target DNA is distributed randomly. In every single droplet a PCR reaction takes place. The target DNA is marked by the fluorescence probe and the number of positive droplets is measured by a droplet reader. All ddPCR reactions were run in triplicates with a positive control (tissue), an extraction blank and an NTC for each 96 well PCR plate. Each 22 µl reaction contained 11 µl of ddPCR Supermix for Probes (Bio Rad), 1 µl of the probe (5.5 µM), 1 µl of each primer (19.8 µM), 6 µl of DEPC treated H_2O and 3 µl of template DNA. Of each 22 µl reaction 20 µl were transferred to a BioRad QX200 droplet generator, which partitioned each reaction mixture into nanodroplets by combining 20 µl of the reaction mixture with 70 µl of BioRad droplet oil for Probes. After processing, this resulted in a total nanodroplet volume of 40 µl, which was transferred to a 96 well PCR plate for amplification on Bio Rad C1000 Touch™ thermal cycler using the following cycling protocol: hold at 95°C for 10 min, 40 cycles of 94°C for 30 s (denaturation), 58°C (*A. torrentium*) or 60°C (*A. pallipes*) for 30 s (annealing), 60°C for 30 s (elongation), and a final enzyme deactivation step at 98°C for 10 min. The plate was then analyzed on a QX200 droplet reader. The DNA copies in each reaction were calculated (absolute quantification) using the manufactures software (QuantaSoft™ Analysis Pro 1.0.596),

which separates negative from positive droplets. A ddPCR replicate was scored as positive, when two or more droplets were positive. The QuantaSoft™ software automatically calculates the number of copies per µl of the final PCR Mix, which was subsequently converted to copies per µl of the starting sample using the following formula: number of copies per µl × 25 µl (the initial volume of the PCR Mix used for each reaction)/ 3 µl (the volume of template DNA used for each reaction). As for the endpoint PCR, a water sample was considered as positive, when one or more of the three technical ddPCR replicates were positive. Finally, the mean number of DNA copies per µl was calculated for each water sample by averaging the three technical ddPCR replicates.

Population Abundance and Size

To assess whether eDNA-concentration obtained by ddPCR was related to upstream crayfish population size, the size of six *A. pallipes* and *A. torrentium* populations was estimated from point abundance data and the population extent upstream of the sampling site. Abundance (indiv./m^2) of crayfish was estimated with the "removal capture"-method (two-pass depletion approach) in August 2020 (Gouin et al., 2011). For this, a defined stretch of the stream was searched twice for crayfish by carefully inspecting potential shelters (e.g. stones, submerged roots) with a standardized effort. After the first pass, captured animals were temporarily removed from the stream until the second pass was undertaken. From the number of crayfish caught in the first and the second pass and the sampled area an estimate for the abundance (±confidence interval, CI) was calculated using the *R* package FSA (Ogle et al., 2020). Upstream population extent was assessed as in-stream distance between the respective eDNA sampling site and the upper distribution limit of the population. The distribution limit was taken from recent crayfish surveys in the course of the Habitats Directive (survey years: 2017–2019, fish and crayfish database of the federal state of Baden-Württemberg, FiAKa). Upstream population size was then calculated from the point abundance estimate and the upstream population extent, with confidence intervals propagated from the CI of the removal capture estimate. All crayfish samplings were done in agreement with the federal fisheries and nature conservation laws.

Statistical Analyses

All statistical analyses were performed using the software R 4.0.3 (R Core Team, 2019). Prior to statistical analyses, all variables were checked for normality and nonparametric tests were used when appropriate. Multiple linear mixed-effect models (LMM) were used to assess the effects of the spatio-temporal candidate predictors (distance to upstream population, season, and stream size) on eDNA detection rate (endpoint PCR) and eDNA-concentration (ddPCR), respectively. For the endpoint PCR, the proportion of positive PCRs per sampling site (detection rate) and for the ddPCR, the mean eDNA concentration per sampling site was used as dependent response variable. The stream ID was included as a random factor, as samplings sites were not independent from each other. Distance to upstream population (Dist. [m]), season (factor with four levels) and stream size (ordered factor with four levels) were included as independent explanatory variables. To identify the optimal model, four candidate models containing different combinations of the predictor variables were compared based on the Akaike information criterion, corrected for small sample size (AICc). The AICc is a model selection tool that measures model fit based on likelihood, with a penalty for model complexity (Chambers and Hastie, 1992; Zuur et al., 2009). Then the model with the highest Akaike weight (wAICc) and the highest relative likelihood (rel. LL) was selected and its performance was tested against a null model that only included a constant predictor as a fixed effect. For all model comparisons, individual models were fitted with maximum likelihood (ML) (Zuur et al., 2009). To examine the optimal model in detail, it was fitted with restricted maximum likelihood (REML) and predictor effects were assessed by analysis of deviance (Type II Test) (Zuur et al., 2009). As a measure of goodness of fit of the optimal model, marginal R^2 were calculated following Nakagawa et al., 2017. To test for a correlation between estimated population size and eDNA concentration a Pearson's product-moment correlation was undertaken. Then a linear model (LM) was used to assess the effect of estimated population size on eDNA concentration.

RESULTS

Primer Evaluation

In silico evaluation of the primers for the six target crayfish species showed high specificity (no amplification of non-target species at zero nucleotide mismatches for all primers). When allowing for 1–3 mismatches amplification of $N = 0$ (*A. torrentium* primers), $N = 7$ (*A. pallipes* primers), $N = 6$ (*A. astacus* primers), $N = 19$ (*F. limosus* primers), $N = 4$ (*F. immunis* primers), and $N = 285$ (*P. leniusculus* primers) non-target species occurred, none of which are known to inhabit European freshwaters. *In vitro* validation with tissue samples confirmed that all six primer pairs amplified

TABLE 3 | Results of species-specific eDNA detection from sampling sites used for primer evaluation (see **Figure 1** and **Table 2** for location and characteristics of water bodies). Detection rate is given as proportion of positive endpoint PCRs (including 95% confidence interval).

No.	Species	Habitat	Pos. # PCR	Total # PCR	Detection rate (95 %CI)
1	*A. astacus*	stream	5	9	0.56 (0.21–0.86)
2	*A. pallipes*	stream	15	15	1.00 (0.78–1.00)
3	*A. pallipes*	small stream	12	12	1.00 (0.73–1.00)
4	*A. pallipes*	stream	12	12	1.00 (0.73–1.00)
5	*A. torrentium*	small stream	12	12	1.00 (0.73–1.00)
6	*A. torrentium*	small stream	12	12	1.00 (0.73–1.00)
7	*A. torrentium*	stream	12	12	1.00 (0.73–1.00)
8	*P. leniusculus*	river	4	12	0.33 (0.09–0.65)
9	*P. leniusculus*	small river	9	18	0.50 (0.26–0.74)
10	*P. leniusculus*	stream	18	18	1.00 (0.81–1.00)
11	*P. leniusculus*	large lake	3	18	0.17 (0.03–0.41)
12	*F. limosus*	large lake	6	18	0.33 (0.13–0.59)
13	*F. limosus*	small lake	9	9	1.00 (0.66–1.00)
13	*F. immunis*	small lake	3	9	0.33 (0.07–0.70)

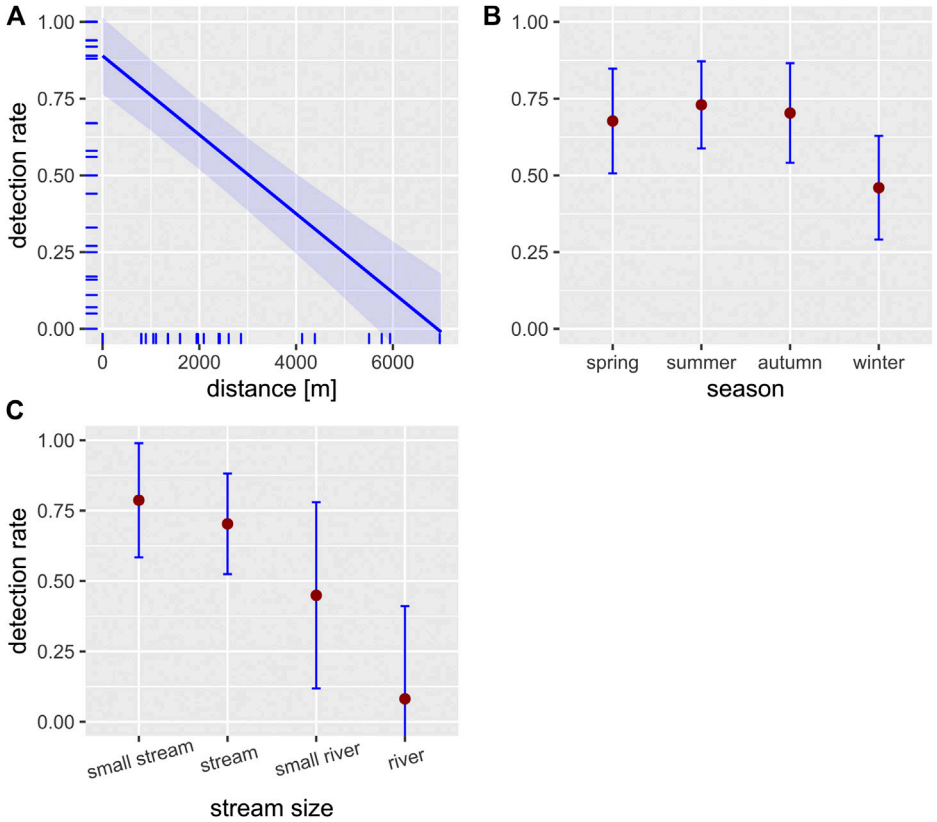

FIGURE 2 | Effect plots of the LMM used to predict the effect of the three independent variables distance **(A)**, season **(B)** and stream size **(C)** on the detection rate (proportion of positive PCRs; blue-shaded areas and error bars represent the 95% confidence intervals; *N* = 40, *see* **Table 5** for model statistics). Small ticks on the axes indicate the marginal distribution of the data.

TABLE 4 | Ranking of the three best performing candidate models to explain the detection rate (proportion of positive PCRs) and eDNA concentration in relation to the respective null model (with a constant predictor as fixed effect).

Response	*N*	Model	Predictors	δAICc	ωAICc	Rel. LL
detection rate	40	LMM_1	distance + season + streamsize	0.00	1.00	0.98
		LMM_2	distance + season	7.63	0.02	0.02
		LMM_3	distance	15.18	0.00	0.00
		null mod.	constant predictor	47.14	0.00	0.00
eDNA concentration	30	LMM_A	distance + season	0.00	1.00	0.73
		LMM_B	distance + season + streamsize	2.00	0.37	0.27
		LMM_C	distance	9.28	0.00	0.00
		null mod.	constant predictor	30.17	0.00	0.00

Stream ID was included in all models as random factor. AICc refers to the Akaike information criterion, ωAICc indicates the Akaike weight, and rel. LL gives the relative likelihood for each model.

their target species successfully, with no cross-amplification of tissue from other crayfish species (*A. torrentium, A. pallipes, A. astacus, P. leniusculus, F. limosus, F. immunis, P. virginalis*). In environmental water samples, taken *in situ*, detection of target-DNA was also successful for all tested crayfish species, but detectability varied across species and habitats (**Table 3**). None of the negative controls (extraction blank or NTC) was positive. Moreover, sanger sequencing of a subset of PCR products from water samples confirmed the correct amplicon for each species.

Spatio-Temporal Effects on eDNA Detectability

Multiple mixed regression analysis revealed a significant effect of distance, season and stream size on eDNA detectability (**Figure 2**). A ranking of the best performing candidate models is shown in **Table 4**. LMM 1 and LMM A were considered optimal models based on the applied model selection criteria (δAICc, ωAICc, and rel. LL). Both models were highly significant and performed better than the

TABLE 5 | Fixed effects of spatio-temporal variables on the detection rate (proportion of positive PCRs) and eDNA concentration as assessed by mixed-model analysis with stream ID as a random factor (*see* **Table 4** for model statistics).

Response	*N*	Model	Fixed effects	χ^2	*P*	
detection rate	40	LMM_1	distance	79.50	<0.001	***
			season	13.24	<0.01	**
			stream size	14.41	<0.01	**
eDNA concentration.	30	LMM_A	distance	47.49	<0.001	***
			season	16.61	<0.001	***

*Chi-square statistics were calculated by drop-one hypothesis testing using likelihood as measure of model fit, and asterisks denote significant effects (*p < 0.05, **p < 0.01, and ***p < 0.001).*

respective null models with a constant predictor as a fixed effect (chi-square test: $\chi^2 = 61.14$, $p < 0.001$ and $\chi^2 = 38.17$, $p < 0.001$ for LMM 1 and LMM A, respectively). Goodness of fit of the optimal models, as assessed by marginal R^2, ranged between 0.69 (LMM A) and 0.72 (LMM 1).

The fixed effects of the spatio-temporal predictors on detection rate and eDNA concentration, as contained in the optimal model, are summarized in **Table 5**. Distance had a strong negative effect on detection rate and eDNA concentration (**Figures 2**,**3**, respectively). Season showed a clear effect on both response variables, with a lower detection rate and eDNA concentration in winter compared to the other seasons (**Figures 2**,**3**, respectively). Stream size was negatively associated with detection rate (**Figure 2**). The detection rate was independent of the PCR method (endpoint PCR *vs.* ddPCR) (Wilcoxon Signed Rank Test, $N = 30$, $p > 0.3$).

Relationship Between eDNA Concentration and Estimated Population Size

There was a significant correlation between estimated upstream population size and eDNA concentration in the water from sampling sites within populations (Pearson's product-moment correlation, $N = 6$, $R = 0.93$, $p < 0.01$). Linear regression analysis revealed a positive effect of estimated population size on eDNA concentration (**Figure 4**, adj. $R^2 = 0.83$, $p < 0.01$).

DISCUSSION

Primer Evaluation and eDNA Detection

We designed a novel set of specific assays for all native (*A. torrentium, A. pallipes, A. astacus*) and the most relevant invasive crayfish species (*P. leniusculus, F. limosus, F. immunis*) in Europe. To ensure specificity and sensitivity of our assays we used a consistent multiple step approach, consisting of *in silico*, *in vitro* and *in situ* evaluation as well as Sanger sequencing to confirm correct amplification of each amplicon.

Our approach is largely consistent to the recently proposed validation scale for targeted eDNA assays (Thalinger et al., 2020). According to this scale there are five levels ranging from "incomplete" to "operational." Within this scale our assays can be classified from level 4 "substantial" to level 5 "operational." Except for establishing a limit of detection (LOD), which is not possible when using endpoint PCR, our assays met all criteria propagated by the validation scale (Thalinger et al., 2020).

Our study shows successful eDNA detection of all investigated native and invasive crayfishes in a variety of habitats. Detection efficiency was 100%, *i.e.* crayfish were successfully detected at all sites where they have been known to occur (*c.f.* **Table 3**). This is in line with results of previous studies that investigated the suitability of eDNA as monitoring tool for freshwater crayfish (Tréguier et al., 2014; Dougherty et al., 2016; Ikeda et al., 2016; Agersnap et al., 2017; Mauvisseau et al., 2017; Ikeda et al., 2019; Troth et al., 2019; Rusch et al., 2020). However, detection efficiency varied among studies. For example, Treguier et al. (2014) detected the invasive crayfish *Procambarus clarkii* in 59% of ponds where it was trapped. Mauvisseau et al. (2017) investigated ponds in the same region but with a different assay and detected *P. clarkii* in 70% and *F. limosus* in 66% of the ponds, where presence was confirmed.

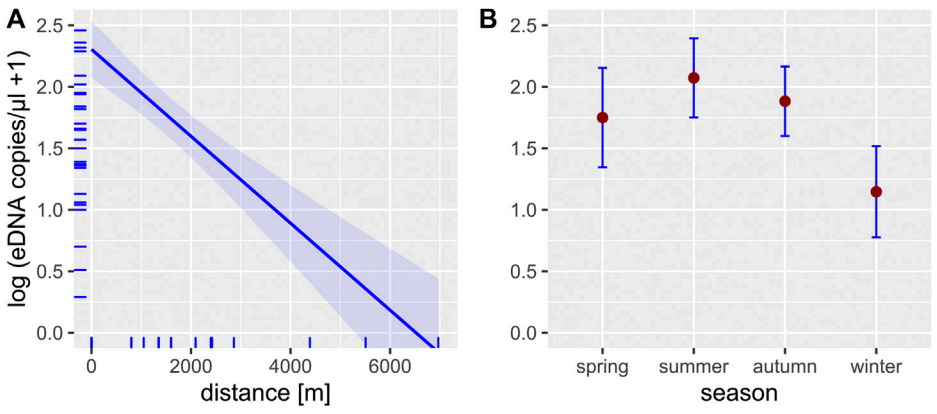

FIGURE 3 | Effect plots of the LMM used to predict the effect of the two independent variables distance **(A)** and season **(B)** on the eDNA concentration (blue-shaded area and error bars represent the 95% confidence intervals; $N = 30$, *see* **Table 5** for model statistics). Small ticks on the axes indicate the marginal distribution of the data.

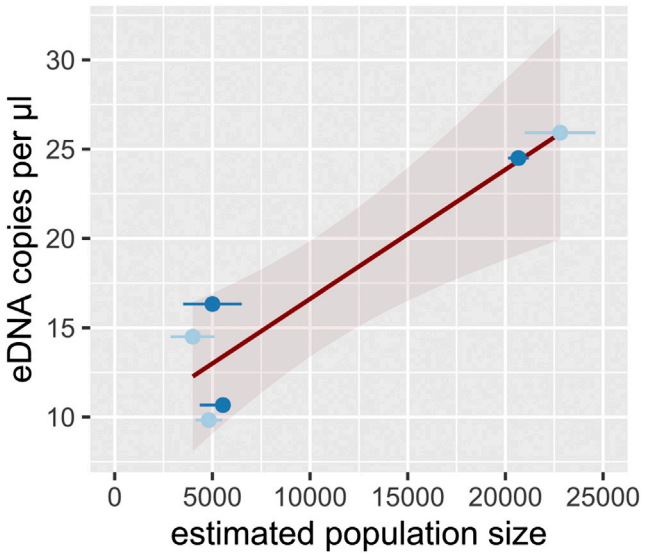

FIGURE 4 | eDNA concentration within populations (median per sampling site) in relation to estimated upstream population size. Trend line is given by linear regression ($R^2 = 0.83$, $p < 0.01$, red-shaded area represents the 95% confidence interval). Light-blue and dark-blue dots represent populations of *A. pallipes* and *A. torrentium*, respectively. Horizontal error bars indicate the lower and upper 95% CI for the population estimate.

Dougherty et al. (2016) investigated eDNA detection of *Faxonius rusticus* at low abundances in inland lakes of North America and showed a 100% accordance with conventional methods. A recent study of Rusch et al. (2020) investigated eDNA detection of native (*A. astacus*) and invasive (*P. leniusculus, F. limosus* and *P. virginalis*) crayfishes in a wide range of habitats in Central Europe and found crayfish in 95% of the habitats, where presence was confirmed.

Detection rate, *i.e.* the number of positive PCRs, in our study ranged between 17 and 100% for the respective sampling sites (**Table 3**). Ikeda et al. (2016), by comparison, showed a lower eDNA detection rate (range 12–50%) for their sampling sites in small headwater streams. This difference might be explained by the lower filter volume (0.25 L vs 2 L) used by Ikeda et al. (2016), the differences in extraction methods (DNeasy Blood & Tissue kit vs. DNeasy Power Water Kit) and the polymerase used (Taq Man Environmental Master Mix 2.0 vs. Platinum™ *Taq* DNA-Polymerase High Fidelity).

Spatio-Temporal Effects on eDNA Detectability

Our study is among the first to coherently assess eDNA detectability of crayfish using a spatial and temporal sampling design. Previous field studies on eDNA detection of crayfish have primarily focused on either determining presence/absence (Tréguier et al., 2014; Harper et al., 2018; Mauvisseau, 2019) or on correlations between abundance/biomass and eDNA concentrations (Dougherty et al., 2016; Larson et al., 2017; Rice et al., 2018).

Our results show successful eDNA detection of crayfish species up to 7 km downstream of the source population (**Figures 2**, **3**), whereby detection rate was independent of the PCR method. As expected, both detection rate and eDNA concentration were highest within or directly downstream of the source population and decreased with increasing in-stream distance. This represents a known effect, as eDNA in lotic waters is transported downstream through advection, until settlement and decay processes lead to a complete vanishing of eDNA from the water column (Sansom and Sassoubre, 2017). Over which distances detectable eDNA persists, is variable and depends on hydraulic properties of the water body (e.g., flow rate) as well as species-specific eDNA shedding rates (Nukazawa et al., 2018) and upstream population size (this study, Rice et al., 2018). Observed eDNA distances in previous studies vary therefore between less than 1 km in mesocosm and field experiments (Pilliod et al., 2014; Jane et al., 2015; Wilcox et al., 2016) to more than 100 km for a large river, where eDNA of a lake-dwelling fish species was detected (Pont et al., 2018). For freshwater crayfish, specific eDNA transport distance have not been assessed until now but are presumably more comparable with distances reported for other benthic invertebrates rather than fish and amphibians. Indeed, eDNA detection distances observed for *Unio tumidus*, a lake-dwelling benthic freshwater mussel, were with a maximum distance of 9 km relatively similar to detection distances in our study (Deiner and Altermatt, 2014). Generally, it seems that each species or taxon has its own spatial eDNA footprint, depending on individual eDNA shedding rates that are determined by the biology of the investigated species (Deiner and Altermatt, 2014; Wacker et al., 2019). Compared to fish and amphibians, freshwater crayfish are expected to have low eDNA shedding rates, because crayfish lack mucous producing structures present in fish and amphibians. Additionally, crayfish exhibit a hard exoskeleton that limits the release of extracellular eDNA into the water (Tréguier et al., 2014; Cai et al., 2017).

Detection of crayfish was successful year-round. However, LMM analysis indicated that there is a difference in eDNA detectability between winter and summer samples both with regard to detection rate and eDNA concentration (**Figures 2**, **3**). This result is in line with previous studies, showing that eDNA detectability can be affected by season, depending on the biology of the target species (*e.g.*, reproduction period, activity) (Buxton et al., 2018; Curtis et al., 2020). For crayfish, reduced detection rates in winter were an anticipated effect, since all European crayfish species show reduced activity and metabolism during winter (Bubb et al., 2002), which likely results in reduced eDNA shedding rates. Nonetheless, detection rate in winter never dropped below a threshold of 50% (range 50–100%), even at the most downstream sites, suggesting a reliable eDNA detection. Previous studies that analyzed winter samples for eDNA detection of crayfish showed contrasting results (Ikeda et al., 2016; Harper et al., 2018; Rusch et al., 2020). A Scottish study from Harper et al. (2018) completely failed to detect eDNA of *P. leniusculus* in three streams in winter. Another study from Japan was able to detect *Cambaroides japonicus* at one site with a low detection rate (12.5%), but failed at two other sites, where it was presumed to occur (Ikeda et al., 2016). A recent study from Rusch

et al. (2020) investigated two lakes and two streams in winter, three of them with syntopic occurrences of *F. limosus* and *P. virginalis* and one of them with an abundant *F. limosus* population. eDNA detection was successful in all habitats, where species presence was currently confirmed, but failed in one habitat, where historic presence was documented. Differences in detectability among studies might be in part related to varying filter volumes and/or differences in extraction methods. For example, Harper et al. (2018) used a filter volume of 15 ml combined with precipitation as extraction method, whereas Rusch et al. (2020) filtered a total volume of 10 L and used the CTAB method for extraction (Strand et al., 2019).

Finally, stream size also had a negative effect on eDNA detection rate (**Figure 2**). In addition, the lakes examined in this study also tended to show a lower detection rate with increasing water body size (*cf.* **Table 3**). This was an expected outcome as the eDNA signal is probably diluted in a larger volume of water. Rusch et al. (2020) made a similar observation in a large river in Hungary, where the detection of an abundant *F. limosus* population failed. In contrast to that, Pont et al. (2018) were able to successfully detect a fish species in a large river habitat. This difference is probably due to the fact that crayfish are benthic littoral species that usually only colonize the area near the shore due to the increased flow rate in the middle of streams (Bohl, 1999). Population size of crayfish is therefore not linearly related to water body size and the eDNA signal is presumably disproportionally more diluted in large water bodies. This is a marked difference to pelagic taxa, whose population size can be expected to be more closely related to water body size, suggesting similar eDNA concentrations across different sized habitats.

Relationship Between eDNA Concentration and Estimated Population Size

eDNA concentration correlated positively with estimated upstream population size (**Figure 4**). This result met our expectations as eDNA concentrations in lotic environments probably depend on both abundance and length of the populated stretch. According to Rice et al. (2018) it is very likely that the eDNA signal integrates with the populated stretch through downstream transport of water and accumulates with increasing stream distance. For that reason, it might be difficult to infer abundance from eDNA concentration in lotic environments, in particular when population extent is unknown and not accounted for. In line with this reasoning, Rice et al. (2018) found that the likelihood to detect *Faxonius eupunctus* in a large lotic system was independent from local crayfish abundance but increased with the upstream length of the populated stretch. In lentic situations, previous studies mostly reported a relationship between eDNA copy number and relative abundance of crayfish, estimated by trapping (Dougherty et al., 2016; Larson et al., 2017) or visual counts (Cai et al., 2017), although a recent study found no correlation between trapping data and eDNA concentration (Johnsen et al., 2020). In our study, manual capture was used as reference method, which is known to have a very high capture probability in streams of the study region (*see* Chucholl and Schrimpf, 2016), and, which is also known as the least biased conventional sampling method (Peay, 2004; Hilber et al., 2020). In general, rough quantification of crayfish abundance using eDNA copy numbers seems therefore possible (*cf.* Yates et al., 2019), provided that population extent and habitat type (lotic vs lentic) as well as sampling season are accounted for.

CONCLUSION

Our results highlight that eDNA is a suitable tool for year-round detection of native and invasive crayfish species in a wide variety of habitats. Opposed to most conventional methods, eDNA based monitoring allows detection of crayfish independent of their activity pattern, even in natural habitats with abundant or inaccessible shelters, where detection with conventional methods is difficult and labor intensive (Peay, 2004). Typical applications could include non-invasive monitoring of native populations, for instance following reintroductions or population bottlenecks, and control of functionality of invasive species barriers (Cowart et al., 2018). Moreover, eDNA detection allows to scan large sections of running waters with only few sampling sites, which can be used for the targeted search of as-yet unrecorded or newly emerging populations. This feature makes eDNA an excellent tool for initial large-scale surveys, whereby sampling effort should be higher in large water bodies and winter to maximise the detection rate and, thus, detection probability. Subsequent validation and fine-scale localization of populations should include conventional monitoring methods, though (*c.f.* Johnsen et al., 2020). Finally, eDNA might allow for a rough quantification of upstream population size. However, further research across different habitats and natural settings is needed to refine our ability to predict population size or abundance from eDNA surveys.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in the **Supplementary Material (Supplementary Tables S1-S3)**.

AUTHOR CONTRIBUTIONS

FC designed the study with input from LE and GS, conducted the data analysis, visualized analysed data and wrote the manuscript. FC and FF collected samples and carried out laboratory work. LE and GS supervised the study and contributed to the article.

FUNDING

This study was funded by the scholarship program of the German Federal Environmental Foundation (DBU) to FC. Lab work was enabled through funding by the Young Scholar Fund of the University of Konstanz to LE.

ACKNOWLEDGMENTS

We thank L. Gutbrod (University of Constance) for laboratory assistance and C. Chucholl (Fisheries Research Station BW) for crayfish distribution data, field work assistance and helpful comments on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.639380/full#supplementary-material.

REFERENCES

Agersnap, S., Larsen, W. B., Knudsen, S. W., Strand, D., Thomsen, P. F., Hesselsøe, M., et al. (2017). Monitoring of noble, signal and narrow-clawed crayfish using environmental DNA from freshwater samples. *PLoS One* 12, e0179261. doi:10.1371/journal.pone.0179261

Almeida, D., Ellis, A., England, J., and Copp, G. H. (2014). Time-series analysis of native and non-native crayfish dynamics in the Thames River Basin (south-eastern England): dynamics of native and non-native crayfish. *Aquat. Conserv. Mar. Freshw. Ecosyst.* 24, 192–202. doi:10.1002/aqc.2366

Atkinson, S., Carlsson, J. E. L., Ball, B., Kelly-Quinn, M., and Carlsson, J. (2019). Field application of an eDNA assay for the threatened white-clawed crayfish *Austropotamobius pallipes*. *Freshw. Sci.*, 35, 503–509. doi:10.1086/704712

Bohl, E. (1999). Motion of individual noble crayfish *Astacus astacus* in different biological situations: *in-situ* studies using radio telemetry. *Freshw. Crayfish* 12, 677–687.

Bubb, D. H., Lucas, M. C., and Thom, T. J. (2002). "Winter movements and activity of signal crayfish *Pacifastacus leniusculus* in an upland river, determined by radio telemetry," in *Aquatic telemetry*, Editors E. B. Thorstad, I. A. Fleming, and T. F. Næsje, (Dordrecht, Netherlands: Springer), 111–119. doi:10.1007/978-94-017-0771-8_13

Buxton, A. S., Groombridge, J. J., and Griffiths, R. A. (2018). Seasonal variation in environmental DNA detection in sediment and water samples. *PLoS One* 13, e0191737. doi:10.1371/journal.pone.0191737

Cai, W., Ma, Z., Yang, C., Wang, L., Wang, W., Zhao, G., et al. (2017). Using eDNA to detect the distribution and density of invasive crayfish in the Honghe-Hani rice terrace World Heritage site. *PLoS One* 12, e0177724. doi:10.1371/journal.pone.0177724

Chambers, J. M., and Hastie, T. J. (1992). *Statistical models in S*. Pacific Grove, CA: Wadsworth & Brooks. Cole advanced books & software.

Champlot, S., Berthelot, C., Pruvost, M., Bennett, E. A., Grange, T., and Geigl, E.-M. (2010). An efficient multistrategy DNA decontamination procedure of PCR reagents for hypersensitive PCR applications. *PLoS One* 5, e13042. doi:10.1371/journal.pone.0013042

Chucholl, C., and Dehus, P. (2011). Flusskrebse in Baden-Württemberg. Fischereiforschungsstelle Baden-Württemberg (LAZBW), Baden-Württemberg, Langenargen: Ministerium für Ländlichen Raum und Verbraucherschutz.

Chucholl, C., and Schrimpf, A. (2016). The decline of endangered stone crayfish (*Austropotamobius torrentium*) in southern Germany is related to the spread of invasive alien species and land-use change. *Aquat. Conserv. Mar. Freshw. Ecosyst.* 26, 44–56. doi:10.1002/aqc.2568

Cowart, D. A., Breedveld, K. G. H., Ellis, M. J., Hull, J. M., and Larson, E. R. (2018). Environmental DNA (eDNA) applications for the conservation of imperiled crayfish (Decapoda: Astacidea) through monitoring of invasive species barriers and relocated populations. *J. Crustac. Biol.* 38, 257–266. doi:10.1093/jcbiol/ruy007

Curtis, A. N., Tiemann, J. S., Douglass, S. A., Davis, M. A., and Larson, E. R. (2020). High stream flows dilute environmental DNA (eDNA) concentrations and reduce detectability. *Divers. Distrib.* 00, 1–14. doi:10.1111/ddi.13196

Deiner, K., and Altermatt, F. (2014). Transport distance of invertebrate environmental DNA in a natural river. *PLoS One* 9, e88786. doi:10.1371/journal.pone.0088786

Dougherty, M. M., Larson, E. R., Renshaw, M. A., Gantz, C. A., Egan, S. P., Erickson, D. M., et al. (2016). Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes rusticus* at low abundances. *J. Appl. Ecol.*, 53, 722–732. doi:10.1111/1365-2664.12621

Dunn, N., Priestley, V., Herraiz, A., Arnold, R., and Savolainen, V. (2017). Behavior and season affect crayfish detection and density inference using environmental DNA. *Ecol. Evol.* 7, 7777–7785. doi:10.1002/ece3.3316

Ficetola, G., Coissac, E., Zundel, S., Riaz, T., Shehzad, W., Bessière, J., et al. (2010). An in silico approach for the evaluation of DNA barcodes. *BMC Genomics* 11, 434. doi:10.1186/1471-2164-11-434

Ficetola, G. F., Miaud, C., Pompanon, F., and Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biol. Lett.* 4, 423–425. doi:10.1098/rsbl.2008.0118

Geerts, A. N., Boets, P., Van den Heede, S., Goethals, P., and Van der heyden, C. (2018). A search for standardized protocols to detect alien invasive crayfish based on environmental DNA (eDNA): a lab and field evaluation. *Ecol. Indic.* 84, 564–572. doi:10.1016/j.ecolind.2017.08.068

Goldberg, C. S., Strickler, K. M., and Pilliod, D. S. (2015). Moving environmental DNA methods from concept to practice for monitoring aquatic macroorganisms. *Biol. Conserv.* 183, 1–3. doi:10.1016/j.biocon.2014.11.040

Gouin, N., Grandjean, F., and Souty-Grosset, C. (2006). Population genetic structure of the endangered crayfish *Austropotamobius pallipes* in France based on microsatellite variation: biogeographical inferences and conservation implications. *Freshw. Biol.* 51, 1369–1387. doi:10.1111/j.1365-2427.2006.01570.x

Gouin, N., Souty-Grosset, C., Bórquez, J., Bertin, A., and Grandjean, F. (2011). Disentangling the impact of demographic factors on population differentiation of an endangered freshwater crayfish (*Austropotamobius pallipes*) using population density and microsatellite data: genetic drift in *A. pallipes* populations. *Freshw. Biol.* 56, 2105–2118. doi:10.1111/j.1365-2427.2011.02629.x

Grandjean, F., Harris, D. J., Souty-Grosset, C., and Crandall, K. A. (2000). Systematics of the european endangered crayfish species *Austropotamobius pallipes* (Decapoda: Astacidae). *J. Crustac. Biol.* 20, 522–529. doi:10.1651/0278-0372(2000)020[0522:SOTEEC]2.0.CO;2

Harper, K., Anucha, P., Turnbull, J., Bean, C., and Leaver, M. (2018). Searching for a signal: environmental DNA (eDNA) for the detection of invasive signal crayfish, *Pacifastacus leniusculus* (Dana, 1852). *Manag. Biol. Invasions* 9, 137–148. doi:10.3391/mbi.2018.9.2.07

Hilber, T., Oehm, J., Effenberger, M., and Maier, G. (2020). Evaluating the efficiency of three methods for monitoring of native crayfish in Germany. *Limno.* 85, 125821. doi:10.1016/j.limno.2020.125821

Holdich, D. M., Reynolds, J. D., Souty-Grosset, C., and Sibley, P. J. (2009). A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. *Knowl. Manag. Aquat. Ecosyst.* 11, 394–395. doi:10.1051/kmae/2009025

Ikeda, K., Doi, H., Tanaka, K., Kawai, T., and Negishi, J. N. (2016). Using environmental DNA to detect an endangered crayfish *Cambaroides japonicus* in streams. *Conserv. Genet. Resour.* 8, 231–234. doi:10.1007/s12686-016-0541-z

Ikeda, K., Doi, H., Terui, S., Kato, A., Mitsuzuka, T., Kawai, T., et al. (2019). Estimating native and invasive crayfish distributions in relation to culvert barriers with environmental DNA. *Freshw. Sci.*, doi:10.1086/704998

Jane, S. F., Wilcox, T. M., McKelvey, K. S., Young, M. K., Schwartz, M. K., Lowe, W. H., et al. (2015). Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Mol. Ecol. Resour.* 15, 216–227. doi:10.1111/1755-0998.12285

Jerde, C. L., Mahon, A. R., Chadderton, W. L., and Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA: eDNA surveillance of rare aquatic species. *Conserv. Lett.* 4, 150–157. doi:10.1111/j.1755-263X.2010.00158.x

Johnsen, S. I., Strand, D. A., Rusch, J. C., and Vrålstad, T. (2020). Environmental DNA (eDNA) monitoring of noble crayfish *Astacus astacus* in lentic environments offers reliable presence-absence surveillance - but fails to predict population density. *Front. Environ. Sci.* 8, 612253. doi:10.3389/fenvs.2020.612253

Kouba, A., Petrusek, A., and Kozák, P. (2014). Continental-wide distribution of crayfish species in Europe: update and maps. *Knowl. Manag. Aquat. Ecosyst.* 413, 05. doi:10.1051/kmae/2014007

Larson, E. R., Abbott, C. L., Usio, N., Azuma, N., Wood, K. A., Herborg, L.-M., et al. (2012). The signal crayfish is not a single species: cryptic diversity and invasions in the Pacific Northwest range of *Pacifastacus leniusculus*: the signal crayfish is not a single species. *Freshw. Biol.* 57, 1823–1838. doi:10.1111/j.1365-2427.2012.02841.x

Larson, E. R., Renshaw, M. A., Gantz, C. A., Umek, J., Chandra, S., Lodge, D. M., et al. (2017). Environmental DNA (eDNA) detects the invasive crayfishes *Orconectes rusticus* and *Pacifastacus leniusculus* in large lakes of North America. *Hydrobiologia* 800, 173–185. doi:10.1007/s10750-017-3210-7

Mauvisseau, Q., Coignet, A., Delaunay, C., Pinet, F., Bouchon, D., and Souty-Grosset, C. (2017). Environmental DNA as an efficient tool for detecting invasive crayfishes in freshwater ponds. *Hydrobiologia*. 805, 163–175. doi:10.1007/s10750-017-3288-y

Mauvisseau, Q. (2019). Early detection of an emerging invasive species: eDNA monitoring of a parthenogenetic crayfish in freshwater systems. *Manag. Biol. Invasions* 10, 461–472. doi:10.3391/mbi.2019.10.3.04

Nakagawa, S., Johnson, P. C., and Schielzeth, H. (2017). The coefficient of determination R2 and intra-class correlation coefficient from generalized linear mixed-effects models revisited and expanded. *J. R. Soc. Interf.* 14, 20170213. doi:10.1098/rsif.2017.0213

Nukazawa, K., Hamasuna, Y., and Suzuki, Y. (2018). Simulating the advection and degradation of the environmental DNA of common carp along a river. *Environ. Sci. Technol.* 52, 10562–10570. doi:10.1021/acs.est.8b02293

Nunes, M. S. With contributions from T., Heuer, C., Marshall, J., Sanchez, J., Thornton, R., Reiczigel, J., et al. (2020). epiR: tools for the analysis of epidemiological data. Available at: https://CRAN.R-project.org/package=epiR.

Ogle, D. H., Wheeler, P., and Dinno, A. (2020). FSA: fisheries stock analysis. Available at: https://github.com/droglenc/FSA.

OIE (2019). Infection with Aphanomyces astaci (crayfish plague): manual of diagnostic tests for aquatic animals. Available at: https://www.oie.int/index.php?id=2439&L=0&htmfile=chapitre_aphanomyces_astaci.htm. (Accessed December 31, 2020).

Olarte, N., García-Arberas, L., and Antón, A. (2019). Evaluation of different kinds of disturbance on the efficiency of sampling methods employed for an endangered freshwater crayfish, *Austropotamobius pallipes* Lereboullet, 1858 (Decapoda: Astacidea: Astacidae), in the northern Iberian Peninsula. *J. Crustac. Biol.* 39, 342–348. doi:10.1093/jcbiol/ruz037

Peay, S. (2004). A cost-led evaluation of survey methods and monitoring for white-clawed crayfish- lessons from the UK. *Bull. Fr. Pêche Piscic.* 335–352. doi:10.1051/kmae:2004008

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., and Waits, L. P. (2014). Factors influencing detection of eDNA from a stream-dwelling amphibian. *Mol. Ecol. Resour.* 14, 109–116. doi:10.1111/1755-0998.12159

Pont, D., Rocle, M., Valentini, A., Civade, R., Jean, P., Maire, A., et al. (2018). Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. *Sci. Rep.* 8, 10361. doi:10.1038/s41598-018-28424-8

R Core Team (2019). *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. Available at: https://www.R-project.org/.

Rice, C. J., Larson, E. R., and Taylor, C. A. (2018). Environmental DNA detects a rare large river crayfish but with little relation to local abundance. *Freshw. Biol.* 63, 443–455. doi:10.1111/fwb.13081

Robinson, C. V., Uren Webster, T. M., Cable, J., James, J., and Consuegra, S. (2018). Simultaneous detection of invasive signal crayfish, endangered white-clawed crayfish and the crayfish plague pathogen using environmental DNA. *Biol. Conservation* 222, 241–252. doi:10.1016/j.biocon.2018.04.009

Rusch, J. C., Mojžišová, M., Strand, D. A., Svobodová, J., Vrålstad, T., and Petrusek, A. (2020). Simultaneous detection of native and invasive crayfish and *Aphanomyces astaci* from environmental DNA samples in a wide range of habitats in Central Europe. *NeoBiota* 58, 1–32. doi:10.3897/neobiota.58.49358

Sansom, B. J., and Sassoubre, L. M. (2017). Environmental DNA (eDNA) shedding and decay rates to model freshwater mussel eDNA transport in a river. *Environ. Sci. Technol.* 51, 14244–14253. doi:10.1021/acs.est.7b05199

Souty-Grosset, C., Holdich, D. M., and Noël, P. Y. (2006). *Atlas of crayfish in Europe*. Paris, France: Publ. Scientifiques du Muséum National d'Histoire Naturelle

Strand, D. A., Johnsen, S. I., Rusch, J. C., Agersnap, S., Larsen, W. B., Knudsen, S. W., et al. (2019). Monitoring a Norwegian freshwater crayfish tragedy: eDNA snapshots of invasion, infection and extinction. *J. Appl. Ecol.* 56, 1661–1673. doi:10.1111/1365-2664.13404

Thalinger, B., Deiner, K., Harper, L. R., Rees, H. C., Blackman, R. C., Sint, D., et al. (2020). A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. bioRxiv preprint. doi:10.1101/2020.04.27.063990 (Accessed November 27, 2020)

Tréguier, A., Paillisson, J.-M., Dejean, T., Valentini, A., Schlaepfer, M. A., and Roussel, J.-M. (2014). Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. *J. Appl. Ecol.* 51, 871–879. doi:10.1111/1365-2664.12262

Trontelj, P., Machino, Y., and Sket, B. (2005). Phylogenetic and phylogeographic relationships in the crayfish genus Austropotamobius inferred from mitochondrial COI gene sequences. *Mol. Phylogenet. Evol.* 34, 212–226. doi:10.1016/j.ympev.2004.09.010

Troth, C. R., Burian, A., Mauvisseau, Q., Bulling, M., Nightingale, J., Mauvisseau, C., et al. (2019). Development and application of eDNA-based tools for the conservation of white-clawed crayfish. *Ecology* doi:10.1101/732941

Wacker, S., Fossøy, F., Larsen, B. M., Brandsegg, H., Sivertsgård, R., and Karlsson, S. (2019). Downstream transport and seasonal variation in freshwater pearl mussel (*Margaritifera margaritifera*) eDNA concentration. *Environ. DNA* 1, 64–73. doi:10.1002/edn3.10

Wilcox, T. M., McKelvey, K. S., Young, M. K., Sepulveda, A. J., Shepard, B. B., Jane, S. F., et al. (2016). Understanding environmental DNA detection probabilities: a case study using a stream-dwelling char *Salvelinus fontinalis*. *Biol. Conserv.* 194, 209–216. doi:10.1016/j.biocon.2015.12.023

Yates, M. C., Fraser, D. J., and Derry, A. M. (2019). Meta-analysis supports further refinement of eDNA for monitoring aquatic species-specific abundance in nature. *Environ. DNA* 1, 5–13. doi:10.1002/edn3.7

Zuur, A., Ieno, E. N., Walker, N., Saveliev, A. A., and Smith, G. M. (2009). *Mixed effects models and extensions in ecology with R*. Berlin, Germany: Springer Science & Business Media.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 17 June 2021
doi: 10.3389/fevo.2021.623814

First Detection of the Crayfish Plague Pathogen *Aphanomyces astaci* in Costa Rica: European Mistakes Should Not Be Repeated

*Laura Martín-Torrijos[1], Angel Jose Correa-Villalona[1], Juan Carlos Azofeifa-Solano[2], Fresia Villalobos-Rojas[2], Ingo S. Wehrtmann[2] and Javier Diéguez-Uribeondo[1]**

[1] Department of Mycology, Real Jardín Botánico-CSIC, Madrid, Spain, [2] Unidad de Investigación Pesquera y Acuicultura (UNIP), Centro de Investigación en Ciencias del Mar y Limnología (CIMAR), Universidad de Costa Rica, San José, Costa Rica

The crayfish plague pathogen *Aphanomyces astaci* is one of the main factors responsible for the decline in European and Asian native crayfish species. This pathogen was transported to these regions through its natural carriers, North American crayfish species, which were introduced during the last century. Since then, the carrier species and the pathogen have spread worldwide due to globalization and the highly invasive nature of these species. In Europe, five carrier species have been categorized as high-risk as they are responsible for the loss of provisioning services, which endangers freshwater ecosystems. The red swamp crayfish *Procambarus clarkii*, in particular, is currently one of the most concerning species as its spread threatens crayfish biodiversity and freshwater ecosystems worldwide. In this study, we describe the first detection of *A. astaci* in an introduced population of *P. clarkii* in Central America, specifically in Costa Rica. Using molecular approaches, we analyzed 48 crayfish samples collected from Reservoir Cachí and detected the presence of *A. astaci* in four of these samples. The introduction of *P. clarkii* and the incorrect management of the species (related to its fishery and the commercialization of live specimens) over the past decades in Europe are mistakes that should not be repeated elsewhere. The detection of the pathogen is a warning sign about the dangerous impact that the introduction of this invasive crayfish may have, not only as a carrier of an emerging disease but also as a direct risk to the invaded ecosystems. Our results may serve to (1) assess current and future consequences, and (2) direct future research activities, such as determining the potential impacts of *A. astaci* on native decapod species, or on other introduced crayfish species that are used for aquaculture purposes, such as *Cherax quadricarinatus*.

Keywords: biodiversity, invasive alien species, crayfish plague, *Procambarus clarkii*, *Cherax quadricarinatus*, mtDNA, conservation

OPEN ACCESS

Edited by:
Leopold Füreder, University of Innsbruck, Austria

Reviewed by:
Elena Tricarico, University of Florence, Italy
Adam Petrusek, Charles University, Czechia

****Correspondence:***
Javier Diéguez-Uribeondo
dieguez@rjb.csic.es

Specialty section:
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *30 October 2020*
Accepted: *17 May 2021*
Published: *17 June 2021*

Citation:
Martín-Torrijos L, Correa-Villalona AJ, Azofeifa-Solano JC, Villalobos-Rojas F, Wehrtmann IS and Diéguez-Uribeondo J (2021) First Detection of the Crayfish Plague Pathogen Aphanomyces astaci in Costa Rica: European Mistakes Should Not Be Repeated. Front. Ecol. Evol. 9:623814. doi: 10.3389/fevo.2021.623814

INTRODUCTION

Freshwater crayfishes are a highly diverse group of aquatic organisms that comprises more than 650 described species (Crandall and Buhay, 2008; Crandall and De Grave, 2017). These crustacean decapods are threatened by habitat destruction, water diversion, pollution, and invasive alien species, particularly other crayfish species (Kawai and Crandall, 2016). For example, native Eurasian

crayfishes (i.e., species of the genera *Astacus, Austropotambius, Pontastacus,* and *Cambaroides*) are keystone species that have alarmingly declined in the last few decades (Kouba et al., 2014; Martín-Torrijos et al., 2018). The introduction and spread of North American crayfish species is one of the main factors responsible for this decline (reviewed in Rezinciuc et al., 2015). The rapid life cycle, dispersal capacities, burrowing activities, high population densities, and aggressive behavior of crayfishes destabilize trophic chains (Souty-Grosset et al., 2016). In addition, North American crayfish species act as vectors for the crayfish plague agent *Aphanomyces astaci* (see Huang et al., 1994). This pathogen, one of the 100 world's worst invasive alien species (Lowe et al., 2000), is responsible for the European crayfish decline during the past century (reviewed in Holdich et al., 2009; Rezinciuc et al., 2015).

Aphanomyces astaci belongs to the class Oomycetes, which includes important pathogenic species of both plants and animals (Beakes et al., 2012). This pathogen chronically infects its natural hosts, North American crayfish species, by establishing a balanced host-pathogen interaction (Unestam, 1969, 1972; Cerenius et al., 2003). The first documented introductions of North American crayfish species to Europe, and subsequent crayfish plague outbreaks, occurred during the 19th century (Cornalia, 1860; Bott, 1950). The ensuing large-scale and worldwide importation of these species, and their spread by illegal translocations, has caused outbreaks throughout Europe (Taugbøl et al., 1993; Huang et al., 1994; Diéguez-Uribeondo et al., 1997; Lilley et al., 1997; Machino and Diéguez-Uribeondo, 1998; Vennerström et al., 1998; Diéguez-Uribeondo and Söderhäll, 1999; Oidtmann et al., 1999; Diéguez-Uribeondo, 2006; Kozubíková et al., 2009; Rezinciuc et al., 2014; Jussila et al., 2015; Martín-Torrijos et al., 2019) and other biogeographical regions of the world (Kawai and Crandall, 2016; Martín-Torrijos et al., 2018). The introduced North American crayfish species pose a real threat to European crayfish biodiversity, public health, and the economy. Indeed, five introduced crayfish species (*Faxonius limosus, Faxonius virilis, Pacifastacus leniusculus, Procambarus clarkii,* and *Procambarus virginalis*) have already been included in the "List of Invasive Alien Species of Union concern" (the Union list). This legislation, which aims to prevent, control and/or eradicate invasive alien species, has been adopted by all member states (EUR-lex, 2014, 2016; Kopf et al., 2017).

Over the past few decades, several studies have assessed the devastation caused by introduced North American crayfishes on native European crayfish populations (Huang et al., 1994; Diéguez-Uribeondo et al., 2006; Kouba et al., 2014; Martín-Torrijos et al., 2019). In particular, the red swamp crayfish *P. clarkii* is a well-known threat, not only as a vector of *A. astaci* but also for its countless impact on freshwater ecosystems (Gherardi et al., 2011; Arce and Dieguez-Uribeondo, 2015; Souty-Grosset et al., 2016). The natural distribution of *P. clarkii* includes northern Mexico, and the southern and southeastern United States (Nagy et al., 2020). This species, however, has been introduced to all continents, except Australia and Antarctica (Kawai and Crandall, 2016); furthermore, it has also been seized from Australian pet trade (Queensland Government, 2019). To date, all *A. astaci* haplotypes analyzed from *P. clarkii* belong to the same lineage, also named D-haplogroup (Makkonen et al., 2018; Martín-Torrijos et al., 2018, 2019, 2021a,b). Some strains of the D-haplogroup can grow, sporulate, and disperse their zoospores at higher temperatures (by more than 5°C) than those belonging to other genetic groups, suggesting that they are better adapted to warmer freshwater environments compared with other strains (Diéguez-Uribeondo et al., 1995). The presence of both *P. clarkii* and *A. astaci* has been confirmed in several tropical regions, including Brazil (Peiró et al., 2016) and Indonesia (Putra et al., 2018). Although several Central American countries, including Costa Rica, Nicaragua, Guatemala, and Belize, have reported the presence of the invasive species *P. clarkii* (Wehrtmann et al., 2016), the presence of *A. astaci* in its Central American populations has not yet been tested.

Several cases of the introduction of alien animal species have been reported for Costa Rica (e.g., Barrientos-Llosa and Monge-Nájera, 2010; Barquero and Araya, 2016), a known biodiversity hotspot (Myers et al., 2000). However, to date, only two invasive crayfish species, *P. clarkii* and *Cherax quadricarinatus,* have been reported for the country (**Figure 1**; Torres and Álvarez, 2012; Wehrtmann et al., 2016; Azofeifa-Solano et al., 2017). *Procambarus clarkii* was introduced to Costa Rica from Louisiana in 1966 (Huner, 1977). The population present in the Reservoir Cachí, Province of Cartago, likely represents the oldest and largest population in the country (**Figure 1**). The crayfish resource in this reservoir is harvested and commercialized by locals for human consumption; however, it is suspected that individuals from this population are also captured for the pet trade or for their translocation to other water bodies (FV-R, CIMAR, *personal communication*).

The high ecological plasticity of both *P. clarkii* and *A. astaci* in tropical environments makes the presence of *P. clarkii* in Costa Rica a matter of great concern (Villalobos-Rojas, 2019), as does their continued introduction to other parts of the world, despite the well-documented negative impacts that both species have had, and continue to have, on European biodiversity. In this study, we aimed to determine whether the crayfish plague pathogen *A. astaci* is already present in the *P. clarkii* population at Reservoir Cachí. By using a specific mitochondrial marker [the ribosomal small subunit (rnnS) (Makkonen et al., 2018)], we confirm the presence of the pathogen *A. astaci* in Costa Rica, and provide information about its genetic diversity. Finally, we discuss our results in light of the European experience as a lesson learned to emphasize the potential danger of the introduction of both invasive alien species (*P. clarkii* and *A. astaci*) to tropical freshwater ecosystems.

MATERIALS AND METHODS

Crayfish Sampling

In May 2019, we collected a total of 48 live crayfishes from a population of *P. clarkii* introduced into Reservoir Cachí (Province of Cartago, Costa Rica) (**Table 1**, **Figure 1**, and **Supplementary Table 1**). The specimens were transferred into aquaria at the Centro de Investigación en Ciencias del Mar y Limnología (CIMAR) of the Universidad de Costa Rica, located

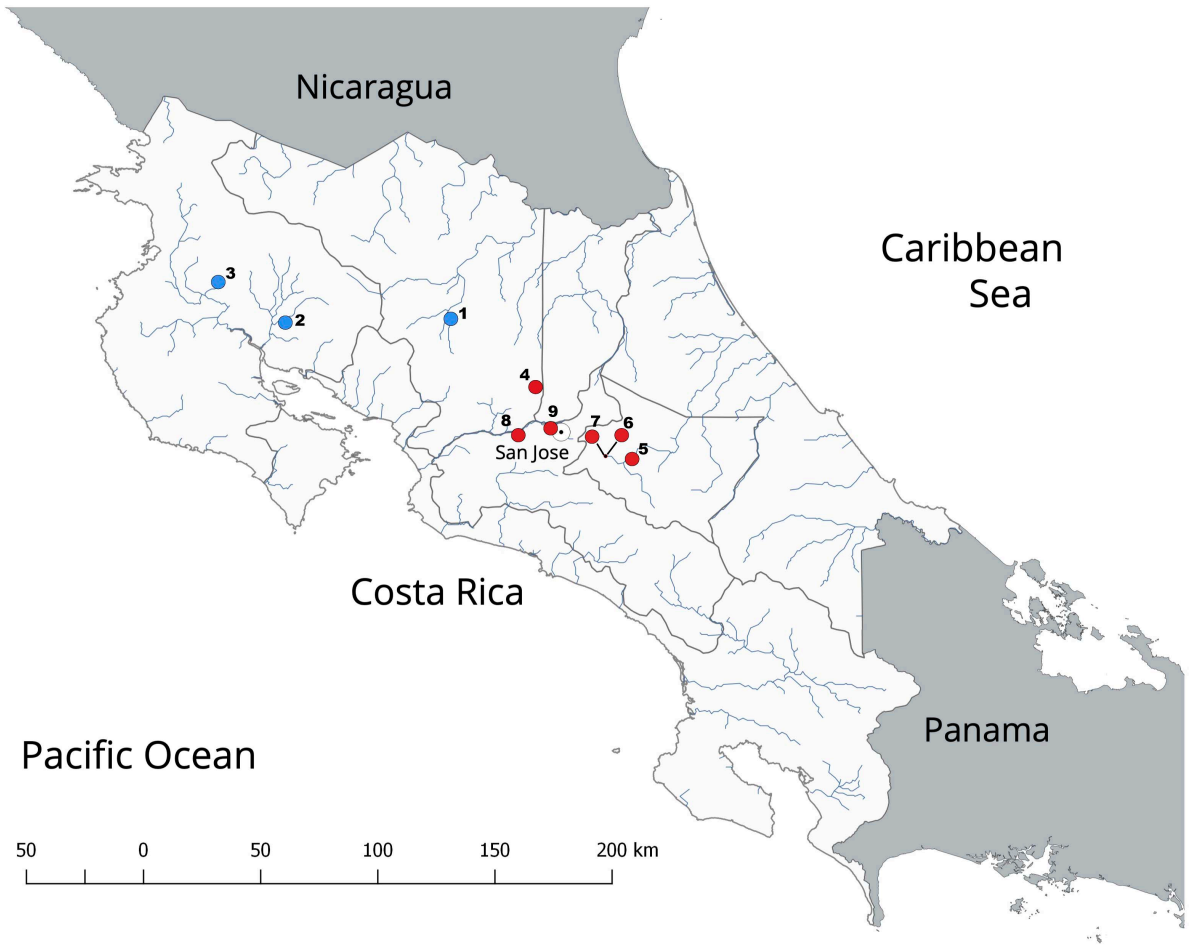

FIGURE 1 | Map showing the locations of the reported invasive alien crayfishes in Costa Rica. Red circles indicate *Procambarus clarkii* populations; blue circles indicate *Cherax quadricarinatus* populations. Numbers (1–9) indicate the following locations: (1) Río Balsa, Ciudad Quesada, Alajuela Province; (2) Universidad Técnica Nacional, Bagaces, Guanacaste Province; (3) Irrigation Channels in Bagatzi, Guanacaste Province; (4) Fraijanes Lagoon, Alajuela Province; (5) Hydroelectric Dam Cachí, Cartago Province; (6) Jardín Botánico Lankester Lagoons, Cartago Province; (7) Instituto Tecnológico Costarricense Lagoons, Cartago Province; (8) Peace Park, El Rodeo, San José Province; (9) La Sabana Lake, San José Province.

in San José (Costa Rica). The aquaria were maintained at 25°C, and crayfishes were checked daily for the presence of molts. Molts were maintained in sterile distilled water for 3 days prior to their examination under a microscope to detect for the presence of *A. astaci*, as described in Martín-Torrijos et al. (2021b). Finally, molts were individually preserved in 96% ethanol for further examination and the molecular analyses.

Macroscopic and Microscopic Examination

Each collected individual was examined macroscopically for the presence of melanized areas or spots in the soft cuticle and appendages (common indications of potential *A. astaci* infection). Because crayfish can regenerate pereiopods (Shull, 1909), we collected one pereiopod per individual (suspected to be infected) and individually preserved them in 96% ethanol for the molecular analyses. Prior to these analyses, each pereiopod was examined microscopically for the presence of melanized hyphae using an Olympus CKX41SF inverted microscope (Olympus Optical, Tokyo, Japan). Light micrographs of colonizing hyphae were captured using a QImaging Micropublisher 5.0 digital camera (QImaging, Burnaby, BC, Canada).

Molecular Analyses and Phylogenetic Approximations

All samples were washed with TE buffer (TRIS 10 mM/EDTA 1 mM, pH 8) prior to DNA extraction. Samples were transferred into individual 2-ml Eppendorf tubes, frozen at −80°C and then lyophilized in a VirTis BenchTop K freeze dryer for 24 h (≤−50°C; ≤20 mTorr). Subsequently, samples were mechanical ruptured using a TissueLyser (QIAGEN, Venlo, Netherlands). Genomic DNA was isolated using the E.Z.N.A.® Insect DNA Kit (Omega Bio-Tek, Norcross, GA, United States).

In order to detect the presence and diversity of *A. astaci*, we attempted to amplify fragments of the mitochondrial ribosomal small (rnnS) and large (rnnL) subunits using two primer pairs

TABLE 1 | Locations of populations of invasive alien crayfish species in Costa Rica.

Location	Latitude	Longitude	Crayfish species
1 Río Balsa, Ciudad Quesada, Alajuela Province	10.3625	−84.51418	*Cherax quadricarinatus*
2 Universidad Técnica Nacional, Bagaces, Guanacaste Province	10.33497	−85.13975	*Cherax quadricarinatus*
3 Irrigation Channels in Bagatzi, Guanacaste Province	10.37946	−85.27321	*Cherax quadricarinatus*
4 Fraijanes Lagoon, Alajuela Province	10.12471	−84.19105	*Procambarus clarkii*
5 Hydroelectric Dam Cachí, Cartago Province	9.8316	−83.80968	*Procambarus clarkii*
6 Jardín Botánico Lankester Lagoons, Cartago Province	9.839553	−83.888691	*Procambarus clarkii*
7 Instituto Tecnológico Costarricense Lagoons, Cartago Province	9.854629	−83.91052	*Procambarus clarkii*
8 Peace Park, El Rodeo, San José Province	9.91911	−84.2768	*Procambarus clarkii*
9 La Sabana Lake, San José Province	9.934766	−84.10359	*Procambarus clarkii*

described in Makkonen et al. (2018). The positive control for the rnnS and rnnL primers was the *A. astaci* strain SAP-Malaga 5 (which originated from the Iberian Peninsula) (Martín-Torrijos et al., 2019); milliQ water was used as the negative control. All PCR products were checked on 1% agarose gels containing 0.5 μM of SYBR Safe. Both strands were sequenced using an automated sequencer (Applied Biosystems 3730xl DNA) by Macrogen (Netherlands).

Sequences were assembled and edited using the program Geneious® 10.2.3 (Kearse et al., 2012). Two phylogenetic approximations, Bayesian Inference (BI) and Maximum Likelihood (ML), were used to reconstruct phylogenetic relationships, following Makkonen et al. (2018). Reference sequences were obtained from Makkonen et al. (2018) and Martín-Torrijos et al. (2019). *Aphanomyces frigidophilus* was used as the outgroup.

RESULTS

Macroscopic and Microscopic Examination

We obtained a total of two molts (1CR and 23CR) from the collected *P. clarkii* specimens (**Supplementary Table 1**). Macroscopic observations of these molts revealed that both exhibited melanized areas in the soft abdominal cuticle and pereiopods characteristic of infection by *A. astaci*. Moreover, under microscopic observation, we found melanized hyphae growing within the pereiopods of specimen 1CR (**Figure 2**).

Molecular Analyses and Phylogenetic Approximations

The rnnS subunit was amplified from four of the 48 crayfish specimens (3CR, 5CR, 8CR, and 10CR; **Supplementary Table 1**). The obtained sequences showed 100% identity to the d1/d2-haplotypes of the D-haplogroup (GenBank accession numbers MW181669–MW181672) (**Supplementary Figure 1** and **Supplementary Table 1**). However, we could not amplify the rnnL subunit from these specimens. Also, neither of the subunits could be amplified from either of the two collected molts.

The two phylogenetic approximations (BI and ML) based on the rnnS data (**Supplementary Figure 1**) recovered congruent topologies, and showed the correspondence of the analyzed *A. astaci* sequences from *P. clarkii* in Costa Rica with the D-haplogroup.

DISCUSSION

This is the first reported case of the crayfish plague pathogen *A. astaci* in Central America. The presence of this pathogen was found in Reservoir Cachí in Costa Rica as a consequence of translocations of the invasive alien crayfish species *P. clarkii*. We also determined that the *A. astaci* found in this *P. clarkii* population belongs to the D-haplogroup. Four haplotypes have been identified for *A. astaci* in *P. clarkii*: d1, d2, d3, and usa6 (Makkonen et al., 2018; Martín-Torrijos et al., 2018, 2021b). The four mitochondrial rnnS sequences obtained in this study are all identical to those corresponding to either the d1 or d2 rnnS haplotypes reported by Makkonen et al. (2018). However, to identify a specific *A. astaci* haplotype with confidence, both the rnnS and rnnL regions must be analyzed (Casabella-Herrero et al., 2021). Therefore, the lack of rnnL sequences for these samples makes it impossible to confirm their specific haplotype. Nevertheless, rnnS data are reliable enough to identify both the pathogen and its genetic group (rnnS is a new reliable marker, and can be used to identify genetic variability in order to differentiate *A. astaci* from close-related taxa) (Casabella-Herrero et al., 2021). The fact that the analyzed Costa Rican sequences belong to the D-haplogroup and that some strains of this genetic group appear to be better adapted to warm environments (Diéguez-Uribeondo et al., 1995) suggest an increased likelihood of *A. astaci* transmission to other suitable hosts (freshwater decapods) in this tropical environment.

In Costa Rica, although there are no native crayfish species, there are approximately 26 native species of freshwater decapods (21 shrimp species belonging to the families Atyidae and Palaemonidae, and 15 crab species belonging to Pseudothelphusidae) (Lara and Wehrtmann, 2011; Lara et al., 2013; Magalhães et al., 2015). Despite the seemingly narrow host range of *A. astaci* (Unestam, 1972; Diéguez-Uribeondo et al., 2009), several studies have shown transmission of the pathogen to other freshwater decapods including *Atya gabonensis*, *Atyopsis moluccensis*, *Eriocheir sinensis*, *Macrobrachium dayanum*, *Macrobrachium lanchesteri*, *Neocaridina davidi*, *Palaemon*

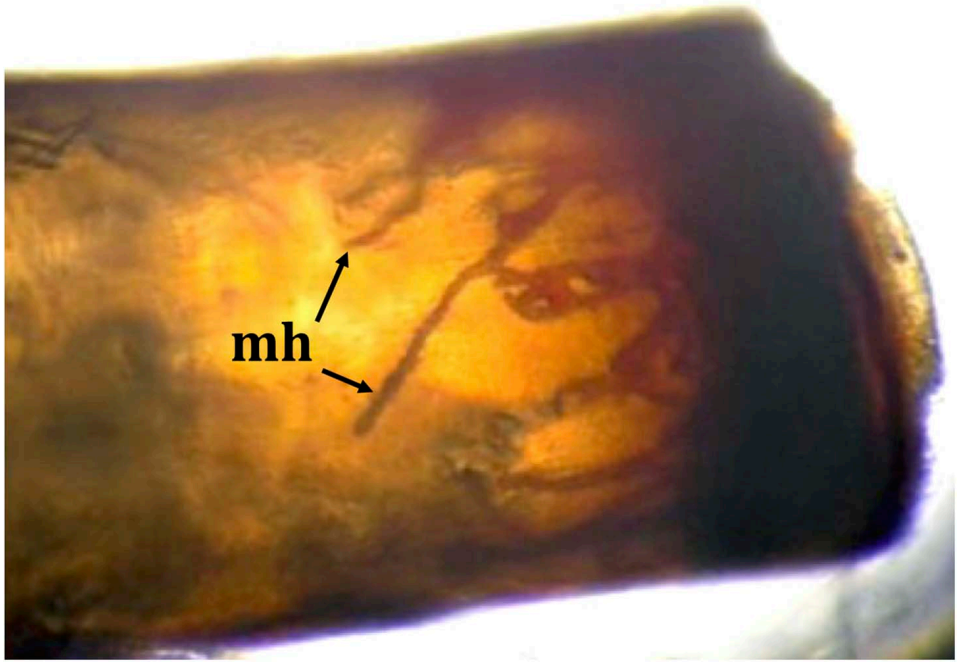

FIGURE 2 | A pereiopod of a *Procambarus clarkii* specimen from Reservoir Cachí, Costa Rica, showing the presence of melanized hyphae (mh), signs of a strong immune reaction against the crayfish plague pathogen *Aphanomyces astaci.*

kadiakensis, and *Potamon potamios* (Schrimpf et al., 2014; Svoboda et al., 2014; Putra et al., 2018; Mrugała et al., 2019; Martín-Torrijos et al., 2021b). Although the coexistence of native freshwater decapods with *P. clarkii* has not been reported yet in Costa Rica, the potential impact of the invasive species presence on freshwater fauna in the country should be evaluated. Also, the occurrence of *P. clarkii* in other Central American countries (Wehrtmann et al., 2016) indicates a need for genetic studies of these other populations to determine the extent of *A. astaci* distribution in the region and assess any possible threats to the local native fauna. Moreover, the accessibility to live specimens by people makes the translocation and further spread of *P. clarkii* and the pathogen highly probable. It is already clear that once North American crayfish species have been introduced, their expansion becomes difficult to control. The main mechanisms of spread of this invasive crayfish and the disease in Europe have been identified as fishery-related activities, fishing as a means to control increasing populations, and the commercialization of live crayfishes (Alonso et al., 2000; Diéguez-Uribeondo, 2006). Therefore, control and biosecurity measures aimed at these particular activities, among others, should be implemented in other countries or regions to prevent the dispersion of the crayfish plague pathogen (e.g., prevent the movement of potentially infected live or dead crayfish and potentially contaminated water and equipment from sites with a potential or known presence of the pathogen to uninfected sites) [OIE (World Organization for Animal Health), 2019].

Furthermore, the effects of *P. clarkii* as an invasive species has been extensively studied in Europe, where the species has been recorded in at least 16 territories (Souty-Grosset et al., 2016). In these areas, freshwater biodiversity has been severely affected due to constant predation of the invasive species on fishes (Ilhéu et al., 2007; Reynolds, 2011), shrimps (Banha and Anastácio, 2011), amphibians (Ficetola et al., 2012; Nunes et al., 2014), mollusks (Correia et al., 2005; Chucholl, 2013), and other macroinvertebrates (Correia et al., 2005; Correia and Anastácio, 2008). Therefore, at management level, *P. clarkii* is considered an agricultural pest, and a threat to water drainage systems and the restoration of several European water bodies (Souty-Grosset et al., 2016). Thus, *P. clarkii* is considered a high-risk species, responsible for the loss of provisioning services (associated with wide changes in ecological communities and increased costs to agriculture and water management) (Souty-Grosset et al., 2016).

Additionally, in 1985, another invasive crayfish, the Australian species *C. quadricarinatus*, was introduced to Costa Rica for aquaculture purposes (Luis Rolier Lara *personal communication* in Wehrtmann et al., 2016). Since its introduction, accidental releases have occurred (INCOPESCA, *personal communication*), and in 2017, the presence of this species in both Pacific and Caribbean freshwater drainages was reported (Azofeifa-Solano et al., 2017) (see **Figure 1**), making the situation in Costa Rica even more complex. These unintentional releases from holding facilities (Azofeifa-Solano et al., 2017) highlight the problematic use of invasive alien species for aquaculture purposes. *Cherax quadricarinatus,* as with other Australian crayfish species, has

been reported as highly susceptible to the crayfish plague pathogen (Unestam, 1975; Marino et al., 2014; Hsieh et al., 2016; Mazza et al., 2018). In fact, some of these studies have even shown that invasive alien species such *P. clarkii* can eradicate other invasive alien species (e.g., *C. quadricarinatus* or *Cherax destructor*) due to the transmission of pathogens, and not direct competition or predation (Marino et al., 2014; Mazza et al., 2018).

Considering Costa Rica is a biodiversity hotspot, the presence of high-risk species such as *P. clarkii* and *C. quadricarinatus* should be a cause of concern for national authorities. The native freshwater fauna may become threatened if more translocations of these invasive alien crayfish species occur. This may be especially true for amphibians: Brannelly et al. (2015) found evidence of *Batrachochytrium dendrobatidis* (*Bd*) infections in farmed and natural populations of *Procambarus* spp. These authors describe how even a low prevalence of *Bd* infection could have implications for global amphibian conservation. Zumbado-Ulate et al. (2019) summarized the data on prevalence of *Bd* in Costa Rica and how it has irreversibly affected amphibian populations since the 1980s. Low-intensity *Bd* infections in amphibian, below the threshold associated with mortalities, seem common in the country. However, populations of *P. clarkii* present in Costa Rica (see **Table 1** and **Figure 1**) could act as additional reservoirs for *Bd* and contribute to its further spread.

In 2014, the European Union implemented legislation to prevent and regulate the introduction and spread of invasive alien species that focused on control and eradication (EUR-lex, 2014, 2016; Kopf et al., 2017). In this context, the Council of Europe has drafted a series of voluntary codes of conduct based on the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention). These codes of conduct and other guidelines aim to make industries and institutions that handle or encounter non-native species aware of the risks that these species can have on native biodiversity [European Alien Species Information Network (EASIN), 2021]. Similarly, in 2017, Costa Rica implemented the Regulations to the Wildlife Conservation Law (Decreto No. 26435 MINAE), which deals with invasive alien species (República de Costa Rica, 2005). However, the importation of live, captive bred invasive alien species (fauna and flora) and products of inland fisheries is still permitted under this legislation (República de Costa Rica, 2005; Young, 2006). If ever released, these species could negatively impact Costa Rican freshwater biodiversity. Currently, there are no official lists of introduced invasive alien species in Costa Rica; however, as of 2017, at least 461 vertebrate species (6 amphibians, 68 birds, 23 mammals, 12 reptiles, and 352 teleost fishes) are considered to have been introduced (Eduardo Chacón-Madrigal, Universidad de Costa Rica, *personal communication*).

The bad experience and mistakes made by European countries in their treatment of invasive crayfish species offer a lesson for other countries starting to experience the impact of introduced crayfish species, such as Costa Rica. The sooner that these countries are aware and concerned about the detrimental impact crayfish invasions can have on native ecosystems and their biodiversity, the greater the chance they can act to minimize the impact. Moreover, wildlife managers, conservationists, aquaculture companies, and authorities should also be made aware of the threats that each invasive alien species (crayfish and pathogen) may have on already threatened species. To better understand the scope of these threats, additional studies are necessary to assess their potential impact on the native freshwater fauna in Costa Rica and other tropical regions worldwide.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

LM-T, IW, and JD-U contributed to the design, supervision of the project, and writing of the manuscript. AC-V conducted the field sampling, performed the molecular analyses (DNA extraction and haplotyping), and helped with the writing of the manuscript. JA-S conducted the field sampling and helped with the writing of the manuscript. FV-R contributed to the supervision of the project and writing of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI). JD-U was supported by Project CGL2016-80526-R of the Ministerio de Economía y Competitividad, Spain, and AC-V was supported by the Máster en Biodiversidad en Áreas Tropicales y su Conservación, Universidad Internacional Menéndez Pelayo, and Consejo Superior de Investigaciones Científicas, UIMP–CSIC. Additional support for this study was made available through the project 808-B7-285 financed by the Universidad de Costa Rica.

ACKNOWLEDGMENTS

We thank the assistance of the Unidad de Investigación Pesquera y Acuicultura (UNIP) from CIMAR for providing us with the facilities to maintain the *Procambarus clarkii* specimens. We also thank María Martinez-Ríos and Gloria Casabella-Herrero from the Real Jardín Botánico–Consejo Superior de Investigaciones Científicas (RJB-CSIC) for their technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.623814/full#supplementary-material

REFERENCES

Alonso, F., Temiño, C., and Diéguez-Uribeondo, J. (2000). Status of the white clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858) in Spain: distribution and legislation. *B. Fr. Pêche Piscic.* 356, 31–54. doi: 10.1051/kmae:2000003

Arce, J., and Dieguez-Uribeondo, J. (2015). Structural damage caused by the invasive crayfish *Procambarus clarkii* (Girard, 1852) in rice fields of the Iberian Peninsula: a study case. *J. Fundam. Appl. Limnol.* 186, 259–269. doi: 10.1127/fal/2015/0715

Azofeifa-Solano, J. C., Naranjo-Elizondo, B., Rojas-Carranza, A. H., and Cedeño-Fonseca, M. (2017). Presence of the Australian redclaw crayfish *Cherax quadricarinatus* (Von Martens, 1868) (Parastacidae, Astacoidea) in a freshwater system in the Caribbean drainage of Costa Rica. *Bioinvasions Rec.* 6, 351–355. doi: 10.3391/bir.2017.6.4.08

Banha, F., and Anastácio, P. M. (2011). Interactions between invasive crayfish and native river shrimp. *Knowl. Manag. Aquat. Ecosyst.* 401:17. doi: 10.1051/kmae/2011033

Barquero, M. D., and Araya, M. F. (2016). First record of the Greenhouse frog, *Eleutherodactylus planirostris* (Anura: Eleutherodactylidae), in Costa Rica. *Herpetol. Notes* 9, 145–147.

Barrientos-Llosa, Z., and Monge-Nájera, J. (2010). Especies introducidas en la Meseta Central de Costa Rica. *Biocenosis* 23, 32–37.

Beakes, G. W., Glockling, S. L., and Sekimoto, S. (2012). The evolutionary phylogeny of the oomycete "fungi". *Protoplasma* 249, 3–19. doi: 10.1007/s00709-011-0269-2

Bott, R. (1950). Die Flußkrebse Europas (Decapoda, Astacidae). *Abh. Senckenberg. Naturforsch. Gesell.* 483, 1–36.

Brannelly, L. A., McMahon, T. A., Hinton, M., Lenger, D., and Richards-Zawacki, C. L. (2015). *Batrachochytrium dendrobatidis* in natural and farmed Louisiana crayfish populations: prevalence and implications. *Dis. Aquat. Org.* 112, 229–235. doi: 10.3354/dao02817

Casabella-Herrero, G., Martínez-Ríos, M., Viljamaa-Dirks, S., Martín-Torrijos, L., and Diéguez-Uribeondo, J. (2021). *Aphanomyces astaci* mtDNA: insights into the pathogen's differentiation and its genetic diversity from other closely related oomycetes. *Fungal Biol.* 125, 316–325. doi: 10.1016/j.funbio.2020.11.010

Cerenius, L., Bangyeekhun, E., Keyser, P., Söderhäll, I., and Söderhäll, K. (2003). Host prophenoloxidase expression in freshwater crayfish is linked to increased resistance to the crayfish plague fungus, *Aphanomyces astaci*. *Cell. Microbiol.* 5, 353–357. doi: 10.1046/j.1462-5822.2003.00282.x

Chucholl, C. (2013). Invaders for sale: trade and determinants of introduction of ornamental freshwater crayfish. *Biol. Invasions* 15, 125–141. doi: 10.1007/s10530-012-0273-2

Cornalia, E. (1860). Sulla malattia dei gamberi. *Atti Soc. Ital. Sci. Nat.* 2, 334–336.

Correia, A. M., and Anastácio, P. M. (2008). Shifts in aquatic macroinvertebrate biodiversity associated with the presence and size of an alien crayfish. *Ecol. Res.* 23, 729–734. doi: 10.1007/s11284-007-0433-5

Correia, A. M., Bandeira, N., and Anastácio, P. M. (2005). Predator-prey interactions of *Procambarus clarkii* with aquatic macroinvertebrates in single and multiple prey systems. *Acta Oecol.* 28, 337–343. doi: 10.1016/j.actao.2005.06.002

Crandall, K. A., and Buhay, J. E. (2008). Global diversity of crayfish (Astacidae, Cambaridae, and Parastacidae-Decapoda) in freshwater. *Hydrobiologia* 595, 295–301. doi: 10.1007/s10750-007-9120-3

Crandall, K. A., and De Grave, S. (2017). An updated classification of the freshwater crayfishes (Decapoda: Astacidea) of the world, with a complete species list. *J. Crustac. Biol.* 37, 615–653. doi: 10.1093/jcbiol/rux070

Diéguez-Uribeondo, J. (2006). The dispersion of the *Aphanomyces astaci*-carrier *Pacifastacus leniusculus* by humans represents the main cause of disappearance of the indigenous crayfish *Austropotamobius pallipes* in Navarra. *B. Fr. Pêche Piscic.* 380-381, 1303–1312. doi: 10.1051/kmae:2006036

Diéguez-Uribeondo, J., and Söderhäll, K. (1999). RAPD evidences for the origin of an outbreak of aphanomycosis in Spain. *Freshw. Crayfish* 12, 313–318.

Diéguez-Uribeondo, J., Cerenius, L., Dyková, I., Gelder, S. R., Henttonen, P., Jiravanichpaisal, P., et al. (2006). "Pathogens, parasites and ectocomensals," in *Atlas of Crayfish in Europe*, eds C. Souty-Grosset, D. M. Holdich, P. Y. Noël, J. D. Reynolds, and P. Haffner (Paris: Muséum national d' Histoire naturelle, Patrimoines naturelle), 133–149.

Diéguez-Uribeondo, J., García, M. A., Cerenius, L., Kozubíková, E., Ballesteros, I., Windels, C., et al. (2009). Phylogenetic relationships among plant and animal parasites, and saprotrophs in *Aphanomyces* (Oomycetes). *Fungal Genet. Biol.* 46, 365–376.

Diéguez-Uribeondo, J., Huang, T.-S., Cerenius, L., and Söderhäll, K. (1995). Physiological adaptation of an *Aphanomyces astaci* strain isolated from the freshwater crayfish *Procambarus clarkii*. *Mycol. Res.* 99, 574–578. doi: 10.1016/S0953-7562(09)80716-8

Diéguez-Uribeondo, J., Temiño, C., and Múzquiz, J. L. (1997). The crayfish plague fungus (*Aphanomyces astaci*) in Spain. *B. Fr. Pêche Piscic.* 70, 753–763. doi: 10.1051/kmae/1997051

European Alien Species Information Network (EASIN) (2021). *Codes of conduct and guidelines*. Available online at: https://easin.jrc.ec.europa.eu/easin/Documentation/Codesofconduct (accessed March 27, 2021).

EUR-lex (2014). *Regulation (EU) No 1143/2014 of the European Parliament and of the Council of 22 October 2014 on the Prevention and Management of the Introduction and Spread of Invasive Alien Species. Edited by European Parliament and of the Council of the European Union. No 1143/2014.* Luxembourg: Official Journal of the European Union.

EUR-lex (2016). *Commission Implementing Regulation (EU)2016/1141 of 13 July 2016 COMMISSION IMPLEMENTING REGULATION (EU) 2016/1141 of 13 July 2016 Adopting a List of Invasive Alien Species of Union Concern Pursuant to Regulation (EU) No 1143/2014 of the European Parliame. Edited by European Parliament and of the Council of the European Union. No 1141/2016.* Luxembourg: Official Journal of the European Union.

Ficetola, G. F., Siesa, M. E., Padoa-Schioppa, E., and De Bernard, F. (2012). Wetland features, amphibian communities and distribution of the alien crayfish, *Procambarus clarkii*. *Alytes* 29, 75–87.

Gherardi, F., Aquiloni, L., Diéguez-Uribeondo, J., and Tricarico, E. (2011). Managing invasive crayfish: Is there a hope? *Aquat. Sci.* 73, 185–200. doi: 10.1007/s00027-011-0181-z

Holdich, D. M., Reynolds, J. D., Souty-Grosset, C., and Sibley, P. J. (2009). A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. *Knowl. Manag. Aquat. Ecosyst.* 11, 394–395. doi: 10.1051/kmae/2009025

Hsieh, C. Y., Huang, C. W., and Pan, Y. C. (2016). Crayfish plague *Aphanomyces astaci* detected in redclaw crayfish, *Cherax quadricarinatus* in Taiwan. *Invertebr. Pathol.* 136, 117–123. doi: 10.1016/j.jip.2016.03.015

Huang, T.-S., Cerenius, L., and Söderhäll, K. (1994). Analysis of genetic diversity in the crayfish plague fungus, *Aphanomyces astaci*, by random amplification of polymorphic DNA. *Aquaculture* 126, 1–9. doi: 10.1016/0044-8486(94)90243-7

Huner, J. V. (1977). Introductions of the Louisiana red swamp crayfish, *Procambarus clarkii* (Girard): an update. *Freshw. Crayfish* 3, 193–202. doi: 10.5869/fc.1977.v3.193

Ilhéu, M., Bernardo, J. M., and Fernandes, S. (2007). "Predation of invasive crayfish on aquatic vertebrates: the effect of *Procambarus clarkii* on fish assemblages in Mediterranean temporary streams," in *Biological Invaders in Inland Waters: Profiles, Distribution, and Threats*, ed. F. Gherardi (Dordrecht: Springer), 543–558. doi: 10.1007/978-1-4020-6029-8_29

Jussila, J., Vrezec, A., Makkonen, J., Kortet, R., and Kokko, H. (2015). "8. "Invasive crayfish and their invasive diseases in Europe with the focus on the virulence evolution of the crayfish plague," in *Biological Invasions in Changing Ecosystems*, ed. J. Canning-Clode (Berlin: Sciendo Migration), 183–211.

Kawai, T., and Crandall, K. A. (2016). "Global diversity and conservation of freshwater crayfish (Crustacea: Decapoda: Astacoidea)," in *A Global Overview of the Conservation of Freshwater Decapod Crustaceans*, eds T. Kawai and N. Cumberlidge (Cham: Springer International Publishing), 65–114. doi: 10.1007/978-3-319-42527-6

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., et al. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28, 1647–1649. doi: 10.1093/bioinformatics/bts199

Kopf, R. K., Nimmo, D. G., Humphries, P., Baumgartner, L. J., Bode, M., Bond, N. R., et al. (2017). Confronting the risks of large-scale invasive species control. *Nat. Ecol. Evol.* 1:172. doi: 10.1038/s41559-017-0172

Kouba, A., Petrusek, A., and Kozák, P. (2014). Continental-wide distribution of crayfish species in Europe: update and maps. *Knowl. Manag. Aquat. Ecosyst.* 413:05. doi: 10.1051/kmae/2014007

Kozubíková, E., Filipová, L., Kozák, P., Ďuriš, Z., Martín, M. P., Diéguez-Uribeondo, J., et al. (2009). Prevalence of the crayfish plague pathogen *Aphanomyces astaci* in invasive American crayfishes in the Czech Republic. *Conserv. Biol.* 23, 1204–1213. doi: 10.1111/j.1523-1739.2009.01240.x

Kozubikova, E., Viljamaa-Dirks, S., Heinikainen, S., and Petrusek, A. (2011). Spiny-cheek crayfish *Orconectes limosus* carry a novel genotype of the crayfish plague pathogen *Aphanomyces astaci*. *J. Invertebr. Pathol.* 108, 214–216. doi: 10.1016/j.jip.2011.08.002

Lara, L. R., and Wehrtmann, I. S. (2011). "Diversity, abundance and distribution of river shrimps (Decapoda: Caridea) in the largest river basin of Costa Rica, Central America," in *New Frontiers in Crustacean Biology*, eds A. Asakura and C. Fransen (Leiden: Koninklijke Brill NV), 197–211.

Lara, L. R., Wehrtmann, I. S., Magalhães, C., and Mantelatto, F. L. (2013). Species diversity and distribution of freshwater crabs (Decapoda: Pseudothelphusidae) inhabiting the basin of the río Grande de Térraba, Pacific. *Lat. Am. J. Aquat. Res.* 41, 685–695.

Lilley, J. H., Cerenius, L., and Söderhäll, K. (1997). RAPD evidence for the origin of crayfish plague outbreaks in Britain. *Aquaculture* 157, 181–185. doi: 10.1016/S0044-8486(97)00153-1

Lowe, S., Browne, M., Boudjelas, S., and De Poorter, M. (2000). *100 of the World's Worst Invasive Alien Species a Selection from the Global Invasive Species Database. The Invasive Species Specialist Group (ISSG) a specialist group of the Species Survival Commission (SSC) of the World Conservation Union (IUCN)*. Auckland: ISSG.

Machino, Y., and Diéguez-Uribeondo, J. (1998). Un cas de peste des écrevisses en France dans le bassin de la Seine. *L'Astaciculteur France* 54, 2–11.

Magalhães, C., Wehrtmann, I. S., Lara, L. R., and Mantelatto, F. (2015). Taxonomy of the freshwater crabs of Costa Rica, with a revision of the genus *Ptychophallus* Smalley, 1964 (Crustacea: Decapoda: Pseudothelphusidae). *Zootaxa* 3905, 301–344. doi: 10.11646/zootaxa.3905.3.1

Makkonen, J., Jussila, J., Panteleit, J., Keller, N. S., Schrimpf, A., Theissinger, K., et al. (2018). MtDNA allows the sensitive detection and haplotyping of the crayfish plague disease agent *Aphanomyces astaci* showing clues about its origin and migration. *Parasitology* 145, 1210–1218. doi: 10.1017/S0031182018000227

Marino, F., Pretto, T., Tosi, F., Monaco, S., De Stefano, C., Manfrin, A., et al. (2014). Mass mortality of *Cherax quadricarinatus* (von Martens, 1868) reared in Sicily (Italy): Crayfish plague introduced in an intensive farming. *Freshw. Crayfish* 20, 93–96. doi: 10.5869/fc.2014.v20-1.93

Martín-Torrijos, L., Correa-Villalona, A. J., Pradillo, A., and Diéguez-Uribeondo, J. (2021a). Coexistence of two invasive species, *Procambarus clarkii* and *Aphanomyces astaci,* in Brackish waters of a mediterranean coastal lagoon. *Front. Ecol. Evol.* 8:622434. doi: 10.3389/fevo.2020.622434

Martín-Torrijos, L., Kawai, T., Makkonen, J., Jussila, J., Kokko, H., and Dieguez-Uribeondo, J. (2018). Crayfish plague in Japan: a real threat to the endemic *Cambaroides japonicus*. *PLoS One* 13:e0195353. doi: 10.1371/journal.pone.0195353

Martín-Torrijos, L., Kokko, H., Makkonen, J., Jussila, J., and Diéguez-Uribeondo, J. (2019). Mapping 15 years of crayfish plague in the Iberian Peninsula: the impact of two invasive species on the endangered native crayfish. *PLoS One* 14:e0219223. doi: 10.1371/journal.pone.0219223

Martín-Torrijos, L., Martinez-Ríos, M., Casabella-Herrero, G., Adams, S. B., Jackson, C. R., and Diéguez-Uribeondo, J. (2021b). Chasing the crayfish plague: Southeastern United States as the center for *Aphanomyces astaci* diversity. *Sci. Rep.* 11:9332. doi: 10.1038/s41598-021-88704-8

Mazza, G., Scalici, M., Inghilesi, A. F., Aquiloni, L., Pretto, T., Monaco, A., et al. (2018). The red alien vs. the blue destructor: the eradication of *Cherax destructor* by *Procambarus clarkii* in Latium (Central Italy). *Diversity* 10:126. doi: 10.3390/d10040126

Mrugała, A., Buřič, M., Petrusek, A., and Kouba, A. (2019). May atyid shrimps act as potential vectors of crayfish plague? *NeoBiota* 51, 65–80. doi: 10.3897/neobiota.51.37718

Myers, N., Mittermeier, R. A., Mittermeier, C. G., da Fonseca, G. A. B., and Kent, J. (2000). Biodiversity hotspots for conservation priorities. *Nature* 403, 853–858.

Nagy, R., Fusaro, A., Conard, W., and Morningstar, C. (2020). *Procambarus Clarkii* (Girard, 1852). U.S. Geological Survey, Nonindigenous Aquatic Species Database. Available online at: https://nas.er.usgs.gov/queries/factsheet.aspx?SpeciesID=217 (accessed October 20, 2020).

Nunes, A. L., Orizaola, G., Laurila, A., and Rebelo, R. (2014). Morphological and life-history responses of anurans to predation by an invasive crayfish: an integrative approach. *Ecol. Evol.* 4, 1491–1503. doi: 10.1002/ece3.979

Oidtmann, B., Cerenius, L., Schmid, I., Hoffmann, R., and Söderhäll, K. (1999). Crayfish plague epizootics in Germany–classification of two German isolates of the crayfish plague fungus *Aphanomyces astaci* by random amplification of polymorphic DNA. *Dis. Aquat. Organ.* 35, 235–238. doi: 10.3354/dao035235

OIE (World Organization for Animal Health) (2019). *Manual of Diagnostic tests for Aquatic Animals: Infection with Aphanomyces astaci* (crayfish plague). Available online at: https://www.oie.int/en/standard-setting/aquatic-manual/access-online/?htmfile=chapitre_aphanomyces_astaci.htm (accessed March 27, 2021).

Peiró, D. F., Almerão, M. P., Delaunay, C., Jussila, J., Makkonen, J., Bouchon, D., et al. (2016). First detection of the crayfish plague pathogen *Aphanomyces astaci* in South America: a high potential risk to native crayfish. *Hydrobiologia* 781, 181–190. doi: 10.1007/s10750-016-2841-4

Putra, M. D., Bláha, M., Wardiatno, Y., Krisanti, M., Yonvitner, Jerikho, R., et al. (2018). *Procambarus clarkii* (Girard, 1852) and crayfish plague as new threats for biodiversity in Indonesia. *Aquat. Conserv. Mar. Freshw. Ecosyst.* 28, 1434–1440. doi: 10.1002/aqc.2970

Queensland Government (2019). *Business Queensland. Red swamp crayfish*. Available online at: https://www.business.qld.gov.au/industries/farms-fishing-forestry/agriculture/land-management/health-pests-weeds-diseases/pests/invasive-animals/prohibited/red-swamp-crayfish (accessed March 27, 2021).

República de Costa Rica (2005). *Reglamento a La Ley de Conservación de La Vida Silvestre*. Available online at: http://www.pgrweb.go.cr/scij/Busqueda/Normativa/Normas/nrm_texto_completo.aspx?param1=NRTC&nValor1=1&nValor2=84592&nValor3=109223&strTipM=TC (accessed October 20, 2020).

Reynolds, J. D. (2011). A review of ecological interactions between crayfish and fish, indigenous and introduced. *Knowl. Manag. Aquat. Ecosyst.* 401, 1–21. doi: 10.1051/kmae/2011024

Rezinciuc, S., Galindo, J., Montserrat, J., and Diéguez-Uribeondo, J. (2014). AFLP-PCR and RAPD-PCR evidences of the transmission of the pathogen *Aphanomyces astaci* (Oomycetes) to wild populations of European crayfish from the invasive crayfish species, *Procambarus clarkii*. *Fungal Biol.* 118, 612–620. doi: 10.1016/j.funbio.2013.10.007

Rezinciuc, S., Sandoval-Sierra, J. V., Oidtmann, B., and Diéguez-Uribeondo, J. (2015). "The biology of crayfish plague pathogen *Aphanomyces astaci*: current answers to most frequent questions," in *Freshwater Crayfish: A Global Overview*, eds T. Kawai, Z. Faulkes, and G. Scholtz (Boca Raton, FL: CRC Press), 182–204.

Schrimpf, A., Schmidt, T., and Schulz, R. (2014). Invasive chinese mitten crab (*Eriocheir Sinensis*) transmits crayfish plague pathogen (*Aphanomyces astaci*). *Aquat. Invasions* 9, 203–209. doi: 10.3391/ai.2014.9.2.09

Shull, C. (1909). Some abnormalities and regeneration of pleiopods in cambarus and other decapoda. *Biol. Bull.* 16, 297–312. doi: 10.2307/1536190

Souty-Grosset, C., Anastácio, P. M., Aquiloni, L., Banha, F., Choquer, J., Chucholl, C., et al. (2016). The red swamp crayfish *Procambarus clarkii* in Europe: impacts on aquatic ecosystems and human well-being. *Limnologica* 58, 78–93. doi: 10.1016/j.limno.2016.03.003

Svoboda, J., Mrugała, A., Kozubíková-Balcarová, E., Kouba, A., Diéguez-Uribeondo, J., and Petrusek, A. (2014). Resistance to the crayfish plague pathogen, *Aphanomyces astaci*, in two freshwater shrimps. *J. Invertebr. Pathol.* 121, 97–104. doi: 10.1016/j.jip.2014.07.004

Taugbøl, T., Skurdal, J., and Håstein, T. (1993). Crayfish plague and management strategies in Norway. *Biol. Conserv.* 63, 75–82. doi: 10.1016/0006-3207(93)90076-D

Torres, E., and Álvarez, F. (2012). Genetic variation in native and introduced populations of the red swamp crayfish *Procambarus clarkii* (Girard, 1852) (Crustacea, Decapoda, Cambaridae) in Mexico and Costa Rica. *Aquat. Invasions* 7, 235–241. doi: 10.3391/ai.2012.7.2.009

Unestam, T. (1969). Resistance to the crayfish plague in some American, Japanese and European crayfishes. *Rep. Inst. Freshw. Res.* 49, 202–209.

Unestam, T. (1972). On the host range and origin of the crayfish plague fungus. *Rep. Inst. Freshw. Res.* 52, 192–198.

Unestam, T. (1975). Defence reactions in and susceptibility of Australian and New Guinean freshwater crayfish to European-crayfish-plague fungus. *Aust. J. Exp. Biol. Med. Sci.* 53, 349–359. doi: 10.1038/icb.1975.40

Vennerström, P., Söderhäll, K., and Cerenius, L. (1998). The origin of two crayfish plague (*Aphanomyces astaci*) epizootics in Finland on noble crayfish, *Astacus astacus*. *Ann. Zool. Fenn.* 35, 43–46.

Villalobos-Rojas, F. (2019). *Propuesta de Plan de Manejo Para las Langostas Exóticas de Agua Dulce Cherax Quadricarinatus* (von Martens, 1868) y *Procambarus clarkii (Girard, 1852) Para el Sistema Nacional de Áreas De Conservación (SINAC), Costa Rica*. Master's thesis. San José: Universidad para la Cooperación Internacional.

Wehrtmann, I. S., Ramírez, A., and Pérez-Reyes, O. (2016). "Freshwater decapod diversity and conservation in Central America and the Caribbean," in *A Global Overview of the Conservation of Freshwater Decapod Crustaceans*, eds T. Kawai and N. Cumberlidge (Cham: Springer Nature), 267–301. doi: 10.1007/978-3-319-42527-6

Young, T. R. (2006). *National and Regional Legislation for Promotion and Support to the Prevention, Control, and Eradication of Invasive Species. Biodiversity Series: The International Bank for Reconstruction and Development/THE WORLD BANK*, no108. Washington, DC: The world bank environment department.

Zumbado-Ulate, H., Nelson, K. N., García-Rodríguez, A., Chaves, G., Arias, E., Bolaños, F., et al. (2019). Endemic infection of *Batrachochytrium dendrobatidis* in Costa Rica: implications for amphibian conservation at regional and species level. *Diversity* 11:129. doi: 10.3390/d1108 0129

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

METHODS
published: 28 June 2021
doi: 10.3389/fevo.2021.597585

Real-Time PCR Assays for Rapid Identification of Common *Aphanomyces astaci* Genotypes

Marco Di Domenico[1], Valentina Curini[1], Riccardo Caprioli[1,2], Carla Giansante[1], Agata Mrugała[3,4,5,6], Michaela Mojžišová[3], Cesare Cammà[1] and Adam Petrusek[3]*

[1] Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise G. Caporale, Teramo, Italy, [2] Agenzia Regionale Protezione Ambientale del Lazio, Rome, Italy, [3] Department of Ecology, Faculty of Science, Charles University, Prague, Czechia, [4] Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Berlin, Germany, [5] Department of Biology, Chemistry, Pharmacy, Institute of Biology, Freie Universität Berlin, Berlin, Germany, [6] Berlin-Brandenburg Institute of Advanced Biodiversity Research, Berlin, Germany

OPEN ACCESS

Edited by:
Javier Diéguez-Uribeondo, Real Jardín Botánico (RJB), Spain

Reviewed by:
Diana Minardi, Centre for Environment, Fisheries and Aquaculture Science (CEFAS), United Kingdom
Enrique Lara, Consejo Superior de Investigaciones Científicas (CSIC), Spain

***Correspondence:**
Valentina Curini
v.curini@izs.it

Specialty section:
This article was submitted to Evolutionary and Population Genetics, a section of the journal Frontiers in Ecology and Evolution

Received: *21 August 2020*
Accepted: *28 May 2021*
Published: *28 June 2021*

Citation:
Di Domenico M, Curini V, Caprioli R, Giansante C, Mrugała A, Mojžišová M, Cammà C and Petrusek A (2021) Real-Time PCR Assays for Rapid Identification of Common Aphanomyces astaci Genotypes. Front. Ecol. Evol. 9:597585. doi: 10.3389/fevo.2021.597585

The oomycete *Aphanomyces astaci* is the etiologic agent of crayfish plague, a disease that has seriously impacted the populations of European native crayfish species. The introduction of non-indigenous crayfish of North American origin and their wide distribution across Europe have largely contributed to spread of crayfish plague in areas populated by indigenous crayfish. Tracking *A. astaci* genotypes may thus be a useful tool for investigating the natural history of crayfish plague in its European range, as well as the sources and introduction pathways of the pathogen. In this study, we describe the development of real-time PCR TaqMan assays aiming to distinguish the five genotype groups of *A. astaci* (A–E) previously defined by their distinct RAPD patterns. The method was evaluated using DNA extracts from pure *A. astaci* cultures representing the known genotype groups, and from *A. astaci*-positive crayfish clinical samples collected mostly during crayfish plague outbreaks that recently occurred in Central Italy and Czechia. The assays do not cross-react with each other, and those targeting genotype groups A, B, D, and E seem sufficiently specific to genotype the pathogen from infected crayfish in the areas invaded by *A. astaci* (particularly Europe). The unusual *A. astaci* genotype "SSR-Up" documented from crayfish plague outbreaks in Czechia and chronically infected *Pontastacus leptodactylus* in the Danube is detected by the group B real-time PCR. The assay originally developed to detect group C (one not yet documented from crayfish plague outbreaks) showed cross-reactivity with *Aphanomyces fennicus*; the *A. astaci* genotype "rust1" described in the United States from *Faxonius rusticus* is detected by that assay as well. Analyses of additional markers (such as sequencing of the nuclear internal transcribed spacer or mitochondrial ribosomal subunits) may complement such cases when the real-time PCR-based genotyping is not conclusive. Despite some limitations, the method is a robust tool for fast genotyping of *A. astaci* genotype groups common in Europe, both during crayfish plague outbreaks and in latent infections.

Keywords: *Aphanomyces astaci*, crayfish plague, genotyping, TaqMan assay, real-time PCR, genotype groups

INTRODUCTION

Crayfish plague is a disease of freshwater crayfish caused by the oomycete *Aphanomyces astaci*, which has been endangering the populations of indigenous crayfish throughout Europe and adjacent regions for over 150 years (Alderman, 1996; Holdich et al., 2009; OIE (World Organisation for Animal Health), 2019). Recently, the presence of *A. astaci* in the wild has been documented from Brazil (Peiró et al., 2016), Indonesia (Putra et al., 2018), and Japan (Mrugała et al., 2017a), with confirmed mortalities of the endemic Japanese crayfish *Cambaroides japonicus* (Martín-Torrijos et al., 2018). Furthermore, the disease has caused mortalities in aquacultures of susceptible crayfish hosts (Hsieh et al., 2016). Its negative impacts thus also extend to biogeographic regions other than the Western Palaearctic.

The original hosts of the crayfish plague pathogen *A. astaci* are North American freshwater crayfish species, such as the signal crayfish *Pacifastacus leniusculus*, red swamp crayfish *Procambarus clarkii*, and spiny-cheek crayfish *Faxonius limosus* (Unestam and Weiss, 1970; Vey et al., 1983; Diéguez-Uribeondo et al., 1995) which are particularly widespread as invaders in Europe (Kouba et al., 2014). North American crayfish coevolved with this oomycete and only succumb to crayfish plague under particular conditions (e.g., Unestam, 1969, 1972; Diéguez-Uribeondo and Söderhäll, 1993; Diéguez-Uribeondo et al., 1995), but can act as carriers of the infection [e.g., Persson and Söderhäll, 1983; Diéguez-Uribeondo and Söderhäll, 1993; Jussila et al., 2015; OIE (World Organisation for Animal Health), 2019]. Recently, with the development of suitable molecular methods, chronic infections in populations of crayfish species that are generally considered susceptible but had not experienced mass mortalities or other symptoms of acute crayfish plague were also documented (see a review in Svoboda et al., 2017).

The first wave of crayfish mass mortalities across Europe seem to have occurred in the second half of the nineteenth and early twentieth centuries, spreading through native populations of European crayfish (reviewed in Alderman, 1996) but without any documented information about the presence of the original pathogen carriers. The subsequent introduction of North American crayfish species to Europe and their current wide distribution across the continent (Holdich et al., 2009; Kouba et al., 2014) have been the cause of past and recent spread of crayfish plague in areas populated by indigenous European species (e.g., Huang et al., 1994; Bohman et al., 2006; Diéguez-Uribeondo, 2006; Kozubíková et al., 2011a; Schrimpf et al., 2012; Filipová et al., 2013; Viljamaa-Dirks et al., 2013; Kozubíková-Balcarová et al., 2014; Rezinciuc et al., 2014; Vrålstad et al., 2014; Martín-Torrijos et al., 2019) and have also led to the introduction of pathogen genotypes different from those involved in early crayfish plague outbreaks (Rezinciuc et al., 2015).

Molecular typing by the random amplified polymorphic DNA (RAPD) of *A. astaci* axenic laboratory cultures (Huang et al., 1994) has allowed the identification of five distinct genotype groups, labeled alphabetically from A to E (reviewed in Rezinciuc et al., 2015). The application of genotyping markers to clinical samples (i.e., DNA isolates from infected crayfish), particularly the analysis of microsatellite loci (Grandjean et al., 2014) and sequencing of mitochondrial ribosomal genes (Makkonen et al., 2018), allowed the discovery of additional variation among *A. astaci* strains, both in outbreaks of the disease (Grandjean et al., 2014; Martín-Torrijos et al., 2018) and from non-symptomatic hosts (Mrugała et al., 2017a; Martín-Torrijos et al., 2018; Panteleit et al., 2019; Martín-Torrijos et al., 2021). However, the vast majority of crayfish plague outbreaks in Europe analyzed so far have been linked to one of the four RAPD-defined genotype groups either isolated from one of the widespread crayfish invaders (groups B, D, E; Huang et al., 1994; Diéguez-Uribeondo et al., 1995; Kozubíková et al., 2011b) or associated with historical mortalities (group A; Huang et al., 1994). Tracking *A. astaci* genotypes or genotype groups may thus be a useful tool for investigating the natural history of crayfish plague in its invaded range, as well as the sources and introduction pathways of the pathogen.

The data on the distribution of *A. astaci* genotypes in Europe and adjacent regions, analyzed from various sources including axenic laboratory cultures, crayfish mass mortalities, and chronically infected crayfish hosts, were recently summarized by Ungureanu et al. (2020). Most information available from Europe so far comes from crayfish mass mortalities, which have been analyzed by various molecular approaches that should allow matching results to the original RAPD-defined genotype groups (see below). In particular, causative agents of crayfish plague outbreaks have been genotyped in regions where crayfish plague is considered a major threat to indigenous crayfish conservation, and thus research has been intensive in the past two decades. It is important to keep in mind that the diversity of *A. astaci* in its native range is largely unexplored, and substantial additional variation has been already discovered since this oomycete became studied in other natural hosts (Panteleit et al., 2019; Martín-Torrijos et al., 2021).

The distribution pattern of *A. astaci* genotype groups, as identified by RAPD analysis of axenic cultures or by other DNA-based methods from clinical samples, is uneven across Europe (see map in Ungureanu et al., 2020). Group A, presumably the first to have been introduced there in the 19th century (Huang et al., 1994), has been frequently detected in Fennoscandia and also caused recent mortalities in Czechia, Croatia, Bosnia and Herzegovina, and Central Italy (Caprioli et al., 2013, 2018; Viljamaa-Dirks et al., 2013; Kozubíková-Balcarová et al., 2014; Vrålstad et al., 2014; Maguire et al., 2016; Mrugała et al., 2017b). Other genotype groups, which have been associated with American crayfish host taxa introduced to Europe in the late 19th and 20th centuries, follow to some extent their distribution across the continent (Kouba et al., 2014). Group B, originally isolated from the signal crayfish *P. leniusculus* (Huang et al., 1994), is widespread, being associated with at least some mortalities in most European regions from which genotyping data are available (e.g., Vrålstad et al., 2014; Caprioli et al., 2018; Martín-Torrijos et al., 2019). Group D, isolated from the red swamp crayfish *P. clarkii* (Diéguez-Uribeondo et al., 1995), has mostly been detected in southwestern Europe, where this crayfish invader is particularly widespread (e.g., Caprioli et al., 2018; Martín-Torrijos et al., 2019). Recently, group D strains were also associated with mortalities in Czechia (Mojžišová et al., 2020),

with aquarium-kept crayfish (known to host this group; Mrugała et al., 2017a; Makkonen et al., 2018) implicated as likely sources of the infection. Group E was originally isolated from the spiny-cheek crayfish *F. limosus* (Kozubíková et al., 2011a). Its crayfish host is widespread from France across Central Europe to the Baltic countries (Kouba et al., 2014), which corresponds to regions where mortalities associated with group E have been reported (Grandjean et al., 2014; Kaldre et al., 2017; Mojžišová et al., 2020).

It is worth noting, however, that most mortalities associated with crayfish plague have not yet been examined by pathogen genotyping, and no data are available from some European regions (such as Poland, or most of the Balkans and Eastern Europe). It is thus certain that the distribution of known genotype groups is substantially wider than so far documented. Furthermore, links between *A. astaci* genotypes and the host taxa serving as sources of the infection are rather presumed than well documented, as genotyping of the pathogen from the invasive populations of its American hosts has not been performed frequently (Ungureanu et al., 2020), and horizontal transfer of the pathogen upon contact with non-symptomatic hosts seems possible both in captivity and in the wild (Mrugała et al., 2015; James et al., 2017).

Interestingly, recently obtained data on the *A. astaci* distribution and genotypes from Eastern Europe and Turkey, summarized in Ungureanu et al. (2020), come mostly from chronic infections of the narrow-clawed crayfish *Pontastacus leptodactylus* indigenous to those regions. In this host, at least three different strains have been documented, including those of genotype groups A and B (e.g., Kokko et al., 2018) as well as the enigmatic genotype "SSR-Up" (Panteleit et al., 2018), characterized so far by a specific allele combination of microsatellite markers and otherwise known only from mass mortalities in Czechia (Grandjean et al., 2014; Mojžišová et al., 2020).

As already mentioned, *A. astaci* genotyping has been accomplished using various molecular typing techniques. Two of these, RAPD analysis (Huang et al., 1994; Diéguez-Uribeondo et al., 1995; Kozubíková et al., 2011a) and amplified fragment length polymorphism (AFLP) (Rezinciuc et al., 2014), require axenic cultures of the pathogen. However, the use of high-throughput sequencing and bioinformatic analyses has allowed the development of alternative genotyping methods suitable for processing mixed genomic DNA samples extracted directly from infected crayfish tissues. The first of these was an analysis of microsatellite markers (Grandjean et al., 2014). The variation in these microsatellite loci is sufficient to differentiate among all *A. astaci* genotype groups identified to date in Europe (Grandjean et al., 2014; Panteleit et al., 2018, 2019). The method also allows the retrospective analyses of the disease natural history, pathogen sources, and most likely introduction pathways, based on historical samples preserved from past crayfish plague outbreaks (e.g., Grandjean et al., 2014; Vrålstad et al., 2014; Kaldre et al., 2017; Caprioli et al., 2018).

More recently, Makkonen et al. (2018) published a genotyping method based on the sequencing of phylogenetically informative mitochondrial ribosomal markers (small and large ribosomal subunits, rnnS and rnnL) amplified by conventional PCR; this method allows the distinguishing of *A. astaci* from other known related oomycete species, as well as the differentiation of known RAPD-defined genotype groups except for group C (Makkonen et al., 2018; Casabella-Herrero et al., 2021). Whole-genome analysis of *A. astaci* strains representing the five currently known genotype groups allowed Minardi et al. (2018) to identify genomic regions presumably unique to each representative strain and to develop genotype-specific primers for a conventional PCR-based genotyping assay. In order to improve the sensitivity, the same group published an alternative method based on restriction fragment length polymorphism (RFLP) of mitochondrial markers (Minardi et al., 2019).

In this study, we describe the development and application of a new method based on real-time PCR as an alternative approach to currently available methods for the quick identification of common *A. astaci* genotype groups causing crayfish plague outbreaks in Europe. This method is applicable on both pure cultures and clinical crayfish samples. Since its appearance two decades ago, real-time PCR has been used as a powerful tool for genotyping (Alker et al., 2004; Birdsell et al., 2014). This method is a closed-tube system requiring no post-PCR processing, reducing the likelihood of laboratory cross-contamination by amplified products. Moreover, real-time PCR often has high specificity and sensitivity. Considering all these benefits, we developed TaqMan real-time PCR assays targeting all five genotype groups, designed on the genomic regions previously described by Minardi et al. (2018). The method was evaluated using DNA extracts from pure *A. astaci* cultures representative of the known genotype groups, and from *A. astaci*-positive clinical crayfish samples collected during crayfish plague outbreaks that recently occurred in Central Italy and Czechia or representing crayfish hosts chronically infected by this pathogen.

MATERIALS AND METHODS

DNA from all *P. clarkii* individuals collected in Italy and used in this study were isolated from the crayfish soft abdominal cuticle by means of a Maxwell 16 Tissue DNA Purification Kit (Promega, Madison, WI) following the manufacturer's guidelines. Other DNA isolates originating from infected crayfish or from axenic laboratory cultures of oomycetes were reused from previous studies (see below).

The anonymous locus sequences (MH016383, MH016384, MH016385, MH016386, MH016387) published by Minardi et al. (2018) as being specific for the *A. astaci* strains representing genotype groups A–E, respectively, were used to design five TaqMan real-time PCR assays in Primer Express software 3.0.1 (Applied Biosystems, Foster City, CA). These assays are further referred to by the respective letter, although some of them amplify a wider range of targets (see "Results"). The sequences of primer pairs and probes of each of the assays are listed in **Table 1**.

PCR reactions were performed on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) in fast mode with the following protocol: initial denaturation at 95°C for 20 s, followed by 45 cycles of denaturation at 95°C for 1 s and

TABLE 1 | Sequences of primers and probes for the real-time PCR assays designed to amplify specific genomic regions of strains representing *Aphanomyces astaci* genotype groups A–E.

Assay	Oligoname	Sequence (5′–3′)	Amplicon size
A	Aast_A_fwd	CAGTCCCACGAGCCGAAA	71 bp
	Aast_A_rev	CCATCGCTGCTTGCAAGAC	
	Aast_A_probe	JOE-TCGCGCTTCTAGTGCTTCTA TTCGATCCA-TAMRA	
B	Aast_B_fwd	AACAAGCGACCTTCCATTTCA	66 bp
	Aast_B_rev	TTTCGGAGCAGCCAGATTG	
	Aast_B_probe	FAM-AACGCAACTCGCACAATGGAACGA-TAMRA	
C	Aast_C_fwd	CAACATACCAGTTGCGAACGA	77 bp
	Aast_C_rev	GATTTCATCTACCGAGCTTCAACA	
	Aast_C_probe	FAM-CTTGCGTTCATGCTCGATTTCG TCTAATTC-TAMRA	
D	Aast_D_fwd	AATTTGACGATGTGCAATGGAA	67 bp
	Aast_D_rev	TCCGCTTTCATTTTGCAAATATT	
	Aast_D_probe	FAM-CCGTTTCAACAAACAGTGG-TAMRA	
E	Aast_E_fwd	TGCCTGGAGTATTGCCTGAAT	74 bp
	Aast_E_rev	TACGCGCAAACCTATCTCTGAA	
	Aast_E_probe	JOE-CGTGCGAGCAAAAGCCT CAACTCG-TAMRA	

annealing/extension at 60°C for 20 s. The 20-µl reaction volume contained final concentrations of 1 × GoTaq Probe qPCR Master Mix (Promega), 0.5 µM forward primer, 0.5 µM reverse primer, 0.2 µM TaqMan probe, 5 µl DNA template, and nuclease-free water up to volume. No Template Control (NTC) was used for every assay. The fluorescent signal was measured during the annealing step of the reaction.

Specificity was tested on DNA isolates from axenic laboratory strains of genotype groups A–E (**Table 2**), which were also used as positive controls in every PCR run, and on DNA isolates from axenic cultures of various other oomycete taxa isolated from crayfish. These included, in particular, multiple *Aphanomyces* strains: *A. fennicus* (M6/1), a recently described apparently avirulent species closely related to *A. astaci*, which cross-reacts with the real-time PCR-based *A. astaci* detection (Viljamaa-Dirks and Heinikainen, 2019), *A. laevis-repetans* (strain SAP761; Kozubíková-Balcarová et al., 2013), six strains of two *Aphanomyces* lineages (T10, T2S1, T2S2, T2S3, T2UN1, T2UN2) isolated from Lake Tahoe signal crayfish *P. leniusculus* by Makkonen et al. (2019), and *A. frigidophilus* (SAP233, SAP472) isolated from Spanish white-clawed crayfish *Austropotamobius pallipes* (Ballesteros et al., 2006; Diéguez-Uribeondo et al., 2009). We also included DNA from strains of *Saprolegnia ferax* (SAP691, Li19), *S. hypogyna* (Li16), *S. parasitica* (SAP694), *S. australis* (SAP684), *Pythium* sp. (Li18, Li20), and an unidentified Saprolegniaceae strain (Li01), all of these originally cultivated from crayfish bodies (Kozubíková-Balcarová et al., 2013). Furthermore, two additional *Aphanomyces* species parasitizing other organisms, a plant pathogen *A. cochlioides* and a fish pathogen *A. invadans* (SAP308; Diéguez-Uribeondo et al., 2009), were tested for the specificity of the assays.

The method was then applied to DNA isolates from *A. astaci*-infected crayfish of various origins (**Table 3**): 13 DNA isolates from 11 crayfish plague outbreaks affecting noble crayfish *Astacus astacus* or stone crayfish *Austropotamobius torrentium* recorded recently in Czechia (Kozubíková et al., 2008; Kozubíková-Balcarová et al., 2014; Mojžišová et al., 2020), on 26 DNA isolates of white clawed crayfish (*A. pallipes*) from Italian outbreaks (mostly reported in Caprioli et al., 2018), 26 DNA isolates from infected crayfish alien to Italy (*P. leptodactylus*, *P. clarkii*), and a DNA isolate from an infected individual of the rusty crayfish *Faxonius rusticus* from the United States (Panteleit et al., 2019). The presence of *A. astaci* DNA in these samples was confirmed and quantified by real-time PCR according to Vrålstad et al. (2009), with details of the protocol provided in Caprioli et al. (2018). In samples with sufficiently high agent levels (from A3 to A7), the pathogen had also been genotyped by microsatellite markers following Grandjean et al. (2014).

The tested samples also included DNA isolates from crayfish infected by two unusual *A. astaci* genotypes that differ at microsatellite markers from so far characterized strains of known RAPD-defined genotype groups. The first was the specific "SSR-Up" genotype from an outbreak in Úpořský brook (Czechia), which is more similar to strains of RAPD-defined group B at the studied microsatellite loci (Grandjean et al., 2014; Mojžišová et al., 2020), but assigned to mitochondrial haplogroup A based on rnnS and rnnL sequences (Makkonen et al., 2018). The second such genotype is "rust1," also belonging to haplogroup A, documented from two US populations of *F. rusticus* and isolated to axenic cultures from one of them (Panteleit et al., 2019).

Finally, assays A and B, labeled with different dyes (**Table 1**), were optimized for duplex real-time PCR. The same approach was taken for assays D and E, while assay C was performed separately. The ROX dye (6-carboxy-X-rhodamine) was used as passive reference in every reaction mix. For the duplex reactions, the 20-µl reaction volume contained final concentrations of 1 × GoTaq Probe qPCR Master Mix (Promega), 0.5 µM of both forward primers, 0.5 µM of both reverse primers, 0.2 µM of both TaqMan probes, 5 µl DNA template, and nuclease-free water to reach the final volume. The PCR cycling conditions were identical to those described above. A paired *t*-test was used to compare simplex and duplex real-time PCR results.

RESULTS

All the reference axenic cultures of *A. astaci* were assigned to their respective genotype group by the newly developed real-time PCR assays (**Table 2**). Moreover, no-template controls and DNA from all but one isolate of non-target taxa did not produce any detectable fluorescence signal, demonstrating the high specificity of the test (data not shown). The exception was the DNA isolate from *A. fennicus*, which yielded a positive signal with the real-time PCR assay originally developed for group C.

TABLE 2 | Axenic cultures of *A. astaci* strains used to validate the new real-time PCR assays.

Isolate	Origin	Crayfish host	Genotype group	ITS[a] (Ct)	Real-time PCR genotyping[b] (Ct)				
					A	B	C	D	E
Al7	Armenia[c]	*Pontastacus leptodactylus*	A	27.7	33.5				
Pec14	Czechia	*Astacus astacus*	B	27.0		34.6			
Kv1	Canada[d]	*Pacifastacus leniusculus*	C	21.7			28.5		
Fin183	Ornamental trade[e]	*Procambarus virginalis*	D	18.6				22.5	
Li10	Czechia	*Astacus astacus*	E	23.7					33

[a]*Ct values obtained from the real-time PCR targeting the nuclear internal transcribed spacer (ITS) region (Vrålstad et al., 2009).*
[b]*Ct values obtained from the real-time PCR genotyping assays described in this study.*
[c]*Isolated in Czechia from narrow-clawed crayfish presumably originating from Armenia (Becking et al., 2015).*
[d]*Isolated in Sweden from signal crayfish that originated from Pitt Lake, Canada (Huang et al., 1994).*
[e]*Isolated in Finland from a crayfish purchased in a German pet shop (Mrugała et al., 2015).*

The genotypes determined by the five assays on DNA extracted directly from crayfish cuticles corresponded with assignments to genotype groups based on the microsatellite analysis. The two "SSR-Up" samples yielded positive signals in the assay developed in this study for genotype group B. The sample from *F. rusticus* infected by the *A. astaci* "rust1" genotype yielded a positive signal with assay C (**Table 3**).

The novel real-time PCR method succeeded in genotyping the pathogen in four DNA isolates from infected crayfish specimens for which the microsatellite analysis failed. However, for 17 samples with low pathogen DNA concentrations that were detectable by the real-time PCR assay targeting the ITS region (Vrålstad et al., 2009), neither our real-time PCR assays nor microsatellite analyses were able to determine the genotype group (**Table 3**).

Combining the real-time PCR assays in duplex reactions was successful. The genotype of all 12 samples tested by the duplex assays was correctly determined without any cross-amplification, and with no significant drop in cycle threshold (Ct) values [$t(11) = -0.45$; $p = 0.32$] when compared to those obtained by simplex assays (**Table 4**).

DISCUSSION

Molecular typing of *A. astaci* strains, which differentiates distinct genotype groups, contributes to a better understanding of the relationship between this pathogen and its host taxa. It also has practical implications, as the identification of *A. astaci* genotypes can assist in tracing sources of infection during crayfish plague outbreaks (e.g., Vrålstad et al., 2014; Maguire et al., 2016; Mrugała et al., 2017b; Martín-Torrijos et al., 2019) and in chronically infected populations (e.g., Kokko et al., 2018).

RAPD analysis was the first genotyping approach applied to *A. astaci* isolates (Huang et al., 1994) but could be applied only to axenic laboratory cultures. Since then, the challenge has been to develop genotyping tools that can be applied directly to DNA isolates from crayfish tissues, avoiding the time-consuming and often unsuccessful isolation of the agent (Oidtmann et al., 1999; Cammà et al., 2010). Based on microsatellite analysis, Grandjean et al. (2014) provided a method to distinguish the five currently known genotype groups and uncover some additional variation. This technique is PCR-based, so even intermediate quantities of template DNA occurring in asymptomatic carriers can be genotyped. However, microsatellite analysis can suffer from inter-laboratory reproducibility, including a failure to establish a successful workflow or inconsistent allele scoring. Indeed, recent papers have reported minor differences in genotyped allele sizes from reference strains (compare Panteleit et al., 2018, 2019 with Mojžišová et al., 2020). In addition, the presence of unusual patterns may lead to uncertain results, especially when applied directly to field-collected samples. This may be caused by apparently novel genotypes (Kozubíková-Balcarová et al., 2014; Panteleit et al., 2019), presumed minor variation within the known RAPD-defined genotype groups (James et al., 2017; Mrugała et al., 2017a; Caprioli et al., 2018), or *A. astaci* coinfections by different strains (Maguire et al., 2016). Possibly, the presence of DNA of other related oomycetes may also contribute to inconclusive results, as some of the markers are not species-specific (Grandjean et al., 2014; but note the typesetting error in the Aast10 locus rectified in Mojžišová et al., 2020). The method also suffers from low success once the pathogen DNA concentrations drop to agent level A3 and below (Grandjean et al., 2014; Caprioli et al., 2018).

Recently, in order to improve the genotyping sensitivity, two alternative methods based on multicopy mitochondrial DNA markers have been successfully developed and applied (Makkonen et al., 2018; Martín-Torrijos et al., 2018, 2021; Minardi et al., 2019; Butler et al., 2020; Casabella-Herrero et al., 2021). Sequence analysis of the two ribosomal subunits (rnnS and rnnL) did not allow differentiating between RAPD-defined genotype groups A and C (which belong to the same haplogroup A), but it did reveal additional variation within group D (Makkonen et al., 2018; Martín-Torrijos et al., 2018). The sensitivity of this haplotyping approach has not been quantified, but it has been successfully applied to agent level A3 isolates, i.e., with relatively low levels of the pathogen DNA in the sample (Kokko et al., 2018; Panteleit et al., 2018). The RFLP-based assay targeting genotype-specific single-nucleotide polymorphisms in mitochondrial DNA (Minardi et al., 2019) demonstrated high sensitivity but lacked specificity. In particular, *A. astaci* genotype group D, widespread from southwestern to central Europe (Ungureanu et al., 2020) and also causing crayfish plague outbreaks in Japan (Martín-Torrijos et al., 2018), showed

TABLE 3 | Direct genotyping of mixed DNA isolates from field samples.

Host	Rivers/streams (country code)	Year	Microsatellite genotypes[a]	ITS[b] (Ct)	Agent level[b]	Real-time PCR genotyping[c] (Ct)				
						A	B	C	D	E
A. astacus	Olše (CZ)	2004	SSR-A	27.5	A4	36.9				
A. astacus	Besének (CZ)	2009	SSR-A	21.3	A5	34.6				
A. astacus	Brook in Horní Pěna (CZ)	2007	SSR-B	14.2	A7		22.6			
A. astacus	Černý brook (CZ)	2014	SSR-B	11.8	A7		21.2			
A. astacus	Litavka (CZ)	2011	SSR-E	18.3	A6					27.1
A. torrentium	Úpořský brook (CZ)	2005	SSR-Up	16.4	A7		24.1			
A. torrentium	Úpořský brook (CZ)	2005	SSR-Up	20.4	A6		25.5			
A. astacus	Žebrákovský potok (CZ)	2008	SSR-B	17.1	A7		24.5			
A. astacus	Rožnovská Bečva (CZ)	2018	SSR-D	19.8	A6				26.8	
A. torrentium	Kublovský brook (CZ)	2018	SSR-D	13.7	A7				28.3	
A. astacus	Stroupinský brook (CZ)	2018	SSR-D	14.9	A7				22.1	
A. astacus	Blanice (CZ)	2018	SSR-A	16.6	A7	24.8				
A. torrentium	Radotínský brook (CZ)	2017	SSR-E	16.6	A7					25.6
A. pallipes	Zingano brook (IT)	2011	SSR-A	31.0	A3	38.0				
A. pallipes	Zingano brook (IT)	2011	SSR-A	20.3	A6	27.0				
A. pallipes	Zingano brook (IT)	2011	SSR-A	25.9	A4	33.0				
A. pallipes	Zingano brook (IT)	2011	SSR-A	27.9	A4	34.0				
A. pallipes	Zingano brook (IT)	2011	SSR-A	26.3	A4	33.0				
A. pallipes	Zingano brook (IT)	2011	SSR-A	28.3	A3	35.0				
A. pallipes	Castelnuovo brook (IT)	2011	SSR-D	19.5	A6				27.0	
A. pallipes	Castelnuovo brook (IT)	2011	SSR-D	16.9	A7				24.0	
A. pallipes	**Gamberale brook (IT)**	**2013**	**nd**	**31.4**	**A3**				**39.0**	
A. pallipes	Rio Verde brook (IT)	2013	SSR-D	16.8	A7				25.0	
A. pallipes	Rio Verde brook (IT)	2013	SSR-D	24.0	A5				33.1	
A. pallipes	Rio Verde brook (IT)	2013	SSR-D	19.9	A6				27.0	
A. pallipes	Rio Verde brook (IT)	2013	SSR-D	14.7	A7				21.9	
A. pallipes	San Leo brook (IT)	2009	SSR-B	27.0	A4		36.0			
A. pallipes	San Leo brook (IT)	2009	SSR-B	29.1	A3		37.5			
A. pallipes	Tirino river (IT)	2016	SSR-B	26.2	A4		35.0			
A. pallipes	Tirino river (IT)	2016	SSR-B	18.4	A6		28.2			
A. pallipes	Tirino river (IT)	2016	SSR-B	20.0	A6		29.1			
A. pallipes	Tirino river (IT)	2016	SSR-B	28.5	A3		36.2			
A. pallipes	Tirino river (IT)	2016	SSR-B	27.0	A4		35.6			
A. pallipes	Tirino river (IT)	2016	SSR-B	16.0	A7		26.4			
A. pallipes	Rio Fosse brook (IT)	2017	SSR-D	17.8	A6				27.2	
A. pallipes	**Rio Fosse brook (IT)**	**2017**	**nd**	**31.6**	**A3**				**39.0**	
A. pallipes	Rio Fosse brook (IT)	2017	SSR-D	29.7	A3				36.9	
A. pallipes	Rio Fosse brook (IT)	2017	SSR-D	18.5	A6				27.0	
A. pallipes	Rio Fosse brook (IT)	2017	SSR-D	26.9	A4				35.1	
P. leptodactylus	Tirino river (IT)	2016	SSR-A	26.0	A4	36.0				
P. leptodactylus	Tirino river (IT)	2016	SSR-A	25.5	A4	35.2				
P. leptodactylus	Tirino river (IT)	2016	SSR-A	26.8	A4	34.5				
P. clarkii	Crayfish farming* (IT)	2017	nd	36.2	A2					
P. clarkii	Crayfish farming* (IT)	2017	nd	35.5	A2					
P. clarkii	Crayfish farming* (IT)	2017	nd	37.0	A2					
P. clarkii	Crayfish farming* (IT)	2017	nd	33.5	A3					
P. clarkii	Crayfish farming* (IT)	2017	SSR-D	29.5	A3				39.9	
P. clarkii	Tevere river (IT)	2018	nd	34.3	A3					
P. clarkii	Tevere river (IT)	2018	nd	35.6	A2					
P. clarkii	Tevere river (IT)	2018	nd	36.4	A2					

(Continued)

TABLE 3 | Continued

Host	Rivers/streams (country code)	Year	Microsatellite genotypes[a]	ITS[b] (Ct)	Agent level[b]	Real-time PCR genotyping[c] (Ct)				
						A	B	C	D	E
P. clarkii	Papacqua lake (IT)	2018	nd	33.0	A3					
P. clarkii	Papacqua lake (IT)	2018	nd	33.6	A3					
P. clarkii	**Crayfish farming* (IT)**	**2018**	**nd**	**31.8**	**A3**				**38.4**	
P. clarkii	**Crayfish farming* (IT)**	**2018**	**nd**	**31.4**	**A3**				**42.0**	
P. clarkii	Crayfish farming* (IT)	2018	SSR-D	30.4	A3				38.1	
P. clarkii	Crayfish farming* (IT)	2018	nd	33.4	A3					
P. clarkii	Crayfish farming* (IT)	2018	SSR-D	30.9	A3				37.3	
P. clarkii	Crayfish farming* (IT)	2018	nd	31.4	A3					
P. clarkii	Crayfish farming* (IT)	2018	nd	34.7	A2					
P. clarkii	Crayfish farming* (IT)	2018	nd	36.7	A2					
P. clarkii	Crayfish farming* (IT)	2018	nd	35.2	A2					
P. clarkii	Crayfish farming* (IT)	2018	nd	31.5	A3					
P. clarkii	Crayfish farming* (IT)	2018	nd	34.4	A3					
P. clarkii	Crayfish farming* (IT)	2018	SSR-D	25.5	A4				35.8	
P. clarkii	Crayfish farming* (IT)	2018	nd	33.5	A3					
F. rusticus	Big lake (WI)	2016	SSR-rust1	22.6	A5			30.8		

**Crayfish farming Rocchetta al Volturno.*
[a]*Microsatellite genotypes obtained according to the protocol described by Grandjean et al. (2014).*
[b]*Ct values and Agent levels obtained from the ITS real-time PCR (Vrålstad et al., 2009).*
[c]*Ct values defined by the real-time PCR genotyping method described in this study.*
Samples marked in bold are those for which genotyping was successful by real-time PCR only.

cross-reactivity with *Leptolegnia caudata* and *Phoma*-like isolates (Minardi et al., 2019).

Based on the data reported here, our real-time PCR assays seem specific for the RAPD-defined genotype groups A, D, and E (or DNA isolates with corresponding microsatellite multilocus genotypes) that are known to cause crayfish plague outbreaks in Europe; no cross-reactions were observed, either between different *A. astaci* isolates, in other oomycete strains isolated from crayfish, or in DNA extracted from field samples. The real-time PCR assay B identified all clinical samples assigned to SSR-B by microsatellite analysis, but also detected two samples classified as SSR-Up by microsatellite markers (Grandjean et al., 2014; Mojžišová et al., 2020).

The genotype group C assay amplified not only DNA of the respective *A. astaci* strain (Huang et al., 1994) but also DNA of *A. fennicus* and a DNA isolate obtained from *F. rusticus* infected by the "rust1" genotype (Panteleit et al., 2019). However, as group C has not been documented in Europe since its original discovery (Ungureanu et al., 2020), the lack of specificity of assay C should be considered less relevant; in fact, rather than for the identification of a particular *A. astaci* genotype group, this assay can be used as an indicator of specific cases requiring further attention (i.e., the presence of unusual *A. astaci* genotypes or *A. fennicus* in a sample). Unfortunately, we failed to obtain any PCR products from *A. fennicus* that would allow sequencing of the anonymous nuclear marker targeted by this assay, so it is not possible to assess the extent of similarity of this genomic region between the respective strains of *A. astaci* and *A. fennicus*. In case of doubt, however, *A. astaci* genotype group C can be distinguished from *A. fennicus* by differences in the sequences of other molecular markers such as ITS (Viljamaa-Dirks and Heinikainen, 2019) and

TABLE 4 | Results of the duplex real-time PCR, with a comparison of the sensitivity expressed as Ct values with separate simplex reactions.

Host	Microsatellite genotypes	Simplex (Ct)	Duplex A–B (Ct)		Duplex D–E (Ct)	
			A	B	D	E
A. astacus	SSR-A	24.8	25.5			
P. leptodactylus	SSR-A	35.2	35.8			
A. pallipes	SSR-A	27.0	28.7			
A. pallipes	SSR-B	35.0		35.1		
A. pallipes	SSR-B	29.1		28.8		
A. pallipes	SSR-B	36.2		36.3		
A. pallipes	SSR-D	33.1			33.2	
A. pallipes	SSR-D	27.2			29.1	
P. clarkii	SSR-D	35.8			38.2	
A. astacus	SSR-E	27.1				29.2
A. astacus	SSR-E	nd				Nd
A. torrentium	SSR-E	25.6				25.8

Assays A and D were labeled with JOE, while B and E were labeled with FAM at the 5′ end.
Differences between simplex and duplex Ct values were not significant [paired t-test, $t(11) = -0.45$; $p = 0.32$].
Microsatellite genotypes obtained according to the protocol described by Grandjean et al. (2014).

both mitochondrial ribosomal subunits (rnnS, rnnL; Casabella-Herrero et al., 2021).

The original reference strain of the RAPD-defined group C of *A. astaci* was isolated from *Pacifastacus leniusculus* (Huang et al., 1994). However, isolates showing some level of genetic similarity to this strain have been obtained from cambarid crayfish in the United States: axenic cultures matching the nuclear marker that Minardi et al. (2018) considered specific to group C have been isolated from *Faxonius obscurus* from Pennsylvania by Butler et al. (2020). Our results suggest that the "rust1" strain infecting *F. rusticus* (Panteleit et al., 2019) in Wisconsin also carries a marker sufficiently similar to be amplified by a TaqMan assay designed to detect the group C. This is in line with analyses of "rust1" laboratory cultures: they have a distinct profile of microsatellite nuclear markers (sharing most alleles with groups C and B), and the sequences of mitochondrial rnnS and rnnL ribosomal subunits and a nuclear chitinase gene are identical with the reference strain of group C (Panteleit et al., 2019). Thus, although originally developed as specific for *A. astaci* genotype group C, the real-time PCR assay developed by us may be rather used as a screening method for *A. fennicus* and for a wider range of unusual *A. astaci* genotypes. In any case, samples with positive detection by our assay C require additional detailed analyses, and the same is likely true when using the genotyping method described in Minardi et al. (2018). If *A. fennicus* is not involved, microsatellite markers from Grandjean et al. (2014), which fail to amplify in *A. fennicus* (Viljamaa-Dirks and Heinikainen, 2019), may be further used to characterize *A. astaci* strains positively reacting with our assay C.

In terms of sensitivity, our real-time PCR assays allowed the genotyping of four samples for which microsatellite analysis failed (**Table 4**). These included a sample with agent level A3 from the 2013 outbreak in the Gamberale brook (near Agnone, Molise region), for which the causative genotype group was previously unknown. Unlike other outbreaks from Central Italy, only two dead crayfish were available from this site, with rather low amounts of pathogen DNA. Although we were unable to genotype this sample previously (Caprioli et al., 2018), the real-time PCR assay indicated that it belonged to group D.

Two samples from Úpořský brook (Czechia) deserve special attention when specificity of the group B assay is considered. The *A. astaci* genotype that caused crayfish plague outbreak in that brook in 2005, since then also detected in chronically infected *P. leptodactylus* in the Danube (Panteleit et al., 2018) and additional recent outbreaks in Czechia (Mojžišová et al., 2020), is characterized by a consistent microsatellite pattern (SSR-Up). This differs from any known strains isolated to axenic cultures (and thus assigned to genotype groups by RAPD), but its microsatellite allele composition is most similar to strains representing genotype group B (Grandjean et al., 2014). Conversely, mitochondrial markers (rnnS and rnnL) assign this genotype to haplogroup A (Makkonen et al., 2018). Both SSR-Up isolates consistently yielded a positive signal in our group B real-time PCR assay. In these cases, the combination of real-time PCR targeting the nuclear loci and mtDNA sequencing would allow distinguishing this particular *A. astaci* genotype from strains of genotype group B.

Although less important than specificity and sensitivity, it is worth reporting that the time needed to perform the real-time PCR analysis proposed here is greatly reduced, thanks to the use of rapid PCR protocols and duplexing the reactions targeting the four widespread genotype groups causing most crayfish plague outbreaks in European countries (A, B, D, and E).

Despite these benefits, the substantial limitation of the new method remains sensitivity, which apparently does not exceed that obtained by sequencing multicopy mitochondrial markers (Makkonen et al., 2018). Indeed, although our method allowed us to characterize four A3-level samples that we were unable to genotype by microsatellite analysis, other A3 samples could not be characterized. Samples with A3 agent levels fall within a wide range of Ct values (28–35), so the lower the Ct, the higher the probability the sample is successfully genotyped. This is because the ITS target region used in quantitative PCR to assign the agent level is a multicopy target (Vrålstad et al., 2009), whereas the five assays described in this study were designed on presumably single-copy genes. Amplification of the target marker in a qPCR reaction nevertheless allows the detection of smaller amounts of the target DNA than is required for successful characterization in fragment analyses used for microsatellite genotyping.

Overall, however, the use of real-time PCR assays for genotyping purposes has multiple advantages. The method speeds up genetic characterization of *A. astaci* DNA extracted directly from crayfish-infected tissues, it is easy to perform, the interpretation of results is unambiguous (positive/negative) and highly reproducible, and the likelihood of laboratory contamination by PCR products is reduced. Once a genotype group is indicated, samples amplified by group B and C assays may be further investigated, taking advantage of other currently available methods. For example, after a positive detection by our group B real-time PCR assay, samples belonging to the RAPD-defined group B may be differentiated from the "SSR-Up" genotype by fragment analysis of diagnostic microsatellite loci (Grandjean et al., 2014; Mojžišová et al., 2020) and/or by sequencing the mitochondrial rnnS or chitinase genes (Panteleit et al., 2018). In conclusion, the different genotyping methods available for *A. astaci,* including the real-time PCR approach described in this study, may thus conveniently complement each other.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

MDD, VC, and AP: conceptualization. MDD: data curation. MDD, VC, AP, and CC: formal analysis. CC, CG, and AP: funding acquisition. RC, VC, AM, MM, and MDD: investigation.

MDD, VC, AP, CC, CG, MM, AM, and RC: methodology. MDD and VC: software. CC and AP: supervision. RC, VC, and MDD: writing—original draft. AP and MDD: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

FUNDING

The research was supported by the Italian Ministry of Health (project IZS AM 06/16 RC) and Charles University, Prague (project SVV 260569). AM acknowledges financial support from the Alexander von Humboldt Foundation.

ACKNOWLEDGMENTS

We thank Satu Viljamaa-Dirks for providing *A. fennicus* and *A. astaci* isolate Kv1 DNA (with the permission of Kenneth Söderhäll) from the collections of the Finnish Food Authority Ruokavirasto (Kuopio), Javier Diéguez-Uribeondo for permission to analyze DNA isolates of oomycetes from the collections of Real Jardín Botánico, CSIC, Madrid (isolates with SAP codes), Jenny Makkonen for DNA of *Aphanomyces* spp. cultures isolated from Lake Tahoe signal crayfish, and Anne Schrimpf for a DNA isolate of infected *Faxonius rusticus* from the United States. David Hardekopf is acknowledged for language revisions of the final version of the manuscript.

REFERENCES

Alderman, D. J. (1996). Geographical spread of bacterial and fungal diseases of crustaceans. *Rev. Sci. Tech.* 15, 603–632. doi: 10.20506/rst.15.2.943

Alker, A. P., Mwapasa, V., and Meshnick, S. R. (2004). Rapid real-time PCR genotyping of mutations associated with sulfadoxine-pyrimethamine resistance in *Plasmodium falciparum*. *Antimicrob. Agents Chemother.* 48, 2924–2929.

Ballesteros, I., Martín, M. P., and Diéguez-Uribeondo, J. (2006). First isolation of *Aphanomyces frigidophilus* (Saprolegniales) in Europe. *Mycotaxon* 95, 335–340.

Becking, T., Mrugała, A., Delaunay, C., Svoboda, J., Raimond, M., Viljamaa-Dirks, S., et al. (2015). Effect of experimental exposure to differently virulent *Aphanomyces astaci* strains on the immune response of the noble crayfish *Astacus astacus*. *J. Invertebr. Pathol.* 132, 115–124. doi: 10.1016/j.jip.2015.08.007

Birdsell, D. N., Vogler, A. J., Buchhagen, J., Clare, A., Kaufman, E., Naumann, A., et al. (2014). TaqMan real-time PCR assays for single-nucleotide polymorphisms which identify *Francisella tularensis* and its subspecies and subpopulations. *PLoS One* 9:e107964. doi: 10.1371/journal.pone.0107964

Bohman, P., Nordwall, F., and Edsman, L. (2006). The effect of the large-scale introduction of signal crayfish on the spread of crayfish plague in Sweden. *Bull. Fr. Pêche Piscicul.* 380–381, 1291–1302. doi: 10.1051/kmae:2006026

Butler, E., Crigler, P., Robbins, G., and Blair, J. E. (2020). Preliminary survey of *Aphanomyces* sp. associated with native and invasive crayfish in the Lower Susquehanna watershed of South Central Pennsylvania. *J. Freshw. Ecol.* 35, 223–233. doi: 10.1080/02705060.2020.1779141

Cammà, C., Ferri, N., Zezza, D., Marcacci, M., Paolini, A., Ricchiuti, L., et al. (2010). Confirmation of crayfish plague in Italy: detection of *Aphanomyces astaci* in white clawed crayfish. *Dis. Aquat. Org.* 89, 265–268. doi: 10.3354/dao02207

Caprioli, R., Cargini, D., Marcacci, M., Cammà, C., Giansante, C., and Ferri, N. (2013). Self-limiting outbreak of crayfish plague in an *Austropotamobius pallipes* population of a river basin in the Abruzzi region (Central Italy). *Dis. Aquat. Org.* 103, 149–156. doi: 10.3354/dao02571

Caprioli, R., Mrugała, A., Di Domenico, M., Curini, V., Giansante, C., Cammà, C., et al. (2018). *Aphanomyces astaci* genotypes involved in recent crayfish plague outbreaks in central Italy. *Dis. Aquat. Org.* 130, 209–219. doi: 10.3354/dao03275

Casabella-Herrero, G., Martínez-Ríos, M., Viljamaa-Dirks, S., Martín-Torrijos, L., and Diéguez-Uribeondo, J. (2021). *Aphanomyces astaci* mtDNA: insights into the pathogen's differentiation and its genetic diversity from other closely related oomycetes. *Fungal Biol.* 125, 316–325. doi: 10.1016/j.funbio.2020.11.010

Diéguez-Uribeondo, J. (2006). Dispersion of the *Aphanomyces astaci*-carrier, *Pacifastacus leniusculus*, by humans represents the main cause of disappearance of native populations of *Austropotamobius pallipes* in Navarra. *Bull. Fr. Pêche Piscicul.* 4, 1303–1312. doi: 10.1051/kmae:2006036

Diéguez-Uribeondo, J., and Söderhäll, K. (1993). *Procambarus clarkii* as a vector for the crayfish plague fungus *Aphanomyces astaci* Schikora. *Aquac. Fish. Manage.* 24, 761–765.

Diéguez-Uribeondo, J., García, M. A., Cerenius, L., Kozubíková, E., Ballesteros, I., Windels, C., et al. (2009). Phylogenetic relationships among plant and animal parasites, and saprotrophs in *Aphanomyces* (Oomycetes). *Fungal Genet. Biol.* 46, 365–376. doi: 10.1016/j.fgb.2009.02.004

Diéguez-Uribeondo, J., Huang, T., Cerenius, L., and Söderhäll, K. (1995). Physiological adaptation of an *Aphanomyces astaci* strain isolated from the freshwater crayfish *Procambarus clarkii*. *Mycol. Res.* 99, 574–578. doi: 10.1016/s0953-7562(09)80716-8

Filipová, L., Petrusek, A., Matasová, K., Delaunay, C., and Grandjean, F. (2013). Prevalence of the crayfish plague pathogen *Aphanomyces astaci* in populations of the signal crayfish *Pacifastacus leniusculus* in France: evaluating the threat to native crayfish. *PLoS One* 8:e70157. doi: 10.1371/journal.pone.0070157

Grandjean, F., Vrålstad, T., Diéguez-Uribeondo, J., Jelić, M., Mangombi, J., Delaunay, C., et al. (2014). Microsatellite markers for direct genotyping of the crayfish plague pathogen *Aphanomyces astaci* (Oomycetes) from infected host tissues. *Vet. Microbiol.* 170, 317–324. doi: 10.1016/j.vetmic.2014.02.020

Holdich, D. M., Reynolds, J. D., Souty-Grosset, C., and Sibley, P. J. (2009). A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. *Knowl. Manag. Aquat. Ecosyst.* 394–395:11. doi: 10.1051/kmae/2009025

Hsieh, C. Y., Huang, C. W., and Pan, Y. C. (2016). Crayfish plague *Aphanomyces astaci* detected in redclaw crayfish, *Cherax quadricarinatus* in Taiwan. *J. Invertebr. Pathol.* 136, 117–123. doi: 10.1016/j.jip.2016.03.015

Huang, T., Cerenius, L., and Söderhäll, K. (1994). Analysis of genetic diversity in the crayfish plague fungus, *Aphanomyces astaci*, by random amplification of polymorphic DNA. *Aquaculture* 126, 1–9. doi: 10.1016/0044-8486(94)90243-7

James, J., Mrugała, A., Oidtmann, B., Petrusek, A., and Cable, J. (2017). Apparent interspecific transmission of *Aphanomyces astaci* from invasive signal to virile crayfish in a sympatric wild population. *J. Invertebr. Pathol.* 145, 68–71. doi: 10.1016/j.jip.2017.02.003

Jussila, J., Vrezec, A., Makkonen, J., Kortet, R., and Kokko, H. (2015). "Invasive crayfish and their invasive diseases in Europe with the focus on the virulence evolution of the crayfish plague," in *Biological Invasions in Changing Ecosystems. Vectors, Ecological Impacts, Management and Predictions*, ed. J. Canning-Clode (Warsaw: De Gruyter).

Kaldre, K., Paaver, T., Hurt, M., and Grandjean, F. (2017). First records of the non-indigenous signal crayfish (*Pacifastacus leniusculus*) and its threat to noble crayfish (*Astacus astacus*) populations in Estonia. *Biol. Invasions* 19, 2771–2776. doi: 10.1007/s10530-017-1496-z

Kokko, H., Harlioglu, M. M., Aydin, H., Makkonen, J., Gökmen, G., Aksu, Ö, et al. (2018). Observations of crayfish plague infections in commercially important narrow-clawed crayfish populations in Turkey. *Knowl. Manag. Aquat. Ecosyst.* 419:10. doi: 10.1051/kmae/2018001

Kouba, A., Petrusek, A., and Kozák, P. (2014). Continental-wide distribution of crayfish species in Europe: update and maps. *Knowl. Manag. Aquat. Ecosyst.* 413:05. doi: 10.1051/kmae/2014007

Kozubíková, E., Petrusek, A., Ďuriš, Z., Martín, M. P., Diéguez-Uribeondo, J., and Oidtmann, B. (2008). The old menace is back: recent crayfish plague outbreaks in the Czech Republic. *Aquaculture* 274, 208–217. doi: 10.1016/j.aquaculture.2007.11.015

Kozubíková, E., Viljamaa-Dirks, S., Heinikainen, S., and Petrusek, A. (2011a). Spiny-cheek crayfish *Orconectes limosus* carry a novel genotype of the crayfish plague pathogen *Aphanomyces astaci*. *J. Invertebr. Pathol.* 108, 214–216. doi: 10.1016/j.jip.2011.08.002

Kozubíková, E., Vrålstad, T., Filipová, L., and Petrusek, A. (2011b). Re-examination of the prevalence of *Aphanomyces astaci* in North American crayfish populations in Central Europe by TaqMan MGB real-time PCR. *Dis. Aquat. Org.* 97, 113–125. doi: 10.3354/dao02411

Kozubíková-Balcarová, E., Beran, L., Ďuriš, Z., Fischer, D., Horká, I., Svobodová, J., et al. (2014). Status and recovery of indigenous crayfish populations after recent crayfish plague outbreaks in the Czech Republic. *Ethol. Ecol. Evol.* 26, 299–319. doi: 10.1080/03949370.2014.897652

Kozubíková-Balcarová, E., Koukol, O., Martín, M. P., Svoboda, J., Petrusek, A., and Diéguez-Uribeondo, J. (2013). The diversity of oomycetes on crayfish: Morphological vs. molecular identification of cultures obtained while isolating the crayfish plague pathogen. *Fungal Biol.* 117, 682–691. doi: 10.1016/j.funbio.2013.07.005

Maguire, I., Jelić, M., Klobučar, G., Delpy, M., Delaunay, C., and Grandjean, F. (2016). Prevalence of the pathogen *Aphanomyces astaci* in freshwater crayfish populations in Croatia. *Dis. Aquat. Org.* 118, 45–53. doi: 10.3354/dao02955

Makkonen, J., Jussila, J., Panteleit, J., Keller, N. S., Schrimpf, A., Theissinger, K., et al. (2018). MtDNA allows the sensitive detection and haplotyping of the crayfish plague disease agent *Aphanomyces astaci* showing clues about its origin and migration. *Parasitology* 26, 1–9. doi: 10.1017/S0031182018000227

Makkonen, J., Kokko, H., Gökmen, G., Ward, J., Umek, J., Kortet, R., et al. (2019). The signal crayfish (*Pacifastacus leniusculus*) in Lake Tahoe (USA) hosts multiple *Aphanomyces* species. *J. Invertebr. Pathol.* 166:107218. doi: 10.1016/j.jip.2019.107218

Martín-Torrijos, L., Kawai, T., Makkonen, J., Jussila, J., Kokko, H., and Diéguez-Uribeondo, J. (2018). Crayfish plague in Japan: a real threat to the endemic *Cambaroides* japonicus. *PLoS One* 13:e0195353. doi: 10.1371/journal.pone.0195353

Martín-Torrijos, L., Kokko, H., Makkonen, J., Jussila, J., and Diéguez-Uribeondo, J. (2019). Mapping 15 years of crayfish plague in the Iberian Peninsula: the impact of two invasive species on the endangered native crayfish. *PLoS One* 14:e0219223. doi: 10.1371/journal.pone.0219223

Martín-Torrijos, L., Martínez-Ríos, M., Casabella-Herrero, G., Adams, S. B., Jackson, C. R., and Diéguez-Uribeondo, J. (2021). Tracing the origin of the crayfish plague pathogen, *Aphanomyces astaci*, to the Southeastern United States. *Sci. Rep.* 11:9332. doi: 10.1038/s41598-021-88704-8

Minardi, D., Studholme, D. J., Oidtmann, B., Pretto, T., and van der Giezen, M. (2019). Improved method for genotyping the causative agent of crayfish plague (*Aphanomyces astaci*) based on mitochondrial DNA. *Parasitology* 146, 1022–1029. doi: 10.1017/S0031182019000283

Minardi, D., Studholme, D. J., van der Giezen, M., Pretto, T., and Oidtmann, B. (2018). New genotyping method for the causative agent of crayfish plague (*Aphanomyces astaci*) based on whole genome data. *J. Invertebr. Pathol.* 156, 6–13. doi: 10.1016/j.jip.2018.06.002

Mojžišová, M., Mrugała, A., Kozubíková-Balcarová, E., Vlach, P., Svobodová, J., Kouba, A., et al. (2020). Crayfish plague in Czechia: outbreaks from novel sources and testing for chronic infections. *J. Invertebr. Pathol.* 173:107390. doi: 10.1016/j.jip.2020.107390

Mrugała, A., Kawai, T., Kozubíková-Balcarová, E., and Petrusek, A. (2017a). *Aphanomyces astaci* presence in Japan: a threat to the endemic and endangered crayfish species *Cambaroides* japonicus? *Aquat. Conserv.* 27, 103–114. doi: 10.1002/aqc.2674

Mrugała, A., Kozubíková-Balcarová, E., Chucholl, C., Cabanillas Resino, S., Viljamaa-Dirks, S., Vukić, J., et al. (2015). Trade of ornamental crayfish in Europe as a possible introduction pathway for important crustacean diseases: crayfish plague and white spot syndrome. *Biol. Invasions* 17, 1313–1326. doi: 10.1007/s10530-014-0795-x

Mrugała, A., Šanda, R., Petrusek, A., Marić, D., and Vukić, J. (2017b). Recent acute crayfish mortality reveals *Aphanomyces astaci* presence in Bosnia and Herzegovina. *J. Invertebr. Pathol.* 150, 73–75. doi: 10.1016/j.jip.2017.09.004

Oidtmann, B., Cerenius, L., Schmid, I., Hoffmann, R., and Söderhäll, K. (1999). Crayfish plague epizootics in Germany—classification of two German isolates of the crayfish plague fungus *Aphanomyces astaci* by random amplification of polymorphic DNA. *Dis. Aquat. Org.* 35, 235–238. doi: 10.3354/dao035235

OIE (World Organisation for Animal Health) (2019). *Manual of Diagnostic Tests for Aquatic Animals*. Available online at: https://www.oie.int/standard-setting/aquatic-manual (accessed August 06, 2020).

Panteleit, J., Horvath, T., Jussila, J., Makkonen, J., Perry, W., Schulz, R., et al. (2019). Invasive rusty crayfish (*Faxonius rusticus*) populations in North America are infected with the crayfish plague disease agent (*Aphanomyces astaci*). *Freshw. Sci.* 38, 425–433. doi: 10.1086/703417

Panteleit, J., Keller, N. S., Diéguez-Uribeondo, J., and Makkonen, J. (2018). Hidden sites in the distribution of the crayfish plague pathogen *Aphanomyces astaci* in Eastern Europe: relicts of genetic groups from older outbreaks? *J. Invertebr. Pathol.* 157, 117–124. doi: 10.1016/j.jip.2018.05.006

Peiró, D. F., Almerão, M. P., Delaunay, C., Jussila, J., Makkonen, J., Bouchon, D., et al. (2016). First detection of the crayfish plague pathogen *Aphanomyces astaci* in South America: a high potential risk to native crayfish. *Hydrobiologia* 781, 181–190. doi: 10.1007/s10750-016-2841-4

Persson, M., and Söderhäll, K. (1983). *Pacifastacus leniusculus* Dana and its resistance to the parasitic fungus *Aphanomyces astaci* Schikora. *Freshw. Crayfish* 5, 292–298.

Putra, M. D., Bláha, M., Wardiatno, Y., Krisanti, M., Yonvitner, Jerikho, R., et al. (2018). *Procambarus clarkii* (Girard, 1852) and crayfish plague as new threats for biodiversity in Indonesia. *Aquat. Conserv. Mar. Freshw. Ecosys.* 28, 1434–1440. doi: 10.1002/aqc.2970

Rezinciuc, S., Galindo, J., Montserrat, J., and Diéguez-Uribeondo, J. (2014). AFLP-PCR and RAPD-PCR evidences of the transmission of the pathogen *Aphanomyces astaci* (Oomycetes) to wild populations of European crayfish from the invasive crayfish species, *Procambarus* clarkii. *Fungal Biol.* 118, 612–620. doi: 10.1016/j.funbio.2013.10.007

Rezinciuc, S., Sandoval-Sierra, J. V., Oidtmann, B., and Diéguez-Uribeondo, J. (2015). "The biology of crayfish plague pathogen: current answers to most frequent questions," in *Freshwater Crayfish: A Global Overview*, eds T. Kawai, Z. Faulkes, and G. Scholtz (Boca Raton, FL: CRC Press), 182–204.

Schrimpf, A., Pârvulescu, L., Copilaș-Ciocianu, D., Petrusek, A., and Schulz, R. (2012). Crayfish plague pathogen detected in the Danube Delta – a potential threat to freshwater biodiversity in southeastern Europe. *Aquat. Invasions* 7, 503–510. doi: 10.3391/ai.2012.7.4.007

Svoboda, J., Mrugała, A., Kozubíková-Balcarová, E., and Petrusek, A. (2017). Hosts and transmission of the crayfish plague pathogen *Aphanomyces astaci*: a review. *J. Fish. Dis.* 40, 127–140. doi: 10.1111/jfd.12472

Unestam, T. (1969). Resistance to the crayfish plague fungus in some American, Japanese and European crayfish. *Rep. Inst. Freshw. Res. Drottningholm* 49, 202–209.

Unestam, T. (1972). On the host range and origin of the crayfish plague fungus. *Rep. Inst. Freshw. Res. Drottningholm* 52, 192–198.

Unestam, T., and Weiss, D. W. (1970). The host-parasite interaction relationship between freshwater crayfish and the crayfish disease fungus *Aphanomyces astaci*: responses to infection by susceptible and resistant species. *J. Gen. Microbiol.* 60, 77–90. doi: 10.1099/00221287-60-1-77

Ungureanu, E., Mojžišová, M., Tangerman, M., Ion, M. C., Pârvulescu, L., and Petrusek, A. (2020). The spatial distribution of *Aphanomyces astaci* genotypes across Europe: introducing the first data from Ukraine. *Freshw. Crayfish* 25, 77–87. doi: 10.5869/fc.2020.v25-1.077

Vey, A., Söderhäll, K., and Ajaxon, R. (1983). Susceptibility of *Orconectes limosus* Raff. to crayfish plague. *Freshw. Crayfish* 5, 192–291.

Viljamaa-Dirks, S., and Heinikainen, S. (2019). A tentative new species *Aphanomyces fennicus* sp. nov. interferes with molecular diagnostic methods for crayfish plague. *J. Fish. Dis.* 42, 413–422. doi: 10.1111/jfd.12955

Viljamaa-Dirks, S., Heinikainen, S., Torssonen, H., Pursiainen, M., Mattila, J., and Pelkonen, S. (2013). Distribution and epidemiology of genotypes of the

crayfish plague agent *Aphanomyces astaci* from noble crayfish *Astacus astacus* in Finland. *Dis. Aquat. Org.* 103, 199–208. doi: 10.3354/dao02575

Vrålstad, T., Knutsen, A. K., Tengs, T., and Holst-Jensen, A. (2009). A quantitative TaqMan MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. *Vet. Microbiol.* 137, 146–155. doi: 10.1016/j.vetmic.2008.12.022

Vrålstad, T., Strand, D. A., Grandjean, F., Kvellestad, A., Håstein, T., Knutsen, A., et al. (2014). Molecular detection and genotyping of *Aphanomyces astaci* directly from preserved crayfish samples uncovers the Norwegian crayfish plague disease history. *Vet. Microbiol.* 173, 66–75. doi: 10.1016/j.vetmic.2014.07.008

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

POLICY AND PRACTICE REVIEWS
published: 30 July 2021
doi: 10.3389/fevo.2021.648495

Money Kills Native Ecosystems: European Crayfish as an Example

Japo Jussila[1], Lennart Edsman[2], Ivana Maguire[3], Javier Diéguez-Uribeondo[4] and Kathrin Theissinger[5,6]*

[1] Department of Environmental and Biological Sciences, University of Eastern Finland, Kuopio, Finland, [2] Department of Aquatic Resources, Institute of Freshwater Research, Swedish University of Agricultural Sciences, Uppsala, Sweden, [3] Department of Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia, [4] Department of Mycology, Real Jardín Botánico - CSIC, Madrid, Spain, [5] LOEWE Centre for Translational Biodiversity Genomics, Senckenberg Biodiversity and Climate Research Centre, Frankfurt, Germany, [6] Institute for Environmental Sciences, University of Koblenz-Landau, Landau, Germany

Native European crayfish conservation was triggered by invasion of crayfish plague disease agent, *Aphanomyces astaci*, starting 1860s in Northern Italy. Resulting crayfish plague epidemics quickly spread over Continental Europe, then to Finland, Sweden and finally, after running amok around Europe, *A. astaci* was discovered also in Iberian Peninsula, Norway, Ireland, and United Kingdom in 1970s and 1980s. By that time significant proportion of native crayfish stocks had been lost, and while crayfish plague epidemics were still recorded, also industrialization and waterways construction were causing damage to remaining native crayfish stocks. While alien crayfish introductions, at least *Faxonius limosus,* already gave rise to first wave of crayfish plague epidemics in late 19th century, later in 1960s it was decided that introductions of alien *Pacifastacus leniusculus* should be initiated to replace native European crayfish populations. Decisions were based on presumed advantages for fishery, suitable habitat requirements and supposed immunity against *A. astaci*. Furthermore, conservation of native European crayfish species was sidelined and focus shifted toward alien crayfish stocking routine and consumption. Alien crayfish species introductions resulted in repeated waves of crayfish plague epidemics among remaining native crayfish stocks. It was soon discovered that alien crayfish of North American origin were, as suspected, permanent reservoirs for *A. astaci*, that some of those alien species were losing their resistance against selected strains of *A. astaci* and struggled in European aquatic ecosystems. In this article, we introduce numerous motives behind grand mistake of introducing alien crayfish species to Europe and then promoting their stocks instead of focusing on conservation of native crayfish species. We outline how false economical, biological and ecologic assumptions were used to justify a hasty introduction of alien crayfish, which has further devastated native crayfish and also permanently changed European aquatic ecosystems, both with disastrous consequences. Lesson to be learnt is that science-based warnings about alien species damage to native ecosystems and native crayfish must be taken with utmost caution. Protection of native European crayfish should be core issue, not commercial activities.

OPEN ACCESS

Edited by:
Orsolya Valkó,
Centre for Ecological Research, Hungarian Academy of Sciences, Hungary

Reviewed by:
Christoph Chucholl,
Fischereiforschungsstelle Baden-Württemberg, Germany
Antonín Kouba,
University of South Bohemia, Czechia

****Correspondence:***
Japo Jussila
japo.jussila@uef.fi

Specialty section:
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *06 January 2021*
Accepted: *29 June 2021*
Published: *30 July 2021*

Citation:
Jussila J, Edsman L, Maguire I, Diéguez-Uribeondo J and Theissinger K (2021) Money Kills Native Ecosystems: European Crayfish as an Example. Front. Ecol. Evol. 9:648495. doi: 10.3389/fevo.2021.648495

Finally, we summarize main threats and actions needed to protect remaining native freshwater crayfish fauna in Europe.

Keywords: conservation, biased decision making, environmental economics, political ecology, fisheries administration, native and alien crayfish, shortsightedness

INTRODUCTION

The Role of Crayfish in Europe: Ecosystem vs. Economy

Of all ecosystems in the world, freshwaters are among the most diverse and vulnerable, while constituting only 0.8% of the Earth's surface (Strayer and Dudgeon, 2010). Inland waters exhibit a high degree of endemism and extinction rates (Dudgeon et al., 2006) due to their insular nature, frequently small size, and the limited dispersal ability of many freshwater species which commonly results in adaptation to narrow habitat conditions. These characteristics make freshwaters vulnerable to extensive and growing human pressures (Naiman and Turner, 2000; Jackson et al., 2001; Strayer and Dudgeon, 2010). In the past century, the rapid increase of human populations accompanied by economic development resulted in an equally rapid increase in the demand for freshwater provisioning services, such as water for consumptive use, irrigation, power generation or transport as indicated in the Millennium Ecosystem Assessment from 2005. Among many pressures to freshwaters and their rich biodiversity, land-use change, pollution, physical alteration and damming, water abstraction, climate change and introduction of alien species have caused the most severe degradations (Dudgeon et al., 2006; Domisch et al., 2011).

Freshwater biodiversity is significantly influenced by freshwater crayfish, which are keystone species and ecosystem engineers, important components of freshwater food webs, due their relatively large body size, long life span and omnivorous feeding habits (Holdich, 2002; Usio and Townsend, 2008; Weinländer and Füreder, 2016). Owing to these characteristics, crayfish can directly affect ecosystem processes, species abundance and diversity (Pyšek and Richardson, 2010). Thus, crayfish disappearance from an ecosystem can significantly alter freshwater ecosystem processes and services, species abundance and diversity, leading to changes in habitat structure and functioning as well as to watercourse succession and changes in the dynamics of sediment transport (Moorhouse and Macdonald, 2011). Since native freshwater crayfish have a key role in the ecosystem by ensuring its normal functioning, and frequently being economically important, they need to be conserved. In a modern approach to species conservation, apart from habitat conservation, the focus is also on the preservation of species genetic diversity. High genetic diversity enables survival of species through time due to higher adaptive potential and fast evolutionary response to environmental changes (Bickford et al., 2007; Eizaguirre and Baltazar-Soares, 2014).

On the other hand, crayfish are among the most widely translocated aquatic invertebrates, introduced both within and between continents mainly through extensive harvesting for food, aquaculture and aquarium trade (Kouba et al., 2014; Loureiro, 2020) (**Table 1**). Once brought into a new habitat some alien crayfish species frequently become established and invasive. Invasive crayfish species are characterized by advantageous life history traits such as fast growth rate, high fecundity and early maturation (Souty-Grosset et al., 2006), which contribute to their invasive success. Their aggressiveness, e.g., *Pacifastacus leniusculus* (Dana, 1852) (Söderbäck, 1991), *Procambarus clarkii* (Girard, 1852) (Souty-Grosset et al., 2006; Arce and Dieguez-Uribeondo, 2015), enable them to exclude native species in competition for space and food sources (e.g., Söderbäck, 1994a, 1995; Hudina et al., 2014; Pacioglu et al., 2020). Additionally, they may outcompete native species by reproductive interference (Söderbäck, 1994b). Further, their tolerance to a broad range of conditions including pollution or organic enrichment, e.g., *Faxonius limosus* (Rafinesque, 1817) (Souty-Grosset et al., 2006), and transmission of diseases such as crayfish plague, caused by pathogen *Aphanomyces astaci* Schikora (Persson and Söderhäll, 1983; Vey et al., 1983; Diéguez-Uribeondo and Söderhäll, 1993), enhances their potential to drastically affect native crayfish populations. Thus, invasive species are recognized as the second most important factor affecting biodiversity loss worldwide (Lodge et al., 2000; Fahrig, 2003) and determining the factors of their invasive success is a key issue in invasive species management (Capinha et al., 2013) in order to conserve native crayfish populations as well as local biodiversity and ecosystem functioning.

Commercial activity seems to have had a key role in the introduction of alien crayfish and their associated diseases to Europe, and thus to the devastation of the native European crayfish. Crayfish have been traded since the discovery of their worth as food item, either for nutritional purposes as a valuable source of proteins in remote and sometimes poor parts of Europe (Holdich, 2002; Maguire and Gottstein-Matočec, 2004; Gherardi, 2011) and other regions (Andriantsoa et al., 2019), or as a focal point of cultural events (Jussila, 1995; Edsman, 2004; Alonso et al., 2000). They have even been used as food during fasting within religious communities, in order to bypass regulations forbidding the consumption of animals during fasting (e.g., Swahn, 2004; Ackefors, 2005; Patoka et al., 2016b) and also as a source of additional income for people catching and marketing them to bourgeois and religious communities (e.g., Lehtonen, 1975; Bohman et al., 2006; Gherardi, 2011). Trading frequently included crayfish transport over long distances (Anon, 1899; Edsman and Schröder, 2009; Jussila et al., 2013b) resulting in a mix of the natural genetic composition of native species (e.g., Edsman et al., 2002; Makkonen et al., 2015). Moreover, by trading live alien crayfish species within Europe, the spreading of the crayfish plague pathogen *A. astaci* was facilitated around the continent, as crayfish were often placed in water bodies along the way to markets (e.g., Alderman, 1996).

TABLE 1 | Native and alien crayfish in Europe: distribution, introduction motivation and *A. astaci* relationship. Distribution indicates in which European countries species is present (two letter country codes; www.iban.com/country-codes); introduction indicates motivation with AQ, aquaculture; PT, pet trade; WS, wild stock creation; *A. astaci* status is V, vulnerable; C, carrier; n/a, no information on *A. astaci* relation available; background coloration indicative of native species = no color; early introduced aliens = dark gray; late introduced aliens = light gray. The data is based on Kouba et al. (2014) and unless otherwise indicated been updated using data from Invasive Species Compendium website (https://www.cabi.org/isc/search/index?q=crayfish).

Species	Distribution	Introduction[9]	*A. astaci*
Astacus astacus	AL, AD, AT, BY, BE, BA, BG, HR, CY, CZ, DK, GB, EE, FI, FR, DE, GR, HU, IT, LV, LI, LT, LU, MD, ME, NL, NO, PL, RO, RS, RU, SK, SI, SE, CH, UA		C V
Pontastacus leptodactylus	AT, BY, BE, BG, HR, CZ, DE, ES, FI, GB, GR, HU, IT, LV, LT, LU, MT[1], MD, NL, PL, RO, RS, RU, SK, CH, UA		C V
Astacus pachypus	RU, UA		n/a
Austropotamobius pallipes	AT, BA, HR, GB, FR, DE, IE, IT, LI, ME, SI, ES, CH		C V
Austropotamobius torrentium	AL, AT, BA, BG, HR, CZ, FR, DE, HU, IT, LU, MK, ME, RO, RS, SI, SK, ES, CH		V
Austropotamobius bihariensis	RO[16]		
Faxonius limosus	AT, BG[12], BY, BE, HR, CZ, GB, FR, DE, HU, IT, LV, LT, LU, NL, PL, RO, RS, RU, SK, SI[18], ES, CH	WS	C
Pacifastacus leniusculus	AT, BA[15], BE, HR, CY, CZ, DK, GB, EE, FI, FR, DE, GR, HU, IT, LV, LT, LU, MT[1], NL, NO, PL, PT, SK, SI, ES, SE, CH	AQ, WS	C V
Procambarus clarkii	AT, BE[11], CY, GB, FR, DE, HU[2], IT, MT[1], NL, PL[10], PT, ES, CH	AQ, PT, WS	C
Faxonius immunis	FR, DE	AQ, WS	C
Faxonius juvenilis	FR	WS	n/a
Faxonius cf. *virilis*	GB, ME, NL		C
Procambarus cf. *acutus*	BE[12], GB, NL		C
Procambarus virginalis	AT[13], BE[3], CZ[8], DE, EE[17], FR[19], HR[17], HU[2], IT[6], MT[1], NL[17], SE[4], SK, RO[7], UA[5]	PT	C
Procambarus alleni	HU[2]	PT	C
Cherax destructor	ES, IT	AQ, PT, WS	V
Cherax quadricarinatus	ES[14], GB, GR, HU[2], SI, MT[1]	PT, AQ	CV
Cherax holthuisi	HU[2]	PT	n/a
Cherax snowden	HU[2]	PT	n/a
Cherax sp. (2 different)	HU[2]	PT	n/a
Cambarellus patzcuarensis	HU[2]	PT	C

[1]*Deidun et al., 2018*; [2]*Weiperth et al., 2020*; [3]*Scheers et al., 2021*; [4]*Bohman et al., 2013*; [5]*Novitsky and Son, 2016*; [6]*Vojkovská et al., 2014*; [7]*Pârvulescu et al., 2017*; [8]*Patoka et al., 2016a*; [9]*Chucholl, 2015*; [10]*Maciaszek et al., 2019*; [11]*Scheers et al., 2020*; [12]*Todorov et al., 2020*; [13]*Cab Direct UK, 2014*; [14]*Arias Rodríguez and Torralba Burrial, 2021*; [15]*Trožić-Borovac et al., 2019*; [16]*Pârvulescu et al., 2019*; [17]*Vogt, 2020*; [18]*Govedič, 2017*; [19]*Grandjean et al., 2021*.

After struggling for around 100 years with *A. astaci* epidemics, some countries of Northern and Southern Europe, losing more native crayfish stocks and failing with previous introductions of *F. limosus* and *Faxonius virilis* (Hagen, 1870) decided, largely for commercial purposes, that mass introduction of other alien crayfish should start from North America, such as *P. leniusculus* in Sweden 1960s (Svärdson, 1965, 1995; Abrahamsson, 1973), *P. leniusculus* in Finland 1960s (e.g., Westman, 1973), *F. limosus* and *P. leniusculus* in Austria 1960s and 1970s (Spitzy, 1973) and *P. leniusculus* and *P. clarkii* in Spain 1970s (e.g., Diéguez-Uribeondo et al., 1997a). Previous attempts to also introduce other North American species had happened, but not on such a massive scale (Alonso et al., 2000; Souty-Grosset et al., 2006). As mentioned above, the introduction of the alien

P. leniusculus, together with organized hatchery and stockling production, resulted amongst other matters in further waves of *A. astaci* epidemics among native European crayfish stocks. The commercial value of the native crayfish such as *Astacus astacus* (Linnaeus, 1758), *Pontastacus leptodactylus* (Eschscholtz, 1823) and *Austropotamobius pallipes* (Lereboullet, 1858), has partially contributed to protecting their wild stocks via a possibility for their profitable exploitation. When facing extinction or severe decline in Continental Europe and Fennoscandia (*A. astacus*), the Mediterranean countries (*A. pallipes*), Eastern Europe and Turkey (*P. leptodactylus*), their commercial value could be seen as directed against these European native crayfish. This resulted in the potential income from crayfish being used several times, one could say every time, when introductions of alien crayfish were discussed and justified (e.g., Westman, 1973; Söderbäck and Edsman, 1998; Alonso et al., 2000; Sahlin et al., 2010).

Monetary benefits have played a crucial role in the alien crayfish introductions, as there have also been at least temporary economic gains from alien species through aquaculture (e.g., Bohman et al., 2006) and exploiting wild stocks (Jussila and Mannonen, 2004; Bohman et al., 2006). In Fennoscandia, the economic benefits may have initially been obvious (e.g., Kirjavainen and Sipponen, 2004), Spain appears to be the same (e.g., Gutiérrez-Yurrita et al., 2017), while in Central Europe and Balkans the economics gains have been considerably smaller (e.g., Maguire and Gottstein-Matočec, 2004; Souty-Grosset et al., 2006). Even when discussing the initial cumulative benefits, the long term direct and indirect economic gains have so far been negligible or even negative when the whole aquatic ecosystem and society is taken into account (e.g., Gren et al., 2009). In Spain, the Scientific Committee of Spanish Ministry has finally declared that alien *P. leniusculus* and alien *P. clarkii* are not naturalized but invasive and detrimental to local aquatic ecosystems (Díaz, 2021). The entire ecosystem should always be considered when alien species benefits are discussed, even though short-term thinking favors money and ignores intangible benefits.

One crucial issue in the native crayfish conservation has been, and also will be in the future, several contrasting interests promoting either native crayfish conservation or alien species introductions. This issue has been discussed in detail by Biasetti et al. (2021), highlighting the complex network of intangible ideas and values relevant to different interest groups with also strong economic interest involved. Conservation obstinacy has been mentioned as an example of wasting resources to fight lost causes, as conservation outcome could, in some cases, be hard or even impossible to predict (Lehmann et al., 2002; Gontier et al., 2006) while we argue that lost causes can only be species extinctions. Animal welfare issues can also be relevant, since native species might have to be raised under artificial conditions and alien species eradication could be cruel (e.g., Cowan and Warburton, 2011). Finally, social factors of conservation are important and actions would require community acceptance and support with intensive awareness raising campaigns taking place. Ecosystem health or biodiversity as such are complex entities and adding diverse individual attitudes and expectations to considerations when native species conservation acts are planned, the result can easily be counterproductive for native species. The conservation of native crayfish should thus be seen as an attempt to conserve native ecosystem health, as the native crayfish are true keystone species and ecosystem managers in the most positive sense of the phrase.

We have used cases from Fennoscandian policies and practices as an overwhelming example of official management strategies, based often on economical justifications rather than ecological, to highlight the detrimental effects of alien species to native species and ecosystems. In addition, *P. leniusculus* alone is the most widespread alien crayfish species in Europe and is present in at least 28 countries (Kouba et al., 2014; **Table 1**). The magnitude of alien crayfish promotion by governmental institutions has been of such fundamental proportions in Fennoscandia, that several cases have been discussed from a Fennoscandian view point, however, with cases from Continental Europe being introduced. The regions might differ, the species might differ, but the outcome seems to be repeated. Money talks and native European crayfish walks, this time, out. This review on policy and practice is about the dramatic chain of events affecting the fate of European freshwater crayfish.

On the Brink of Extinction: Crayfish Plague Is Wiping Out Native Crayfish Stocks

Over the past 150 years, freshwater crayfish in Europe have faced a severe challenge caused by the pathogen *A. astaci*, probably introduced with alien crayfish species of North American origin, with mass mortalities first reported by Cornalia (1860). Today, the European native crayfish populations are in decline nearing extinction both regionally and species-wise (e.g., Souty-Grosset et al., 2006). Due to its devastating effects on the native crayfish populations of Europe, *A. astaci* is today considered among the world's 100 worst invasive species (Lowe et al., 2004). *A. astaci* belongs to the class of Oomycetes a diverse group of fungus-like organisms, including not only a wide variety of plant and animal pathogens, but also saprophytic species (Diéguez-Uribeondo et al., 2009). *A. astaci* itself is originally a very specific parasite (Unestam, 1969a; Unestam, 1972; Diéguez-Uribeondo et al., 2009) of freshwater crayfish species from North America that have developed some tolerance and resistance whilst also alternative hosts have been reported and speculated (e.g., Unestam and Weiss, 1970; Svoboda et al., 2014; Martín-Torrijos et al., 2021b) (**Table 2**). However, in the European crayfish, the parasite causes a lethal disease known as crayfish plague. The pathogen spreads from host to host by producing free-swimming zoospores; should a suitable host be found, the zoospores then encyst, germinate, and start to grow hyphae into the host tissues, typically resulting in death of the host (Söderhäll and Cerenius, 1999; Cerenius et al., 2009; Rezinciuc et al., 2016). By contrast, in *A. astaci* infected North American crayfish species, there is a continual but low level of sporulation (Strand et al., 2012; Svoboda et al., 2013).

The presence of North American crayfish, and thus most likely the pathogen *A. astaci,* has caused high mortalities and numerous population collapses among all native European crayfish species

TABLE 2 | *Aphanomyces astaci* haplogroups (genotypes) and crayfish carrying these haplotypes as latent infections. Genotypes of *A. astaci* can only be distinguished on a molecular (genetic) level, as no morphological differences exist.

Genotypes			Species dying on crayfish plague outbreaks	Latent infections with genotype characterization	
Haplogroup*	**Haplotype***	**RAPD-PCR based****		**European species**	**North American species**
A (lineage 1)	a	A (As)	*Astacus astacus*[1] *Austropotamobius pallipes*[1] *Austropotamobius torrentium*[1] *Pontastacus leptodactylus*[2]	*Astacus astacus*[5] *Pontastacus leptodactylus*[2] *Austropotamobius torrentium*[9]	*Faxonius rusticus*[6] *Faxonius obscurus*[7] *Faxonius etnieri*[8] *Procambarus hybus*[8] *Procambarus acutus*[8]
		C (PsII)	*Pacifastacus leniusculus*[2]	unknown	
B (lineage 1)	b	B (Psl)	*Pacifastacus leniusculus*[1] *Astacus astacus*[1] *Austropotamobius pallipes*[1] *Austropotamobius torrentium*[1] *Pontastacus leptodactylus*[2]	*Pontastacus leptodactylus*[2]	*Pacifastacus leniusculus*[1]
E (lineage 1)	e	E (Or)	*Astacus astacus*[1] *Austropotamobius torrentium*[4]	Not observed	*Faxonius immunis*[7] *Faxonius limosus*[7]
NEW1 (lineage 1)	Usa-1	Not tested	Not observed	Not observed	*Faxonius tricuspis*[8]
NEW2 (lineage 1)	Usa-2	Not tested	Not observed	Not observed	*Cambarus striatus*[8]
D (lineage 2)	d1	D (Pc)	*Austropotamobius pallipes*[1] *Austropotamobius torrentium*[1]	Not observed	*Cambarellus shufeldtii*[8] *Procambarus clarkii*[1]
	d2		*Austropotamobius pallipes*[1]	Not observed	*Procambarus abiusus*[8] *Procambarus clarkii*[1] *Procambarus fallax*[1]
	d3	Not tested	*Cambaroides japonicus*[3]	Not observed	*Procambarus clarkii*[3]
	Usa-3	Not tested	Not observed	Not observed	*Procambarus raneyi*[8]
	Usa-4	Not tested	Not observed	Not observed	*Faxonius* sp.[8]
	Usa-5	Not tested	Not observed	Not observed	*Procambarus raneyi*[8]
	Usa-6	Not tested	Not observed	Not observed	*Cambarellus shufeldtii*[8] *Procambarus clarkii*[8]

*Based on phylogenetic analyses of mitochondrial DNA regions; **alternative terminology of RAPD-PCR groups; [1]Makkonen et al. (2018); [2]Panteleit et al. (2018); [3]Martín-Torrijos et al. (2018); [4]Mojžišová et al. (2020); [5]Jussila et al. (2021a); [6]Panteleit et al. (2019); [7]Butler et al. (2020); [8]Martín-Torrijos et al. (2021b); [9]Jussila et al. (2017).

(e.g., Souty-Grosset et al., 2006). The invasive North American crayfish species appears to tolerate and resist the crayfish plague infection across their original distribution in North America (e.g., Martín-Torrijos et al., 2021b). This seems to be the result of a balanced relationship arising from coevolution (Unestam, 1969b; Cerenius et al., 2009). Thus, the pathogen was usually unable to freely infect its North American host because their immune system can encase the pathogen within the cuticle (Söderhäll and Cerenius, 1999; Cerenius et al., 2009). However, susceptible crayfish and *A. astaci* seemed to have created a novel and complex relationship, with evidence for a rapid co-evolution of native crayfish and *A. astaci* (Jussila et al., 2015, 2021a). Moreover, the alien crayfish in their newly invaded biogeographic regions in Europe show increased susceptibility with even population collapses reported (e.g., Jussila et al., 2014a; Sandström et al., 2014; Thomas et al., 2020). As a result, among crayfish stocks in Europe, the resistance of both native European and alien crayfish against the crayfish plague has changed, as has the virulence of the disease agent *A. astaci* (e.g., Jussila et al., 2015).

Currently, four distinct haplogroups (i.e., group of strains evolutionarily related as judged by mitochondrial concatenated sequences, *sensu* Makkonen et al., 2018) of *A. astaci* are known to infect native and alien crayfish in Europe and Asia (**Table 2**), named as A, B, D, and E haplogroups (e.g., Kozubíková et al., 2011; Kozubíková-Balcarová et al., 2013; Makkonen et al., 2018; Martín-Torrijos et al., 2018), with haplogroup D consisting of d1, d2, and d3 haplotypes (Makkonen et al., 2018; Martín-Torrijos et al., 2018). These haplogroups and haplotypes can only be distinguished on a genetic level, as no morphological differences exist. Recent studies on *A. astaci* in the south-eastern United States indicate that this region seems to be a center of diversity of the pathogen. In this region, 19 additional North American crayfish species were found to carry *A. astaci* and six new haplotypes of the pathogen were identified

(Martín-Torrijos et al., 2021b). Laboratory infection trials have shown extensive variation in the virulence among different strains of some haplogroups (e.g., Makkonen et al., 2012, 2014; Francesconi et al., 2021). In general, strains of haplogroups B, D and E seem to possess higher virulence (e.g., Makkonen et al., 2012; Jussila et al., 2013a; Francesconi et al., 2021) than those of haplogroup A, which, on the other hand, appear to be more variable in their virulence (Makkonen et al., 2012, 2014). Furthermore, latent crayfish plague infections with *A. astaci* from the haplogroup A, and in some cases also haplogroup B, without mass mortalities have been reported in the native European *A. astacus* (Jussila et al., 2011; Viljamaa-Dirks et al., 2011; Maguire et al., 2016), *P. leptodactylus* (Kokko et al., 2012; Svoboda et al., 2012; Ungureanu et al., 2020), *A. torrentium* (Van Paula Schrank, 1803) (Kušar et al., 2013; Maguire et al., 2016; Jussila et al., 2017) and *A. pallipes* (Manfrin and Pretto, 2014; Maguire et al., 2016) (**Tables 1**, **2**).

Aphanomyces astaci has been under high selective pressure to adapt to the European crayfish hosts and its new environmental conditions since its original introduction to Europe 150 years ago (Jussila et al., 2015, 2016a). After its presumed arrival in Europe in the 1850s (Alderman, 1996), it had access to a variety of host habitats across the European native crayfish spectrum (Souty-Grosset et al., 2006) (**Table 1**). All European crayfish species were susceptible to *A. astaci* of haplogroup A, the first intruder, and the outcome of the crayfish plague epidemic during the first decades was massive mortality among European crayfish populations. If it had not had assistance from humans, the disease might have had a short history in Europe. But the spread of the disease agent was unintentionally aided by transferring it to new water bodies and populations through commercial marketing chains and through natural water ways also along with alien invasive crayfish species (Alderman, 1996). The rapid and efficient spread allowed for both the constant presence of epidemics and opportunities for *A. astaci* to jump from one European crayfish species host to the next and, apparently, to also jump back and forward among crayfish species in close proximity (e.g., Diéguez-Uribeondo, 2006; Jussila et al., 2015; James et al., 2017).

It has long been presumed that *A. astaci* strains of haplogroup A may have been naturally selected by lowered virulence toward a balanced host-pathogen relationship, which has been demonstrated experimentally only during the last decade (e.g., Makkonen et al., 2014; Jussila et al., 2014b; Francesconi et al., 2021). Some wild native European and Turkish crayfish stocks, which are viable and producing commercial catches, have been shown to be latent carriers of *A. astaci* (e.g., Svoboda et al., 2012; Kokko et al., 2018; Jussila et al., 2021a). Laboratory scale infection studies have revealed significant virulence differences among and within *A. astaci* haplogroups and even the existence of very low virulent strains (e.g., Makkonen et al., 2012; Jussila et al., 2017; Francesconi et al., 2021). The invasive crayfish, especially *P. leniusculus*, have lately been shown to be susceptible to *A. astaci*, which points to the high virulence of the infecting *A. astaci* haplogroup B and possibly a lowered resistance of *P. leniusculus* toward *A. astaci* (Aydin et al., 2014; Jussila et al., 2014a; Thomas et al., 2020). Laboratory experiments have demonstrated that the *A. astaci* of haplogroup B, although highly virulent, is also capable of exhibiting a significant but narrow range of virulence variation (Jussila et al., 2013a). This indicates that even *A. astaci* of haplogroup B could be adapting in Europe (Ungureanu et al., 2020), while the presence of a permanent host habitat for the *A. astaci* of haplogroup B allows for the maintenance of high virulence without the immediate threat of the parasite's evolutionary suicide due to the outbreak of a devastating crayfish plague epidemic (Jussila et al., 2015). A similar trend has been observed in *A. astaci* of haplogroup D currently infecting some *Procambarus* species (e.g., Martín-Torrijos et al., 2017; Makkonen et al., 2018). Furthermore, some strains of this haplogroup have shown adaptation to warmer temperatures than other strains from other haplogroups (Diéguez-Uribeondo et al., 1995; Rezinciuc et al., 2014) and tolerance to brackish waters (Martín-Torrijos et al., 2021b). These evolutionary adaptations might be regional between specific populations of crayfish and *A. astaci,* as a consequence of host-parasite co-evolution (e.g., Svoboda et al., 2012; Makkonen et al., 2014; Jussila et al., 2017). It has recently been shown that the *A. astaci* strain carried by *Faxonius rusticus* (Girard, 1852) is genetically different from all other *A. astaci* strains described to date (Panteleit et al., 2019), leading the authors to hypothesize that each North American crayfish species might carry its own *A. astaci* haplogroup, or haplotype, to which it evolved resistance against. In other words, there might be as many strains of *A. astaci* as there are different species of crayfish in North America; over 300 (Mathews et al., 2008). If the mode of action and virulence of *A. astaci* depends on the different host species or populations, this could have a high impact on the invasion success of the invasive species. However, recent studies in North America by Martín-Torrijos et al. (2021b) do not seem to support this hypothesis since no clear species-specific or distributional patterns of the haplotypes and crayfish species were found. Further investigations are needed to enhance our understanding on the phylogeography of *A. astaci*.

The introduction of different *A. astaci* haplogroups into Europe and the repeated introductions of its chronically infected hosts are a classic example of a man-made ecological disaster, stemming from the naive belief that the manipulation of an ecosystem would be straightforward. Currently, the native European crayfish species are on the brink of extinction (Richman et al., 2015). *A. astaci* itself has apparently adapted rather well to European conditions (Jussila et al., 2016a), and seems to be currently co-evolving while maintaining contact with its relatively resistant hosts as new crayfish stocks were imported from North American into Europe (e.g., Jussila et al., 2016a). One could predict that this will inevitably lead to possible total eradication of the remaining native European crayfish stocks. The original introduction of *A. astaci* to Europe, though it was most probably purely accidental, has not only seriously devastated native crayfish populations throughout Europe, but also resulted in further damage due to misguided management attempts such as further introductions of alien crayfish species from North America to rectify the

situation (Souty-Grosset et al., 2006; Jussila et al., 2015, 2016a).

ASSESSMENT OF POLICY AND PRACTICE

Human Impact on the Struggle Between Native and Alien Crayfish

The high financial and cultural values of the freshwater crayfish in Europe, namely *A. pallipes*, *A. astacus,* and *P. leptodactylus*, and the devastation of the native crayfish stocks during 19th and 20th century (Alderman, 1996; Alonso et al., 2000; Jussila et al., 2016a) encouraged fisheries officers and researchers in several European countries to grasp the opportunity to introduce alien freshwater crayfish into Europe (Lodge et al., 2000; Holdich et al., 2009). During the first wave of the crayfish plague epidemics in Europe from the 1860s onward, the cause of mass mortalities among European crayfish populations was not known to the scientific community, not to mention administrators or the common public. It took until the 1930s to discover that *A. astaci* was the organism causing crayfish plague epidemics (Schikora, 1903, 1906; Schäperclaus, 1935; Nybelin, 1936), while it took much longer to fully understand how to halt its spread. Within 50 years from arrival, the crayfish plague epidemic's first wave had permanently changed European aquatic ecosystems by almost wiping out native crayfish from Continental Europe (Jussila et al., 2015) and collapsing native crayfish stocks in Fennoscandia (Jussila and Mannonen, 2004; Bohman et al., 2006; Jussila et al., 2015). Within one century, crayfish plague epidemics were even reported from the Iberian Peninsula to the United Kingdom and Ireland, which were all but the last safe havens for the native European crayfish (Reynolds, 1988; Holdich and Reeve, 1991; Diéguez-Uribeondo et al., 1997a, respectively). Even though in some parts of south-eastern Europe alien crayfish were introduced quite early, e.g., in Greece (Perdikaris et al., 2017), other parts of south-eastern Europe resisted alien crayfish introductions for a long time compared to the rest of Europe (Holdich, 2002). The first record of *F. limosus* in freshwaters of Croatia was in 2003 (Maguire and Gottstein-Matočec, 2004), while *P. leniusculus* was first recorded in 2008 (Maguire et al., 2008). In both cases, alien crayfish species spread naturally through big rivers (Danube and Mura, respectively) from neighboring countries (Hungary and Slovenia, respectively) and continued their expansion toward east (i.e., *F. limosus* to Serbia and Romania) causing irreversible negative impact onto the native astacofauna in the region, i.e., in the south-eastern Europe. It was only in 2011 that *P. leniusculus* was illegally introduced to the Korana River (Hudina et al., 2013) situated in the continental part of Croatia in the karstic region that is known as a hotspot of the *A. torrentium* and *A. astacus* diversity (Klobučar et al., 2013; Lovrenčić et al., 2020; Gross et al., 2021), inflicting great damage to the populations of these vulnerable species distributed in this area.

A common mistake made whenever a large-scale epidemic is happening is the open spreading of the disease agent and organisms that are carrying the disease (Alonso et al., 2000; Bohman et al., 2006; Diéguez-Uribeondo, 2006). In most cases, it can be claimed that due to the lack of prior knowledge, it was not possible to apply strategies or tactics that would have prevented the disease spreading and resulting wide epidemic. When the pandemic started in the late 19th century, the cause of the mass mortalities among native European crayfish populations was not known, while several alternative theories were discussed (Alderman, 1996). Some of those were based on the actual disease agent being responsible, but even then there did not seem to be rational strategies implemented to save the valuable natural resource of European freshwater crayfish (e.g., Fiskeriverket, 1993; Alderman, 1996; Jussila et al., 2016a). The Europeans were caught by surprise regarding *A. astaci,* and proper means of attempting to stop the spreading of *A. astaci* happened only after the whole Continental Europe and large parts Fennoscandia were hit by crayfish plague epidemics.

Repeated introductions of the disease agent carriers, i.e., alien crayfish of North American origin, but also pathogens spreading through natural waterways, made matters even more complicated, as eradication of the permanent disease carriers became impossible and prevention of spreading very hard (e.g., Alonso et al., 2000; Pârvulescu et al., 2012; Peay et al., 2019). Europeans have imported alien crayfish for various reasons from North America since the late 19th century (Henttonen and Huner, 1999; Alonso et al., 2000; Souty-Grosset et al., 2006), but the prime mistake was made during the mid-20th century when decisions to start mass introductions of alien *P. leniusculus* to fill the now mostly empty freshwater courses in Europe were made. The possibility of introducing novel diseases, e.g., *Psorospermium haeckeli*, *Saprolegnia* spp. (Diéguez-Uribeondo and Söderhäll, 1993; Diéguez-Uribeondo et al., 1994), even the possibility of introducing novel strains of *A. astaci,* were played down (e.g., Westman, 1973), while the Swedes had their concerns (e.g., Svärdson, 1995). The decision to introduce crayfish from the region where the crayfish plague disease originated, the disease that had already eradicated most European crayfish stocks, was made (e.g., Kilpinen, 2003). Several North America species have been introduced, but the main focus was on *P. leniusculus* (Abrahamsson, 1973; Souty-Grosset et al., 2006; Holdich et al., 2009). The deliberate large-scale introduction of *P. leniusculus* in Sweden starting in the 1960s resulted in a fivefold increase in the spread of crayfish plague epidemics in the country (Bohman et al., 2006; Bohman, 2020).

It is difficult to appreciate how this decision was made and justified from an ecologically perspective, mainly because it was obvious that the introduction of *P. leniusculus* would result in further spreading of *A. astaci* in Europe (e.g., Kilpinen, 2003). Field introductions during the period of 1960–1967, thus before the massive importation of *P. leniusculus* to Fennoscandia, indicated that *P. leniusculus* was a carrier of *A. astaci* and thus eradicated coexisting *A. astacus* populations (Unestam, 1969b). The import license application of *P. leniusculus* to Finland was first rejected in 1967 by the veterinary administration due to risks of introducing diseases, namely crayfish plague (Kilpinen, 2003). Later the same year, the veterinary administration was bypassed and an import license granted using political maneuvers

and ignoring the risks. Unestam (1969a) already discussed *P. leniusculus* resistance against *A. astaci,* clearly indicating that the possibility of *P. leniusculus* acting as a permanent reservoir existed, yet permits were given to stock 58,100 adult *P. leniusculus* from Lake Tahoe, California, into 64 natural waters in Sweden in 1969 (Svärdson, 1968; Abrahamsson, 1973). Also, alien *P. leniusculus* was introduced as resistant to *A. astaci*, even immune, while the obvious outcome from this resistance, i.e., *P. leniusculus* as a spreading vector for *A. astaci*, was largely ignored.

Promotion of the Harmful Alien Species Is Not Conservation of the Native Species: Cases From Fennoscandia

Soon after the introduction of *P. leniusculus* to Fennoscandia, it was discovered that even the most sophisticated stockling production systems might produce *P. leniusculus* that were carriers of *A. astaci* (e.g., Makkonen et al., 2010), while some *P. leniusculus* stocks remained in disease-free status for a while once established from farm raised stocklings. This did not halt the stocking of the disease carrying *P. leniusculus* into numerous water bodies, even though there were strict national regulations banning introductions of diseased organisms into the wild (e.g., Fiskeriverket, 1993; Ruokonen et al., 2018; Jussila and Edsman, 2020): a case of fisheries administration favoring the distribution of an alien species even if it was suspected or known to carry and spread *A. astaci* (e.g., Kilpinen, 2003). The relaxed attitude among fisheries administrators, even an attitude that favors alien species over native ones (e.g., Ruokonen et al., 2018; Jussila and Edsman, 2020), is bound to cause devastation of the native crayfish, even when it is already claimed to be vulnerable and threatened, as has been the case in Sweden (Bohman and Edsman, 2011).

Initial optimistic assumptions, based sometimes on biased analyses and even wishful thinking, can be hard to correct when new scientific information surfaces. It was discovered some 20 years after the initiation of major alien crayfish stocking campaigns that the *P. leniusculus* wild stocks were declining, even collapsing in some cases (Jussila et al., 2014a, 2016b; Sandström et al., 2014). Sometimes this information was ignored and those managing the wild *P. leniusculus* stocks were kept in the dark, which was evident during meetings with stake holders in Finland and Sweden. The initial inflated information regarding the resistance of *P. leniusculus* against *A. astaci* infection was not corrected when the first stock collapses were observed and reported (e.g., Jussila et al., 2014a; Sandström et al., 2014) and local fisheries managers were wondering how the collapses were even possible. The original assumption of disease resistance was used to justify further spreading of the alien crayfish, even after it was discovered that it was spreading very virulent *A. astaci* of haplogroup B and it was quite obvious that it too was suffering from the infection itself (e.g., Aydin et al., 2014; Jussila et al., 2014a).

It is common to deprecate novel findings indicating that an alien harmful species could have developed new diseases under the new environmental conditions. In Fennoscandia, it was discovered some 20 years after the intensive *P. leniusculus* stocking program that established *P. leniusculus* stocks were showing gross symptoms that were then studied and described as eroded swimmeret syndrome, i.e., ESS (Sandström et al., 2014; Edsman et al., 2015). The suspected disease causes total or partial erosion of the female swimmerets and thus prevents the female from hatching eggs, resulting in reproductive failures. Later it was been discovered that male *P. leniusculus* also show similar gross symptoms including gonopod trauma (Jussila et al., 2016b, 2021b). It took a while before the existence of ESS was admitted to affect populations of *P. leniusculus* in Finland. Even then, the response was based on undermining the possible population level effects of ESS. This was motivated by trying to maintain the suspected good reputation of *P. leniusculus* in Fennoscandia (e.g., Jussila and Edsman, 2020). Despite struggling in some parts of Europe, alien *P. leniusculus* is still the second worst alien crayfish in Europe and still spreading (**Table 1**).

Short-term monetary benefits are sometimes regarded as more valuable than long-term ecological sustainability, and thus the promotion of the alien species could be justified by economic reasons. The designated area for the introductions of the alien *P. leniusculus* in Sweden was originally limited to the south-eastern part of the country (Bohman et al., 2006). The designated area in Finland was originally only the great lakes in southern Finland, too (Ruokonen et al., 2018). Due to intensive promotion of *P. leniusculus* as a commercially lucrative species and initial good development of the introduced stocks (e.g., Ackefors, 1999; Kirjavainen and Sipponen, 2004), the illegal introductions of *P. leniusculus* northwards were commonplace (Bohman et al., 2006; Bohman and Edsman, 2011; Ruokonen et al., 2018). In Finland, fisheries administration, instead of taking a firm stand against illegal introductions, drafted several crayfishery strategies, which all included the regions of illegal introductions within the newly designated region for *P. leniusculus* (Ruokonen et al., 2018). This only encouraged the spread of the alien crayfish and *A. astaci* it is carrying, resulting in further devastation of the remaining native *A. astacus* stocks.

Emphasizing the alien species' economic benefits could sideline conservation attempts of the native species. The start of the crayfish season is one of the widely publicized events in Fennoscandian late summer (Taugbøl et al., 2004; Jussila et al., 2015; Jussila and Edsman, 2020). The premium price for the crayfish is paid during the first few days of the crayfishing season due to high demand, while the prices stay considerably high throughout the season (Jussila, 1995). To boost the start of the crayfish season trade, the ministry in charge of fisheries arranged an importation of alien *P. leniusculus* from England to Finland, an exception to the EU Alien Species Regulation 1143/2014, using an economic justification. This is again an example of trying to boost the economic reputation of the alien species and thus undermine the fundamentals of the EU Alien Species Regulation 1143/2014.

The strategy for alien species eradication can be deliberately and erroneously implemented to actually give an upper hand to the alien harmful species over the native species. Eradication of the harmful alien *P. leniusculus* is one of the EU Alien Species Regulation No 1143/2014 aims and there have been national strategies drafted and also implemented to test different strategies and techniques (Edsman and Schröder, 2009;

Bohman and Edsman, 2013; Huusela-Veistola et al., 2019). In Finland, the National Research Institute for Natural Resources, LUKE, has been asked by Ministry of Agriculture and Forestry to assess the possible gains on relaxing *P. leniusculus* trapping regulations, such as open or early season, no bag limits, no specific trapping licenses, encouragement to removal trapping, etc. (Anon, 2019). The official justification is that the relaxed trapping regulations would encourage recreational trappers to increase their trapping pressure, which would then result in halting the spread, or even cause eradication, of the wild *P. leniusculus* stocks. While in theory this might seem achievable, the reality is different. Anyone understanding motivational aspects of the crayfish trapping would claim that recreational trappers would stop trapping when the catch per unit effort (CPUE) falls below a certain limit, for example 0.5, which is well above any rational CPUE that would result in eradication of the population. The planned relaxation of *P. leniusculus* trapping regulations would make the alien harmful *P. leniusculus* a more tempting target than *A. astacus* for a recreational crayfish trapper as the alien species' stocks would then be easier to access. This would only encourage the general public to spread the alien harmful species even more and it would also give a hidden message of the alien harmful species actually being more desirable than the native species. At four international freshwater crayfish scientific conferences, conclusions and resolutions have been issued: IAA17 in Kuopio, Finland (2008), Crayfish conservation meeting in Olot, Spain (2015), IAA21 in Madrid, Spain (2016) and IAA Gotland, Sweden (2019). At all four meetings it was clearly stated that "*the control of invasive crayfish species by intensive recreational and commercial fisheries does not represent a feasible method for this purpose. Instead, it favors the further spread and increase of these alien populations*" (Furse, 2008; Edsman et al., 2019).

Pet Trade Causing Problems as Means to Spread Alien Crayfish Species and Their Diseases

The ornamental aquatic pet trade is another important but frequently overlooked pathway for the introduction of alien crayfish species and their diseases into Europe (Hänfling et al., 2011; Chucholl and Wendler, 2017). While there are numerous parasitic organisms and viral diseases in crustaceans worldwide (Bojko et al., 2020), some of which might become a serious threat for crayfish in the future, the main disease threatening crayfish in Europe to date is the crayfish plague. Crayfish imported from North America to be sold as pets or kept in private aquaria are often vectors of the crayfish plague pathogen *A. astaci*. In a study by Mrugała et al. (2014) six crayfish species were identified as vectors for the first time, with horizontal transmission of *A. astaci,* i.e., the transmission of the pathogen between crayfish individuals kept in close proximity. These results were confirmed in a study by Panteleit et al. (2017), where a further nine crayfish species were identified as vectors for *A. astaci* for the first time. One of the most problematic crayfish in the pet trade is probably the marbled crayfish, *P. virginalis* Lyko, 2017, due to its parthenogenetic reproduction and its high popularity as a pet (Patoka et al., 2014). It probably evolved from *Procambarus fallax* Hagen, 1870, an American species native to Florida and South Georgia, after triploidization in the German pet trade in the mid 1990s (Vogt et al., 2018). The species was first recorded in the wild in Germany in 2003 (Marten et al., 2004) and has since established numerous populations in at least 17 countries worldwide, mainly in Europe[1] (Vogt, 2020; Scheers et al., 2021) (**Table 1**). However, European mean water temperatures are widely below the optimum for reproduction of *P. virginalis* (Seitz et al., 2005). Consequently it has been suggested that when new *P. virginalis* populations become established, it is to some extent due to the invasive potential of this species, but the location of the occurrences is rather dependent on human-mediated releases (Martin et al., 2010). The pet trade presumably led to the introduction of *P. virginalis* into Sweden, Romania, Ukraine, and many other countries (Marten et al., 2004; Chucholl and Pfeiffer, 2010; Bohman et al., 2013; Novitsky and Son, 2016; Weiperth et al., 2020), and the number of different alien crayfish species could be expected to increase as the pet trade through different channels, e.g., on-line trade, will develop (Kotovska et al., 2016; Vodovsky et al., 2017; Weiperth et al., 2020).

Some countries in Europe have stricter rules for pet trade. Examples are Ireland and Scotland, where keeping alien crayfish is illegal and the crayfish pet trade is strictly regulated (Peay, 2009), yet *P. virginalis* is still for sale on the pet market in Ireland (Faulkes, 2015a). Another example is Sweden where all importation, transport and keeping of any live crayfish species from abroad is banned (Edsman, 2004). Laws and regulations can only be effective if they are also enforced (Faulkes, 2015b). When this is not possible, other methods (e.g., education of pet traders and pet owners) to reduce the negative effects of the pet trade need to be implemented. Recognizing threats that alien species and pet trade pose to native European biodiversity, the EU recently adopted regulations dealing with alien invasive species in Europe, including crayfish (EU Pet Trade Regulation No 2016/1141). However, the list of species of Union concern includes only five alien crayfish species which already have established viable populations in Europe, namely *F. limosus*, *F. virilis*, *P. leniusculus*, and *P. virginalis*. This list does not include species that are imported through international pet trade, but are not yet invasive or established in the European aquatic ecosystems. It is very important, that species which are known to have a high invasive potential or species which are known carriers of *A. astaci*, are added to the invasive species list or, alternatively, to prepare a white list of crayfish that would not present concern to European freshwaters and native astacofauna, supported by scientific evidence.

Twisting the Definitions and Creating New Language to Cause Confusion

One common way to cause confusion among the general public and thus also those pondering conservation issues, is a deliberate erroneous usage of alien species arguments when discussing native species (e.g., Courtine and Willett, 1986; Clavero et al., 2016). The concept of an alien species, in the case of Finland,

[1]https://faculty.utrgv.edu/zen.faulkes/marmorkrebs/

is that the species has spread to Finland later than 1850 and the spreading has been assisted by man (Niemivuo-Lahti, 2012). If arrived later than 1850 by natural spreading to Finland, the species is considered a newcomer, but not an alien species. In the EU Alien Species Regulation No 1143/2014, for matters to be simpler and easier to define, whole nations or geographical regions, such as peninsulas, are considered one entity with regard to alien species spreading. Thus, *A. astacus* in Finland, even though originally considered as a southern species in its spreading (Lehtonen, 1975), is regarded a native species in all regions within Finland. Regardless, to improve the status of alien *P. leniusculus* and to weaken the status of native *A. astacus*, there have been claims that native *A. astacus* is actually an alien species above Jyväskylä (latitude 62°14′), according to information regarding its distribution during early days (e.g., Lehtonen, 1975). In the same way, claims have been made that *A. astacus* was introduced into Sweden during the 1500s by the kings. Later genetic studies have shown that *A. astacus* has inhabited Swedish aquatic ecosystems since the last ice age, thus being native to Sweden (Edsman et al., 2002; Gross et al., 2013; Dannewitz et al., 2021). Similarly, *A. pallipes* has been claimed to have been introduced to Spain from Italy only in the 1500s (Clavero et al., 2016), despite genetic evidence dating the species origin on the Iberian Peninsula from the last glaciation (Matallanas et al., 2011, 2016; Jelić et al., 2016; Martín-Torrijos et al., 2021a). Such scenarios, of course, create confusion and employ everyone to waste resources in order to correct the deliberate misinterpretation in any given case.

Another twisted argument in favor of alien invasive species is the claim that introduced North American crayfish species *P. leniusculus, P. clarkii*, or *F. limosus* are immune against *A. astaci* infection, a definition that is commonly used when justifying the introduction and spreading for example alien *P. leniusculus* in Europe (e.g., Bohman et al., 2006; Jussila et al., 2015). However, it has been shown directly in laboratory tests (Unestam and Weiss, 1970; Persson and Söderhäll, 1983; Vey et al., 1983) and indirectly from wild stock observations, that these alien species, particularly *P. leniusculus,* can be quite often susceptible to *A. astaci* infection and stock collapses due the crayfish plague epidemics have been reported (Jussila et al., 2014a; Sandström et al., 2014; Martín-Torrijos et al., 2019; Thomas et al., 2020). This, again, is one example on how decision makers aim to justify their considerations of the first alien species introductions, and how difficult it is to get the novel message of state of the art facts recognized, even though being supported by reliable data. The debates regarding alien *P. leniusculus* spreading *A. astaci* and thus devastating native crayfish populations, quite often bring up claims that not all alien signal stocks or individuals are chronically infected with *A. astaci,* which also neglects the long term competitive displacement of native species by alien *P. leniusculus*. This argument of exceptions to the rule, i.e., alien *P. leniusculus* being most often infected with *A. astaci*, should bring up the magnitude of the risks when attempting to spread alien *P. leniusculus*, but in most cases a principle of cautious approach is ignored, leading to actions detrimental to native crayfish. The possibility of a favored outcome, a false positive expectation, seems to be a strong motivator sidelining serious ecological considerations and cautious approach.

Means to Make Things Right, Only Too Little and Too Late

Eradication of the Alien Species: Another Disaster Waiting Due to no Proper Eradication Means

Alien species eradication is a very complex task, especially for aquatic species, and thus bound to cause problematic situations. Risks are often unknown both because little data is available on the magnitude of the introduction of alien species and also uncertainty about positive and negative effects of potential measures and actions to be undertaken. Under severe uncertainty about knowledge and value ambiguity in management objectives, the best initial step would be to perform a robust decision analyses (Sahlin et al., 2021).

The eradication of *P. leniusculus* from limited water bodies, such as golf courses or irrigation ponds, has been tried and shown to be successful (Peay et al., 2006; Sandodden, 2019). Successful attempts have also been reported for the eradication of *P. clarkii* and Australian *Cherax destructor* (Clark, 1936) in Spain (Alcorlo and Diéguez Uribeondo, 2014). Biocides, even though discussed controversially, have been used efficiently in the United Kingdom, Sweden, Norway, and Spain to tackle alien crayfish introductions (Peay et al., 2006, 2019; our personal observation). In the Italian project RARITY different approaches (e.g., removal by trapping, pheromones, sterilization of males) have been applied simultaneously, resulting in temporary significant reduction in the *P. clarkii* population size in Italy (RARITY, 2020). Other methods that were applied with more or less success include electrofishing, manual removal of crayfish and introduction of predators or specific diseases into the system, building physical barriers, water body drainage and shock liming (Gherardi et al., 2011; Stebbing et al., 2014; Bohman and Edsman, 2013). A recent study (Krieg et al., 2020) showed that implementation of different drastic measures, e.g., drainage of water body in combination with chemicals or barriers, could reduce alien crayfish population size or even eradicate a whole population. On the other hand, controlling invasive crayfish in big rivers (e.g., Danube and Drava) is almost impossible, and mechanical removal from the water body could only slow down their dispersal (Hudina et al., 2017; Krieg and Zenker, 2020). Still, achievable strategies for alien crayfish management in such systems include a combination of methods that would increase ecosystem resilience and continuous crayfish trapping (both fishermen and authorities) as well as involvement of well-informed citizens, as shown in the study of Lemmers et al. (2021). Also, the application of biological and ecological data on invasive crayfish to develop new tailored approaches to the management of specific invasive populations may improve invasive crayfish control (Hudina et al., 2016).

When eradication attempts are planned in habitats common especially in Fennoscandia, but also elsewhere in Europe, where lakes are interconnected by rivers to form watercourses

stretching hundreds of kilometers, it is soon realized that effective eradication is impossible, or the vanishing of the alien harmful *P. leniusculus* could be an indication of drastic changes in the aquatic ecosystem. In this case, the alien species could be of least concern. Even though roughly 10% of Finnish surface area is freshwater (MMM, 2020), there have been plans for eradication or limiting the spreading of the harmful alien species (Erkamo et al., 2019). However, once *P. leniusculus* is released into the aquatic ecosystem there are very limited possibilities for its practical and effective eradication. In addition to the large size of the watercourses in Finland, most of them are shallow, lacelike structures, allowing basically their whole benthic area for crayfish settling.

The release of diseases targeting alien species has been widely suggested and even used in some cases (e.g., McColl et al., 2018; Wells et al., 2018). They have been shown to function as planned initially in some cases (e.g., Saunders et al., 2010), while some have faced problems right from the start (e.g., Holden, 1995). Pathogens are known to have evolved into diseases targeting different species, as is the case with *A. astaci* (e.g., Jussila et al., 2015; Simmonds et al., 2019). In Finland, the authority responsible of veterinary issues was planning to eradicate a sparse *A. astacus* population, a protected species, suspected to be carriers of *A. astaci,* by using another virulent haplogroup B strain of *A. astaci.* Once the suspected remaining *A. astacus* would have been eradicated, it would have allowed reintroduction of healthy stock into those water bodies. The scheme was introduced in a research proposal and later discussed in a conference in Olot (Girona, Spain, 2015), with a lively interaction between audience and presenter. Luckily, the project was not funded. The idea of fixing an obvious mistake, in this case the introduction of *A. astaci* to Europe several times, by making another, known to be a potential mistake from experience and reports, is a strange human urge.

Exploitation of the Alien Species: Another Form of Alien Species Promotion?

One of the main driving forces behind the spreading of the alien crayfish species and devastation of the native European species has been ongoing exploitation of those alien species stocks that have been illegally introduced to regions which have specifically been allocated for the native species. This has been a common phenomenon in Finland, Sweden, and Spain (Alonso et al., 2000; Sahlin et al., 2017; Ruokonen et al., 2018). Thus, even though there have been attempts to halt the spreading of the alien crayfish species, the actions of the fisheries administrations in all three countries have actually encouraged the spreading of the alien crayfish (e.g., Alonso et al., 2000; Bohman et al., 2006; Ruokonen et al., 2018), despite national laws banning these introductions for various reasons, mostly motivated by conservation (e.g., Edsman and Schröder, 2009; Caffrey et al., 2014; Erkamo et al., 2019) and legislation (e.g., Jussila and Edsman, 2020). In Finland, a partial motivation must have been the alien *P. leniusculus* population crashes (e.g., Jussila et al., 2014a), resulting in the urge to push up the alien *P. leniusculus* catch figures, all in promotion of the alien *P. leniusculus* and at the cost of native *A. astacus*. It should clearly be mentioned here that although population density can be thinned and their spreading slowed down through intense trapping (e.g., Hein et al., 2007), there are no examples of successful eradication of crayfish populations by strong trapping pressure. Intensive fishing with baited traps as well as hand searching and removal is highly unsuccessful since only a minimal fraction of the total population is removed (Chadwick et al., 2020; Krieg et al., 2020). On the contrary, intensive trapping as a control measure may rather be a potential damaging activity by limiting cannibalistic predation pressure on the remaining population (Houghton et al., 2017), increasing fitness in remaining individuals (Moorhouse and Macdonald, 2011), inducing early onset of sexual maturity (Holdich et al., 2014), increasing intentional anthropogenic spread (Edsman, 2004) and by increasing bycatch of non-target species (De Palma-Dow et al., 2020).

The promotion of the alien *P. leniusculus* in Finland has been taken to the level of selecting a Crayfish King annually, namely a person who has done the most to promote alien *P. leniusculus* in Pirkanmaa county in southern Finland and thus causing the most damage to native *A. astacus* in that region, the latter normally not mentioned in this context. The nomination gets wide media coverage, not least because quite often the award is handed over by a minister in charge of fisheries and thus also crayfisheries. This minister should also be in charge of protecting native aquatic resources, such as fish and crayfish, but quite ironically does not see any conflict here.

The Conservation of the Native Species Is a Challenging Task

Timescale Creates Problems: Humans Short-Term Planning

Our attempts to solve ecological problems tend to be based on short-term thinking. While timeframes of decades or even centuries are required to remedy some matters, the lack of immediate personal or corporate benefit (e.g., Wu et al., 2017) and higher levels of uncertainty are seen as difficult to justify. Looking for the quick fix might be practical when trying to show benefits to the general public, but from the natural ecosystem's viewpoint this time frame is negligible. The idea to compensate for the declining native natural resources by introducing alien species while there are still native specimens left is bizarre. On the other hand, it is quite understandable that the time scale of human thinking is rather short and quite often does not stretch over generations, not to mention over decades or millennia. From nature's viewpoint, thousand years is not a long time frame and is in many cases not even long enough for any kind of drastic evolutionary changes. Animal species are normally spreading with variable pace (e.g., Messager and Olden, 2018; Melotto et al., 2020), while introductions of alien crayfish species have happened via quick and violent moments, in the false belief that human actions do not result in negative changes within ecosystems.

How does the obvious human selfishness affect decision making? Are we bound to only look for solutions which allow us to reap the glory for ourselves? Are the decisions actually based on selfish gains (like, e.g., political votes) instead of trying to actually solve the problems in the long run and maintain

rational balance for the foreseeable future? Quite often the best solution would have been to do nothing and let solutions be based on natural progression of matters, even though, in the case of European crayfish, that would have taken a very long time for them to possibly bounce back. At least in Finland and Spain, there are now obvious indications that the native crayfish could be recovering in some water bodies previously considered void of native species (personal observation from Finland and Spain; Diéguez-Uribeondo et al., 1997a; Jussila et al., 2016b; Martín-Torrijos et al., 2017). This often happened in Sweden before the introduction of *P. leniusculus*, and later more frequently, as a result of liming in acidified waters (Bohman, 2020). It is also rather common that after alien *P. leniusculus* introductions, there is a short period when also *A. astacus* turns up in trap catches (e.g., Jussila et al., 2016b; Bohman, 2020), even for several years (e.g., Westman and Savolainen, 2001; Westman et al., 2002), while even in these reported Finnish cases of co-existence *A. astacus* have since disappeared (Erkamo, 2020). Thus, *A. astacus* has been in these water bodies, though at such low densities that trapping them has not been worth the effort. However, *A. astacus* must have been waiting for the moment to bounce back and take a stronger position in the aquatic ecosystem. After the hasty introduction of the alien *P. leniusculus*, *A. astacus* faces little or no chance to recover in the long run. It would have been wiser to wait.

Predicting the outcome of an alien species introduction is quite often made too soon, before it has taken its niche properly, leading to false promotion of the alien species role in the aquatic ecosystem (e.g., Kirjavainen and Westman, 1999; Kirjavainen and Sipponen, 2004; Jussila et al., 2016b). In Fennoscandia and Spain, the promotion of the alien *P. leniusculus* (and also *P. clarkii* in Spain) was based on the period when it was only settling down to aquatic ecosystem and was not properly established yet: populations were growing, there was a lot of free habitat, plenty of resources and as a result stress levels were low. This resulted in rapid spreading of these alien crayfish and the virulent *A. astaci* strains they have been carrying (Diéguez-Uribeondo et al., 1997b; Bohman et al., 2006; Ruokonen et al., 2018; Martín-Torrijos et al., 2019). In Fennoscandia and Spain, only 20 years after introduction, the alien *P. leniusculus* stocks were showing signs of maladaptation (Jussila et al., 2014a, 2016b; Sandström et al., 2014; Larumbe, 2020) and warning signs of not being quite suitable to conditions in their novel habitat. From the native European crayfish perspective this was too late, while this still was only early stages when the spreading of the alien *P. leniusculus* is considered. It is hard to change the positive message later, even though it is obvious that alien *P. leniusculus* stocks are not performing as originally told, especially since the alien *P. leniusculus* promoters do not change their story but largely ignore the bad news.

Money Creates Problems: Human Monetary Thinking

The urge to plan and introduce alien species to new regions has long been a temptation. The fundamental question is why alien species introductions are more tempting than conservation of native species. The already known to be flawed justifications are repeated in order to hide the quite obvious indications of introduction failures and severe negative impact on native species (e.g., Lodge et al., 2000; Westman, 2002; Jussila and Edsman, 2020). In Finland, the catch of the *P. leniusculus* was predicted to double every year due to an increasing number of introduced alien *P. leniusculus* populations being established and starting to produce commercial size crayfish after the early 1990s (e.g., Kirjavainen and Sipponen, 2004; Jussila and Edsman, 2020). This prediction was made early into the introduction scheme, mainly to encourage those managing wild crayfish stocks to introduce alien *P. leniusculus* instead of native *A. astacus*. An annual doubling of catches would have easily resulted in some 30,000,000,000 alien *P. leniusculus* been caught by 2010, which is not exactly what happened, since annual *P. leniusculus* catch briefly peaked at 7 million in mid 2010s and then leveled at around 3 million (Erkamo, 2019). Instead, alien *P. leniusculus* was discovered to be suffering similar population collapses during 1990s and 2000s as *A. astacus* in the past (Jussila et al., 2014a; Sandström et al., 2014) and being affected by *A. astaci* and novel diseases (Jussila et al., 2013b; Edsman et al., 2015). Both consequences were largely ignored and vigorously debated against in public. It took the government research institute LUKE until the mid-2010s to admit that their statistics showed the introductions of the alien *P. leniusculus* actually having only a small, even negligible, impact on the total catch of crayfish in Finland (Erkamo, 2019).

One of the unexpected dangers *A. astacus* is facing is possible restrictions on trapping wild *A. astacus* stocks, as has been suggested in Sweden, due to a fundamentalist view on conservation practices (Edsman, 2020a). Crayfish have been traditionally trapped because of their market value, the beach price for *A. astacus*, a minimum 10 cm long, being between one and two euro each (Jussila, 1995; Jussila and Mannonen, 2004). The income for a crayfish trapper could easily be several thousand euros during the crayfish season, which amongst other matters is a valuable lesson to a young crayfish trapper of the value of the natural resource (Jussila, 1995). If trapping of the *A. astacus* is restricted, one can always illegally introduce *P. leniusculus* in the water body, because trapping of *P. leniusculus* will not be restricted in the foreseeable future and it would thus enable trapping incomes. Even though the beach price of *P. leniusculus* is less than half compared to *A. astacus*, this scheme would work against conservation of the native *A. astacus*. Sometimes it would definitely be worth catching and eating a few endangered crayfish, for the benefit of the rest of the population. Even without the economic argument a carefully managed fishery by many local fishing right owners will be favorable for conservation by increasing the will for local people to protect the native crayfish (Edsman and Śmietana, 2004).

The fast buck ideology might have something to do with the alien species introduction and bluntly ignoring the necessity to conserve native species. It might be easy to predict a bright future in the case of unknown factors affecting the outcome of the alien species introduction. The North American crayfish species considered for introduction to Europe were

thriving in their original distribution area despite *A. astaci* being present there in its most virulent haplotypes (e.g., Makkonen et al., 2019). It must have been tempting to claim that these species would be doing similarly in Europe, despite the fact that only the general climate features would be the same, while there are differences between North American and European aquatic ecosystems in terms of potential pathogens and parasites (e.g., Martiny et al., 2006; Litchman, 2010). Most of the aquatic and geological features would be different from, for example, conditions in Lake Tahoe, which was one of the main sources of *P. leniusculus* being introduced to Europe (Westman, 1973; Henttonen and Huner, 1999). Ignoring the fact that Lake Tahoe is very deep and rather constant in water temperature compared to rather shallow and low volume water courses in Europe is a grave mistake, which was verified by a Swedish research group (e.g., Sandström et al., 2014), showing that one of the main variables explaining alien *P. leniusculus* population crashes was warmer water temperature. In this case, climate change would make matters even worse for alien *P. leniusculus*, while it might not help native *A. astacus* either (e.g., Capinha et al., 2013; Préau et al., 2020).

One cannot ignore recent political changes across Europe and at the national level, with the more populist and nationalistic tendencies gaining support (Aalberg et al., 2016; Scoones et al., 2018; Borras, 2020). Quite often these populist movements tend toward conservative and rightwing policies, which tend to ignore the importance of conservation values (Cortes-Vazquez, 2020). There has been an increase in ideologies and movements characterizing nature conservation and an ecologically sound lifestyle as being detrimental to the well-being of individuals, and even a threat to the western life style as such (Apostolopoulou and Adams, 2015). In this political atmosphere, short-term economic benefits tend to gain the upper hand and indirect or intangible long-term benefits, such as ecosystems with biodiversity and strength, are not considered valid priorities (e.g., McCarthy, 2019). These political tendencies, of course, threaten the existence of vulnerable native species and whole native ecosystems, including native European crayfish struggling with detrimental diseases, such as *A. astaci* infections, and pollutants from industrial activities. The well-being of society, in this context, does not include the well-being of natural, native resources (Cortes-Vazquez, 2020) but rather short-term economic benefits.

Lively and productive native ecosystems, as they can be taken when considering native European crayfish stocks in their prime, offer both intangible benefits in the form of recreation and economic benefits in the form of trapping income and sales of trapping related gear and licenses (Jussila, 1995; Jussila and Mannonen, 2004; Bohman and Edsman, 2011). In Fennoscandia, productive native crayfish stocks have been used in the tourism industry as sites for trapping crayfish and then having crayfish parties on the lakesides (e.g., Jussila et al., 2016a), as have lately also the alien *P. leniusculus* stocks been utilized. In the context of conservation, the general public could be educated during the recreational trapping and the following crayfish parties on the importance of native crayfish stocks from both recreational and economic viewpoints. As most, if not all, of the native *A. astacus* populations are not open access, the hospitality enterprises are important in terms of widening possibilities for positive experiences been offered by the productive native crayfish stocks.

DISCUSSION

The Future for the Native European Crayfish Is Bright, if We Just Try, Right?

We would like to summarize the threats and necessary actions to ensure the maximum conservation outcome for the native European crayfish, with several aspects presented in the **Table 3** and outlined in the following paragraphs (cf. Caffrey et al., 2014).

First of all, if ever again attempting to introduce alien species to substitute declining native species, one should be aware that such actions will cause more damage than benefit to the ecosystem or society (Kouba et al., 2021). In Sweden, from a purely national economic perspective, disregarding the disastrous effects on biodiversity, the massive introduction of *P. leniusculus* resulted in a cost rather than a benefit in the end (Gren et al., 2009). The loss of local native species populations, especially if it is limited to a certain region as opposed to a species extinction, even though a serious issue as such, is not a reason to correct the mistake by making another one. The spreading of alien crayfish in Europe is a classic and sad example of how matters can easily be made worse for the native species by introducing alien species to compensate local or regional losses of native species stocks (Gherardi and Holdich, 1999). The causes for their decline have multiple origins, and some of those must be corrected, such as pollution of water, acid rain and waterways construction (Edsman and Schröder, 2009), which the EU has taken a firm stand against (e.g., Paloniitty, 2016). Then, when habitats and environment have been restored to an ecologically maintainable level, one has to wait and see how ecosystem resilience will do its job. Sometimes less is more. If alien species have been introduced, on purpose or by accident, conclusions regarding the establishment and success of these populations should be reached only after a considerable time period, in the case of *P. leniusculus* in Finland more than 20 years after its introductions. In most cases this is too late and the progress of matters cannot be reversed. Maybe being more cautious and suspicious in the first place and eschewing the introduction of alien crayfish into Europe would have been best.

Alien crayfish should at least be restricted to limited designated regions, as is the general aim of the all European national crayfisheries strategies, and those alien crayfish populations which have been stocked, without permission and in most cases illegally, should be banned from all exploitation (e.g., Edsman et al., 2019). It thus would be strictly pointless to spread alien crayfish, which so far has been common practice and partially encouraged by the fisheries administration at least in Spain, Finland and Sweden (e.g., Alonso et al., 2000; Ruokonen et al., 2018; Jussila and Edsman, 2020). If broadly adopted, by banning introductions and trapping of the illegally established populations of alien crayfish at least some social pressure would be created, potentially resulting in hesitation when planning the

TABLE 3 | Means to secure the conservation of native European crayfish.

Threat	Action
Alien crayfish species	✓ No more stockings to novel water bodies. ✓ No commercialization of live crayfish. ✓ Halt the alien species pet trade. ✓ Early detection and rapid eradication of newly emerging alien crayfish populations. ✓ Full economical responsibility for illegal spreading.
Aphanomyces astaci	✓Halt the spread of alien crayfish. ✓ Awareness that North American crayfish are chronic reservoirs and the source of the crayfish plague. ✓ Increased awareness of fishing gear disinfection. ✓ Fish stockings only if verified disease free. ✓ Understanding of molecular mechanisms for crayfish plague resistance for selective breeding.
Lack of EU level interest	✓Impose EU regulations on halting pet trade of crayfish. ✓ qPCR test for *A. astaci* of imported ballast water. ✓ Funding of native crayfish related management and research.
Impaired communication with national governments	✓Awareness of the validity of science. ✓ Participating in national planning. ✓ Relevant applications of academic output. ✓ Demand for transparent crayfisheries policies. ✓ Stronger law enforcement.
People having wrong, old, and false information	✓Awareness-raising campaigns. ✓ Media releases.
People disconnected from nature	✓Awareness raising campaigns. ✓ Boosting motivation for conservation. ✓ Targeting kids as means to educate general public. ✓ Citizen Sciences programs (e.g., https://alien-csi.eu/).
Interest group inactivity	✓Awareness-raising of the material and intangible benefits of conservation for society.

next illegal introduction. The role of information campaigns should be more focused in this context, as it has been clear in the past that one of the main reasons for the irresponsible spreading of alien crayfish species has been messages which do not clearly state the risks related to alien species. One of the main reasons for this misleading information has been the reluctance of those in charge to admit that expectations have not been met and thus the instructions should be revised in order to avoid repeating mistakes.

While competitive exclusion by alien over native crayfish is a major risk to the long-term persistence of native crayfish in Europe, the hitchhiking disease pathogen that comes along with the alien crayfish possesses a much more immediate threat (e.g., Unestam, 1969a; Jussila et al., 2014b). Now that various *A. astaci* strains from different genotypes are present in various European water bodies (**Table 2**), its spread not only via alien crayfish, but also via fish (Oidtmann et al., 2002) or other species transporting *A. astaci* zoospores, i.e., birds and mammals preying on crayfish (Anastácio et al., 2014) appears inexorable, and we might have to start to think about how to make co-existence of the pathogen and native crayfish possible. It has been suggested that the main mechanism underlying the increased resistance of the North American crayfish species against the crayfish plague is the constant overexpression of prophenoloxidase-related genes, inhibiting pathogen growth and hence infection development. In European crayfish species, the enzymatic activation of the prophenoloxidase-cascade is often too inefficient and slow to successfully combat the disease (Cerenius et al., 2003). Therefore, in European native crayfish the infection leads to death usually within a few days or weeks, depending on the pathogen strain and virulence (Makkonen et al., 2014; Becking et al., 2015). However, recent reports indicate that European native crayfish wild populations exposed to *A. astaci* of haplogroup A, in some cases even haplogroup B and D, can sometimes resist the deadly acute crayfish plague infection (Svoboda et al., 2012; Jussila et al., 2017; Martín-Torrijos et al., 2017). Significant differences in disease resistance have also been observed in controlled infection experiments (Makkonen et al., 2012; Martín-Torrijos et al., 2017; Francesconi et al., 2021). It is thus a major future challenge to identify target genes and molecular pathways, which underlie the defense mechanisms of the crayfish immune system under an *A. astaci* challenge that might be responsible for an increased resistance toward crayfish plague infection. In perspective, such results might become the basis of selective breeding programs focusing on resistance-genes. Subsequently, reintroduction programs could make use of crayfish plague

resistant crayfish to be released into their original habitats. That being said, there would then be a risk of promoting yet another reservoir for *A. astaci* among native crayfish populations. Thus, speeding up the positive selection process by genetic enhancement of resistance against pathogens has to be carefully considered in the context of the conservation of the native crayfish species.

International pet trade polices like EU Pet Trade Regulation 2016/1141 need to be extended to cover more species which have high invasive potential and are known *A. astaci* vectors. More conservatively, instead of a blacklist of forbidden species, which takes too much time on EU level to be extended by additional species, it could be suggested to have a white list of species allowed for trade within the EU. Such a list seems to be more in line with a precautionary principle regarding the prevention of introducing invasive species unintentionally. Additionally, a frequent eDNA test of ballast water and the water used during animal cargo for presence of *A. astaci* spores and other emerging diseases using molecular methods would be highly advisable and definitely compulsory in cases where animal cargo could be entering the EU market (Brunner, 2020). Without effective implementation of national and international biosecurity measures, the occurrence, transboundary spread and serious economic and ecological impact of aquatic animal diseases will continue. In this regard, globally agreed standards for sanitary measures to apply to international trade in live aquatic animals are laid out in the OIE Aquatic Animal Health Code[2] and in the OIE Manual of Diagnostic Tests for Aquatic Animals[3]. Finally, public education is probably the key factor to reduce the risk of alien crayfish to be released into the wild. Education of retailers and pet crayfish owners is an important aspect to alleviate the threat posed by the pet trade.

People's awareness can contribute to public engagement benefiting nature conservation, which would be initiated by environmental education as part of the school curriculum. Recent successful conservation campaigns in Finland and Sweden (e.g., LIFE+ CrayMate and other regional campaigns; Jussila, 2016) have resulted in the common public being more aware of the possible benefits of native *A. astacus* stocks and the dangers of the alien *P. leniusculus* (Jussila, 2016). During the 3-year LIFE+ CrayMate awareness campaign, 2013 – 2016, the targeted fishing rights owners, mostly private persons responsible of the management wild fish and crayfish stock, initiated *A. astacus* restocking programs within carefully selected waters and at least in Southern Karelia region the success rate of the introductions was above 50% (Tiitinen, 2020) similarly to what has been observed in Northern Savo during the 2020s (Kosunen, 2020). At the same time, people became more aware of the role of alien *P. leniusculus* as the main reason for the spreading of *A. astaci* and the devastation of the native *A. astacus* stocks. This came as a surprise since the Finns have a strong tradition of trapping and eating crayfish, while the knowledge regarding the basics of crayfish biology and ecology seemed thin. In Fennoscandia an information campaign called "The Crayfish Myth Buster"[4] (in Swedish, Finnish, and English) was launched on the web in 2006 and people were directed to the website by advertisements in commercial radio jingles, TV, newspapers, flyers, special hats, and information on milk cartons. The campaign dealt with the 21 most common myths, exaggerations and misunderstandings of freshwater crayfish. In a very recent project ("MaNaKa," 2017–2020) German authorities funded an awareness campaign to encourage and instruct fishing clubs, water leaseholders and nature conservation authorities to safely stock suitable water bodies with the endangered native *A. astacus*. The colonization of waters previously free from crayfish plague, if carefully selected, should make an important contribution to the preservation of *A. astacus* in Germany. As an example from the south-east Europe, there have been campaigns in Croatia dedicated to raise awareness of the problems that invasive crayfish cause to freshwater diversity (Pavić et al., 2021). Unfortunately, those activities rarely attained the expected result. Even though there were workshops organized for local inhabitants focusing on the problems that *P. leniusculus* could cause to karstic habitats, *P. leniusculus* was illegally introduced into another karstic river (Una River) bordering Croatia and Bosnia and Herzegovina (Trožić-Borovac et al., 2019). Local education campaigns and workshops are needed in the regions where alien crayfish are present and also where they are not yet present but highly likely to spread. Currently, an action plan for alien crayfish in Croatia is being developed, involving local stake holders (fisherpersons, policy makers, protected areas employees, local inhabitants, school teachers, NGOs, etc.) as well as astacologists and the wider scientific community (Faller, 2020). In Croatia, a mobile application for invasive species alert has recently been developed and is now available for citizens (MGOR, 2020).

Finally, natural resources, such as reproductive native crayfish populations, can be taken as exploitable resources, while this approach should not be applied to all native crayfish populations or their distribution regions. The exploitation, and thus the commercial value of the wild crayfish stock, might be a means to protect and conserve native crayfish populations or even a whole species (e.g., Taugbøl, 2004), providing that exploitation is sustainable and spreading of diseases is prevented. When exploitation is discussed, one should introduce ecology into the debate and bear in mind that exploitation should not result in biodiversity decline or drastic ecosystem changes. Exploitation should thus be based on wide ecosystem sustainability, which would then allow both ecosystem health and thriving variety of species, in this case aquatic species, while also ensuring income and benefits to those attempting to exploit natural resources. Discussions regarding exploitation of natural resources quite often, if not always, focus on maximum economical gain, ignoring the long-term health of the exploited natural resource or the ecosystem where this resource belongs to. It never ceases to surprise how those interested in economic gain tend to forget that conservation is actually a rather selfish activity, in most cases, if successful, allowing the existence of human beings and the cultural frame that we rely on. Thus, conserving native European

[2]https://www.oie.int/standardsetting/aquatic-code/

[3]https://www.oie.int/standard-setting/aquatic-manual/

[4]www.krafta.nu

crayfish, protecting them against alien species and their diseases, works for us, too, allowing us to enjoy natural resources and, in the case of the Swedes and maybe the Finns, also crayfish parties, while having nicely prepared *A. astacus* (e.g., Fürst and Törngren, 2003; Edsman, 2004; Jussila et al., 2016a), but not in excess.

Quite a few of the suggested management and conservations actions have been implemented at least partially and with some success, for example LIFE+ CrayMate awareness campaign in Finland. As more radical actions we suggest that a fundamental principle of *polluter pays* should be enforced in the cases where the alien species are spreading and resulting in damage to the native ecosystems (e.g., Gaines, 1991). In Finland, and many other European countries, spreading of alien species is illegal, while so far the legal system fails to find culprits or ignores the cases as meaningless in their impacts (our observation). If an alien species cannot be eradicated, a *functional eradication* could be limiting or even eliminating ecological damage, as has been observed in the case of *P. rusticus* in North America (Green and Grosholz, 2021). Recent advances in bioengineering would also allow *genetic biocontrol*, which is based on modifications of the organism's genome in a heritable way that would for example disrupt the reproduction of the alien species (e.g., Teem et al., 2020). However, such methods are to date only applicable for some insect and vertebrate model species. In the case of freshwater crayfish, the most basic genomic knowledge required for genetic bioengineering, i.e., a fully annotated reference genome, is still lacking.

In this paper we clearly articulate and showcase that human perception and knowledge, or rather the lack of them, together with plain greed, are the most crucial components of the sad predicament of native freshwater crayfish species in Europe. The alien species threat has to be dealt with resolutely. However, to solve the problem of the invasive freshwater crayfish spread today, the most important thing is to manage people rather than the crayfish themselves (e.g., Robbins, 2011; Edsman, 2020b).

AUTHOR CONTRIBUTIONS

JJ, LE, IM, JD-U, and KT contributed equally in fact finding and writing process. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank our two reviewers for their very constructive comments that significantly improved this manuscript. We would like express our deepest respect to all those who have been fighting an uphill battle to support the native ecosystems below and above water level, especially the inspiring atmosphere of the International Association of Astacology (IAA). Remember, it is never too late and, please, never give up. In loving memory of Vesa Tiitinen's life: he was a good boy.

REFERENCES

Aalberg, T., Esser, F., Reinemann, C., Strömbäck, J., and de Vreese, C. H. (2016). *Populist Political Communication in Europe*. London: Routledge.

Abrahamsson, S. (1973). The crayfish, *Astacus astacus*, in Sweden and the introduction of the American crayfish, *Pacifastacus leniusculus*. *Freshw. Crayfish* 1, 27–40. doi: 10.5869/fc.1973.v1.027

Ackefors, H. (1999). "The positive effects of established crayfish introductions in Europe," in *Crayfish as Alien Species. How to Make the Best of a Bad Situation.* Crustacean Issues, 11, eds F. Gherardi and D. M. Holdich (Rotterdam: A.A. Balkema), 49–62. doi: 10.1201/9781315140469-5

Ackefors, H. (2005). *Kräftdjur i Hav Och I Sjöar*. Nyhamnsläge: Kiviksgårdens förlag.

Alcorlo, P., and Diéguez Uribeondo, J. (2014). El cangrejo señal y el declive de las poblaciones de cangrejo autóctono. *Ambienta* 109, 52–61.

Alderman, D. J. (1996). Geographical spread of bacterial and fungal diseases of crustaceans. *Rev. Sci. Tech.* 15, 603–632. doi: 10.20506/rst.15.2.943

Alonso, F., Temiño, C., and Diéguez-Uribeondo, J. (2000). Status of the white clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858) in Spain: distribution and legislation. *Bull. Fr. Pêche Piscic.* 356, 31–54. doi: 10.1051/kmae:2000003

Anastácio, P. M., Ferreira, M. P., Banha, F., and Capinha, C. (2014). Waterbird-mediated passive dispersal is a viable process for crayfish (*Procambarus clarkii*). *Aquat. Ecol.* 48, 1–10. doi: 10.1007/s10452-013-9461-0

Andriantsoa, R., Tönges, S., Panteleit, J., Theissinger, K., Carneiro, V. C., Rasamy, J., et al. (2019). Ecological plasticity and commercial impact of invasive marbled crayfish populations in Madagascar. *BMC Ecol.* 19:8. doi: 10.1186/s12898-019-0224-1

Anon (1899). Rakogojstvo. *Lovaèko Ribarski Viesnik* 5, 49–52.

Anon (2019). *Maa – Ja Metsätalousministeriön Ja Luonnonvarakeskuksen Tulossopimus 2020-2024*. Helsinki: Maa- ja metsätalousministeriö. .

Apostolopoulou, E., and Adams, W. M. (2015). Neoliberal capitalism and conservation in the post-crisis era: the dialectics of "green" and "un−green" grabbing in Greece and the UK. *Antipode* 47, 15–35. doi: 10.1111/anti.12102

Arce, J., and Dieguez-Uribeondo, J. (2015). Structural damage caused by the invasive crayfish *Procambarus clarkii* (Girard, 1852) in rice fields of the Iberian Peninsula: a study case. *J. Fundam. Appl. Limnol.* 186, 259–269. doi: 10.1127/fal/2015/0715

Arias Rodríguez, A., and Torralba Burrial, A. (2021). First record of the redclaw crayfish *Cherax quadricarinatus* (Von Martens, 1868) on the Iberian Peninsula. *Limnetica* 40, 33–42. doi: 10.23818/limn.40.03

Aydin, H., Kokko, H., Makkonen, J., Kortet, R., Kukkonen, H., and Jussila, J. (2014). The signal crayfish is vulnerable to both the As and the PsI-isolates of the crayfish plague. *Knowl. Manag. Aquat. Ecosyst.* 413:3. doi: 10.1051/kmae/2014004

Becking, T., Mrugała, A., Delaunay, C., Svoboda, J., Raimond, M., Viljamaa-Dirks, S., et al. (2015). Effect of experimental exposure to differently virulent *Aphanomyces astaci* strains on the immune response of the noble crayfish *Astacus astacus*. *J. Invertebr. Pathol.* 132, 115–124. doi: 10.1016/j.jip.2015.08.007

Biasetti, P., Ferrante, L., Bonelli, M., Manenti, R., Scaccini, D., and de Mori, B. (2021). Value-conflicts in the conservation of a native species: a case study based on the endangered white-clawed crayfish in Europe. *Rend. Fish. Acc. Lincei* 32, 389–406. doi: 10.1007/s12210-021-00987-1

Bickford, D., Lohman, D. J., Sodhi, N. S., Ng, P. K., Meier, R., Winker, K., et al. (2007). Cryptic species as a window on diversity and conservation. *Trends Ecol. Evol.* 22, 148–155. doi: 10.1016/j.tree.2006.11.004

Bohman, P. (ed) (2020). *Swedish Crayfish Database (In Swedish: Nationella Kräftdatabasen). Slu, Department Of Aquatic Resources*. Available online at: http://www.slu.se/kraftdatabasen (accessed December 22, 2020).

Bohman, P., and Edsman, L. (2011). Status, management and conservation of crayfish in Sweden: results and the way forward. *Freshw. Crayfish* 18, 19–26. doi: 10.5869/fc.2011.v18.19

Bohman, P., and Edsman, L. (2013). *Marmorkräftan i Märstaån. Riskanalys och åtgärdsförslag. (English title: Marmorkrebs in River Märstaån. Risk Assessment And Action Proposals.) Aqua Reports 2013:17*. Drottningholm: Sveriges lantbruksuniversitet.

Bohman, P., Edsman, L., Martin, P., and Scholtz, G. (2013). The first marmorkrebs (Decapoda: Astacida: Cambaridae) in Scandinavia. *BioInv. Rec.* 2, 227–232. doi: 10.3391/bir.2013.2.3.09

Bohman, P., Nordwall, F., and Edsman, L. (2006). The effect of the large-scale introduction of signal crayfish on the spread of crayfish plague in Sweden. *Bull. Fr. Pêche Piscic.* 380-381, 1291–1302. doi: 10.1051/kmae:2006026

Bojko, J., Jennings, L. A., and Behringer, D. C. (2020). A novel positive single-stranded RNA virus from the crustacean parasite, *Probopyrinella latreuticola* (Peracarida: Isopoda: Bopyridae). *J. Invertebr. Pathol.* 177:107494. doi: 10.1016/j.jip.2020.107494

Borras, S. M. Jr. (2020). Agrarian social movements: The absurdly difficult but not impossible agenda of defeating right-wing populism and exploring a socialist future. *J. Agrar. Change* 20, 3–36. doi: 10.1111/joac.12311

Brunner, J. L. (2020). Pooled samples and eDNA-based detection can facilitate the "clean trade" of aquatic animals. *Sci. Rep.* 10:10280. doi: 10.1038/s41598-020-66280-7

Butler, E., Crigler, P., Robbins, G., and Blair, J. E. (2020). Preliminary survey of *Aphanomyces* sp. associated with native and invasive crayfish in the Lower Susquehanna watershed of South Central Pennsylvania. *J. Freshw. Ecol.* 35, 223–233. doi: 10.1080/02705060.2020.1779141

Cab Direct UK (2014). *Procambarus fallax f. virginalis (Martin et al., 2010), Marmorkrebs. [invasive species]. Procambarus fallax f. virginalis (Martin et al., 2010), Marmorkrebs. [invasive species]., (AQB ISC record)*. Available online at: www.cabdirect.org/cabdirect/abstract/20107601509 [accessed 10 March 2021]

Caffrey, J. M., Baars, J. R., Barbour, J. H., Boets, P., Boon, P., Davenport, K., et al. (2014). Tackling invasive alien species in Europe: The top 20 issues. *Manag. Biol. Inv.* 5, 1–20. doi: 10.3391/mbi.2014.5.1.01

Capinha, C., Larson, E. R., Tricarico, E., Olden, J. D., and Gherardi, F. (2013). Effects of climate change, invasive1021 species, and disease on the distribution of native European crayfishes. *Conserv. Biol.* 27, 731–1022. doi: 10.1111/cobi.12043 doi: 10.1111/cobi.12043

Cerenius, L., Andersson, M. G., and Söderhäll, K. (2009). "*Aphanomyces astaci* and crustaceans," in *Oomycete Genetics and Genomics: Diversity, Interactions and Research Tools*, eds K. Lamour and D. Kamoun (Hoboken, NJ: John Wiley & Sons Inc), 425–433. doi: 10.1002/9780470475898.ch21

Cerenius, L., Bangyeekhun, E., Keyser, P., Söderhäll, I., and Söderhäll, K. (2003). Host prophenoloxidase expression in freshwater crayfish is linked to increased resistance to the crayfish plague fungus, *Aphanomyces astaci*. *Cell. Microbiol.* 5, 353–357. doi: 10.1046/j.1462-5822.2003.00282.x

Chadwick, D. D. A., Pritchard, E. G., Bradley, P., Sayer, C. D., Chadwick, M. A., Eagle, L. J. B., et al. (2020). A novel 'triple drawdown' method highlights deficiencies in invasive alien crayfish survey and control techniques. *J. Appl. Ecol* 29, 316–326. doi: 10.1111/1365-2664.13758

Chucholl, C. (2015). "Marbled crayfish gaining ground in Europe: the role of the pet trade as invasion pathway," in *Freshwater Crayfish – A Global Overview*, eds T. Kawai, Z. Faulkes, and G. Scholtz (London: Taylor and Francis Group), 83–114. doi: 10.1201/b18723-8

Chucholl, C., and Pfeiffer, M. (2010). First evidence for an established Marmorkrebs (Decapoda, Astacida, Cambaridae) population in Southwestern Germany, in syntopic occurrence with *Orconectes limosus* (Rafinesque, 1817). *Aquat. Inv.* 5, 405–412. doi: 10.3391/ai.2010.5.4.10

Chucholl, C., and Wendler, F. (2017). Positive selection of beautiful invaders: long-term persistence and bioinvasion risk of freshwater crayfish in the pet trade. *Biol. Inv.* 19, 197–208. doi: 10.1007/s10530-0161272-5

Clavero, M., Nores, C., Kubersky-Piredda, S., and Centeno-Cuadros, A. (2016). Interdisciplinarity to reconstruct historical introductions: solving the status of cryptogenic crayfish. *Biol. Rev.* 91:10361049. doi: 10.1111/brv.12205

Cornalia, E. (1860). "Sulla malattia dei gamberi," in *Atti Della Societa Italiana Di Scienze Naturali E Del Museo Civili Di Storia Naturale*, Vol. 2, Milano, 334–336.

Cortes-Vazquez, J. A. (2020). In the name of the people: The populist redefinition of nature conservation in post-crisis Spain. *Geoforum* 108, 110–118. doi: 10.1016/j.geoforum.2019.12.004

Courtine, J. J., and Willett, L. (1986). A brave new language: Orwell's invention of 'newspeak' in 1984. *SubStance* 15, 69–74. doi: 10.2307/3684756

Cowan, P., and Warburton, B. (2011). "Animal welfare and ethical issues in island pest eradication," in *Island Invasives: Eradication And Management*, eds C. R. Veitch, M. N. Clout, and D. R. Towns (Gland: IUCN), 418–421.

Dannewitz, J., Palm, S., and Edsman, L. (2021). Colonization history and human translocations explain the population genetic structure of noble crayfish (*Astacus astacus*) in Fennoscandia – implications for management of a critically endangered species. *Aquat. Conserv.* doi: 10.1002/aqc.3632 [Epub ahead of print].

De Palma-Dow, A., \urti, J., and Fergus, E. (2020). It's a Trap! An evaluation of different passive trap types to effectively catch and control the invasive red swamp crayfish (*Procambarus clarkii*) in streams of the Santa Monica Mountains. *Manag. Biol. Inv.* 11, 44–62. doi: 10.3391/mbi.2020.11.1.04

Deidun, A., Sciberras, A., Formosa, J., Zava, B., Insacco, G., Corsini-Foka, M., et al. (2018). Invasion by non-indigenous freshwater decapods of Malta and Sicily, central Mediterranean Sea. *J. Crust. Biol.* 38, 748–753. doi: 10.1093/jcbiol/ruy076

Díaz, M. (2021). *Solicitud de dictamen sobre efectos negativos de Procambarus clarkii Y Pacifastacus leniusculus sobre el medio natural*. Madrid: MITECO.

Diéguez-Uribeondo, J. (2006). Dispersion of the *Aphanomyces astaci*-carrier, *Pacifastacus leniusculus*, by humans represents the main cause of disappearance of native populations of *Austropotamobius pallipes* in Navarra. *Bull. Fr. Pêche Piscic* 4, 1303–1312. doi: 10.1051/kmae:2006036

Diéguez-Uribeondo, J., and Söderhäll, K. (1993). *Procambarus clarkii* as a vector for the crayfish plague fungus *Aphanomyces astaci* Schikora. *Aquacult. Res.* 24, 761–765. doi: 10.1111/j.13652109.1993.tb00655.x

Diéguez-Uribeondo, J., Cerenius, L., and Söderhäll, K. (1994). *Saprolegnia parasitica* and its virulence on three different species of freshwater crayfish. *Aquaculture* 120, 219–228. doi: 10.1016/0044-8486(94)90080-9

Diéguez-Uribeondo, J., García, M. A., Cerenius, L., Kozubíková, E., Ballesteros, I., Windels, C., et al. (2009). Phylogenetic relationships among plant and animal parasites, and saprobionts in *Aphanomyces* (Oomycetes). *Fungal Genet. Biol.* 46, 365–376. doi: 10.1016/j.fgb.2009.02.004

Diéguez-Uribeondo, J., Huang, T.-S., Cerenius, L., and Söderhäll, K. (1995). Physiological adaptation of an *Aphanomyces astaci* strain isolated from the freshwater crayfish *Procambarus clarkii*. *Mycol. Res.* 99, 571–578. doi: 10.1016/S0953-7562(09)80716-8

Diéguez-Uribeondo, J., Rueda, A., Castien, E., and Bascones, J. C. (1997a). A plan of restoration for the native freshwater crayfish species, *Austropotamobius pallipes*, in Navarra. *Bull. Fr. Pêche Piscic.* 347, 625–637. doi: 10.1051/kmae/1997056

Diéguez-Uribeondo, J., Temiño, C., and Muzquiz, J. L. (1997b). The crayfish plague fungus, *Aphanomyces astaci* in Spain. *B. Fr. Pêche Piscic.* 347, 753–763. doi: 10.1051/kmae/1997051

Domisch, S., Jaehnig, S. C., and Haase, P. (2011). Climate−change winners and losers: Stream macroinvertebrates of a submontane region in Central Europe. *Freshw. Biol.* 56, 2009–2020. doi: 10.1111/j.1365-2427.2011.02631.x

Dudgeon, D., Arthington, A. H., Gessner, M. O., Kawabata, Z. I., Knowler, D. J., Leveque, C., et al. (2006). Freshwater biodiversity: importance, threats, status and conservation challenges. *Biol. Rev.* 81, 163–182. doi: 10.1017/S1464793105006950

Edsman, L. (2004). The Swedish story about import of live crayfish. *Bull. Franc. Pêche Piscic.* 372-373, 281288. doi: 10.1051/kmae:2004003

Edsman, L. (2020a). "Flodkräfta," in *Fisk- och skaldjursbestånd i hav och sötvatten 2019: Resursöversikt. Rapport 2020:3*, eds A. Bryhn, A. Sundelöf, A. Lingman, A. B. Florin, E. Petersson, F. Vitale, et al. (Göteborg: Havs- och vattenmyndigheten), 55–57.

Edsman, L. (2020b). "The rise and fall of the introduced invasive signal crayfish in Sweden – sixty years with an alien," in *Proceedings of the Abstract of oral presentation, Ecology in the Anthropocene, The fourth conference of the Nordic Society Oikos*, Reykjavik.

Edsman, L., and Schröder, S. (2009). *Åtgärdsprogram För Flodkräfta 2008–2013 (Astacus Astacus). Report nr. 5955*. Drottningholm: Fiskeriverket och Naturvårdsverket.

Edsman, L., and Śmietana, P. (2004). Exploitation, conservation and legislation. *Bull. Franc. Pêche Piscic.* 372-373, 457–464. doi: 10.1051/kmae:2004019

Edsman, L., Farris, J. S., Källersjö, M., and Prestegaard, T. (2002). Genetic differentiation between noble crayfish, *Astacus astacus* (L.), populations

detected by microsatellite length variation in the rDNA ITS1 region. *Bull. Franç. Pêche Piscic.* 367, 691–706. doi: 10.1051/kmae:2002082

Edsman, L., Gydemo, R., and Jussila, J. (2019). Resolution IAA gotland. *Crayfish News* 41, 10–11.

Edsman, L., Nyström, P., Sandström, A., Stenberg, M., Kokko, H., Tiitinen, V., et al. (2015). Eroded swimmeret syndrome in female crayfish *Pacifastacus leniusculus* associated with *Aphanomyces astaci* and *Fusarium* spp. infections. *Dis. Aquat. Org.* 112, 219–228. doi: 10.3354/dao02811

Eizaguirre, C., and Baltazar-Soares, M. (2014). Evolutionary conservation – evaluating the adaptive potential of species. *Evol. Appl.* 7, 963–967. doi: 10.1111/eva.12227

Erkamo, E. (2019). *Conference Presentation*. Jyväskylä: Kansalliset kalantutkimuspäivät, (In Finnish).

Erkamo, E. (2020). *Oral Communication*. Jokioinen.

Erkamo, E., Tulonen, J., and Kirjavainen, J. (2019). *Kansallinen Rapustrategia 2019–2022*. Helsinki: MMM.

Fahrig, L. (2003). Effects of habitat fragmentation on biodiversity. *Ann. Rev. Ecol. Evol. Syst.* 34, 487–515.

Faller, M. (2020). *Oral Communication*.

Faulkes, Z. (2015a). The global trade in crayfish as pets. *Crust. Res.* 44, 75–92. doi: 10.18353/crustacea.44.0_75

Faulkes, Z. (2015b). A bomb set to drop: parthenogenetic Marmorkrebs for sale in Ireland, a European location without non-indigenous crayfish. *Manag. Biol. Inv.* 6:111. doi: 10.3391/mbi.2015.6.1.09

Fiskeriverket (1993). *Ways Of Increasing Populations Of Noble Crayfish Astacus astacus In Swedish Freshwaters. Information From The Institute Of Freshwater Research*. Drottningholm: Fiskeriverket.

Francesconi, C., Makkonen, J., Schrimpf, A., Jussila, J., Kokko, H., and Theissinger, K. (2021). Elevated resistance and latent infections caused by reduced virulence: marbled crayfish and noble crayfish challenged with the crayfish plague disease agent. *Front. Ecol. Evol.* (in press)

Furse, J. (2008). IAA17 Kuopio 2008 outcome and conclusions. *Freshw. Crayfish* 17:xii.

Fürst, M., and Törngren, K. (2003). *Våra Älskade Kräftor. Från Bur Till Bord*. Stockholm: Bäckströms förlag.

Gaines, S. E. (1991). The polluter-pays principle: from economic equity to environmental ethos. *Tex. Int. Law J.* 26, 463–496.

Gherardi, F. (2011). Towards a sustainable human use of freshwater crayfish (Crustacea, Decapoda, Astacidea). *Knowl. Manag. Aquat. Ecosyst.* 401:2. doi: 10.1051/kmae/2011038

Gherardi, F., and Holdich, D. M. (eds.) (1999). *Crayfish in Europe as Alien Species. How to Make Best of a Bad Situation*. Rotterdam: A. A. Balkema.

Gherardi, F., Aquiloni, L., Diéguez-Uribeondo, J., and Tricarico, E. (2011). Managing invasive crayfish: is there a hope? *Aquat. Sci.* 73, 185–200. doi: 10.1007/s00027-011-0181-z

Gontier, M., Balfors, B., and Mörtberg, U. (2006). Biodiversity in environmental assessment—current practice and tools for prediction. *Environ. Imp. Assessm. Rev.* 26, 268–286. doi: 10.1016/j.eiar.2005.09.001

Govedič, M. (2017). First record of the spiny-cheek crayfish (*Orconectes limosus*) in Slovenia–300 km upstream from its known distribution in the Drava River. *Knowl. Manag. Aquatic Ecosyst.* 418, 7. doi: 10.1051/kmae/2016039

Grandjean, F., Collas, M., Uriarte, M., and Rousset, M. (2021). First record of a marbled crayfish *Procambarus virginalis* (Lyko, 2017) population in France. *Bioinv. Rec.* 10, 341–347. doi: 10.3391/bir.2021.10.2.12

Green, S. J., and Grosholz, E. D. (2021). Functional eradication as a framework for invasive species control. *Front. Ecol. Environ.* 19:98–107. doi: 10.1002/fee.2277

Gren, M., Isacs, L., and Carlsson, M. (2009). Costs of alien invasive species in Sweden. *AMBIO* 38:135140. doi: 10.1579/0044-7447-38.3.135

Gross, R., Jelić, M., Lovrenčić, L., Grandjean, F., Đuretanović, S., Simić, V., et al. (2021). Genetic diversity and structure of the noble crayfish populations in the Balkan Peninsula revealed by mitochondrial and microsatellite DNA markers. *PeerJ* (in press)

Gross, R., Palm, S., Koiv, K., Prestegaard, T., Jussila, J., Paaver, T., et al. (2013). Microsatellite markers reveal clear geographic structuring among threatened noble crayfish (*Astacus astacus*) populations in Northern and Central Europe. *Conserv. Genet.* 14, 809–882. doi: 10.1007/s10592-013-0476-9

Gutiérrez-Yurrita, P. J., Marténez, J. M., Bravo-Utrera, M. Á, Montes, C., Ilhéu, M., and Bernardo, J. M. (2017). "The status of crayfish populations in Spain and Portugal," in *Crayfish as Alien Species. How to Make the Best of a Bad Situation. Crustacean Issues, 11*, eds F. Gherardi and D. M. Holdich (Rotterdam: A.A. Balkema), 161–192. doi: 10.1201/9781315140469-12

Hänfling, B., Edwards, F., and Gherardi, F. (2011). Invasive alien Crustacea: dispersal, establishment, impact and control. *Biocontrol* 56, 573–595. doi: 10.3391/ai.2017.12.1.07

Hein, C. L., Vander Zanden, M. J., and Magnuson, J. J. (2007). Intensive trapping and increased fish predation cause massive population decline of an invasive crayfish. *Freshw. Biol.* 52, 1134–1146. doi: 10.1111/j.1365-2427.2007.01741.x

Henttonen, P., and Huner, J. V. (1999). "The introduction of alien species of crayfish in Europe: A historical introduction," in *Crayfish as Alien Species. How to Make the Best of a Bad Situation. Crustacean Issues, 11*, eds F. Gherardi and D. M. Holdich (Rotterdam: A.A. Balkema), 13–22. doi: 10.1201/9781315140469-2

Holden, C. (1995). Rabbit biocontrol project in disarray. *Science* 270, 1123.

Holdich, D. M. (2002). Distribution of crayfish in Europe and some adjoining countries. *Bull. Fr. Pêche Piscic.* 367, 611–650. doi: 10.1051/kmae:2002055

Holdich, D. M., and Reeve, I. D. (1991). Distribution of freshwater crayfish in the British Isles, with particular reference to crayfish plague, alien introductions and water quality. *Aquat. Conserv.* 1, 139–158. doi: 10.1002/aqc.3270010204

Holdich, D. M., James, J., Jackson, C., and Peay, S. (2014). The North American signal crayfish, with particular reference to its success as an invasive species in Great Britain. *Ethol. Ecol. Evol.* 26, 232–262. doi: 10.1080/03949370.2014.903380

Holdich, D. M., Reynolds, J. D., Souty-Grosset, C., and Sibley, P. J. (2009). A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. *Knowl. Manag. Aquat. Ecosyst.* 394395, 11. doi: 10.1051/kmae/2009025

Houghton, R., Wood, C., and Lambin, X. (2017). Size-mediated, density dependent cannibalism in the signal crayfish *Pacifastacus leniusculus* (Dana, 1852) (Decapoda, Astacidea), an invasive crayfish in Britain. *Crustaceana* 90, 417–435. doi: 10.1163/15685403-00003653

Hudina, S., Galić, N., Kutleša, P., Duplić, A., and Maguire, I. (2016). ""Range expansion of the signal crayfish (*Pacifastacus leniusculus*) in a recently invaded region in Croatia and potential for its control"," in *21st Symposium of the International Association of Astacology – Program and Book of Abstracts*, ed. J. Diéguez-Uribeondo (Madrid: Real Jardín Botánico), 29.

Hudina, S., Hock, K., and Žganec, K. (2014). The role of aggression in range expansion and biological invasions. *Curr. Zool.* 60, 401–409. doi: 10.1093/czoolo/60.3.401

Hudina, S., Kutleša, P., Trgovčević, K., and Duplić, A. (2017). Dynamics of range expansion of the signal crayfish (*Pacifastacus leniusculus*) in a recently invaded region in Croatia. *Aquat. Invasions* 12, 67–75.

Hudina, S., Žganec, K., Lucić, A., Trgovčić, K., and Maguire, I. (2013). Recent invasion of the karstic river systems in Croatia through illegal introductions of the signal crayfish. *Freshw. Crayfish* 19, 21–27. doi: 10.5869/fc.2013.v19.021

Huusela-Veistola, E., Holmala, K., Hyvönen, T., Hyvönen, T., Kauhala, K., Ryttäri, T., et al. (2019). *Ehdotus Haitallisten Vieraslajien Hallintasuunnitelmaksi Ja Leviämisväyliä Koskevaksi Toimintasuunnitelmaksi*. Helsinki: Valtioneuvoston kanslia.

Jackson, D. A., Peres-Neto, P. R., and Olden, J. D. (2001). What controls who is where in freshwater fish communities the roles of biotic, abiotic, and spatial factors. *Can. J. Fish. Aqua. Sci.* 58, 157–170. doi: 10.1139/cjfas-58-1-157

James, J., Mrugała, A., Oidtmann, B., Petrusek, A., and Cable, J. (2017). Apparent interspecific transmission of *Aphanomyces astaci* from invasive signal to virile crayfish in a sympatric wild population. *J. Invertebr. Pathol.* 145, 68–71. doi: 10.1016/j.jip.2017.02.003

Jelić, M., Klobučar, G. I. V., Grandjean, F., Puillandre, N., Franjević, D., Futo, M., et al. (2016). Insights into the molecular phylogeny and historical biogeography of the white-clawed crayfish (Decapoda, Astacidae). *Mol. Phylogenet. Evol.* 103, 26–40. doi: 10.1016/j.ympev.2016.07.009

Jussila, J. (1995). On the cost of crayfish trapping in central Finland. *Freshw. Crayfish* 8, 215–227. doi: 10.5869/fc.1995.v8.215

Jussila, J. (2016). *LIFE12 INF/FI/233 Final Report. LIFE+ RapuKamu, LIFE+ CrayMate*. Kuopio: UEF.

Jussila, J., and Edsman, L. (2020). Relaxed attitude towards spreading of alien crayfish species affects protection of native crayfish species: case studies and lessons learnt from a Fennoscandian viewpoint. *Freshw. Crayfish* 25, 39–46. doi: 10.5869/fc.2020.v25-1.039

Jussila, J., and Mannonen, A. (2004). Crayfisheries in Finland, a short overview. *Bull. Fr. Pêche Piscic.* 372-373, 263–273. doi: 10.1051/kmae:2004001

Jussila, J., Francesconi, C., Theissinger, K., Kokko, H., and Makkonen, J. (2021a). Is *Aphanomyces astaci* loosing its stamina: a latent crayfish plague disease agent from lake Venesjärvi, Finland. *Freshw. Crayfish*. (Submitted).

Jussila, J., Kokko, H., Kortet, R., and Makkonen, J. (2013a). *Aphanomyces astaci* PsI-genotype isolates from different Finnish signal crayfish stocks show variation in their virulence but still kill fast. *Knowl. Manag. Aquat. Ecosyst.* 411:10. doi: 10.1051/kmae/2013077

Jussila, J., Maguire, I., Kokko, H., and Makkonen, J. (2016a). "Chaos and adaptation in the pathogen-host relationship in relation to the conservation. The case of the crayfish plague and the noble crayfish," in *Freshwater Crayfish – A Global Overview*, eds T. Kawai, Z. Faulkes, and G. Scholtz (London: Taylor and Francis Group), 246–274. doi: 10.1201/b18723-15

Jussila, J., Makkonen, J., Kokko, H., and Mäkinen, P. (2014a). Numerous population crashes of wild signal crayfish (*Pacifastacus leniusculus*) in Southern Finland. *Freshw. Crayfish* 20, 73–79. doi: 10.5869/fc.2014.v20-1.73

Jussila, J., Makkonen, J., Vainikka, A., Kortet, R., and Kokko, H. (2011). Latent crayfish plague (*Aphanomyces astaci*) infection in a robust wild noble crayfish (*Astacus astacus*) population. *Aquaculture* 321, 17–20. doi: 10.1016/j.aquaculture.2011.08.026

Jussila, J., Makkonen, J., Vainikka, A., Kortet, R., and Kokko, H. (2014b). Crayfish plague dilemma: how to be a courteous killer? *Boreal Env. Res.* 19, 235–244.

Jussila, J., Tiitinen, V., Edsman, L., Kokko, H., and Fotedar, R. (2016b). Signal crayfish in Lake Saimaa could be maladapted to the local conditions due to *Aphanomyces astaci* infection: a seven-year study. *Freshw. Crayfish* 22, 53–60. doi: 10.5869/fc.2016.v22-1.53

Jussila, J., Tiitinen, V., Fotedar, R., and Kokko, H. (2013b). A simple and efficient cooling method for postharvest transport of the commercial crayfish catch. *Freshw. Crayfish* 19, 15–19. doi: 10.5869/fc.2013.v19.015

Jussila, J., Tiitinen, V., Makkonen, J., Kokko, H., Bohman, P., and Edsman, L. (2021b). Eroded swimmeret syndrome: update of the current knowledge. *Freshw. Crayfish* 26, 63–68. doi: 10.5869/fc.2021.v26-1.63

Jussila, J., Vrezec, A., Jaklič, T., Kukkonen, H., Makkonen, J., and Kokko, H. (2017). *Aphanomyces astaci* isolate from latently infected stone crayfish (*Austropotamobius torrentium*) population is virulent. *J. Invertebr. Pathol.* 149, 15–20. doi: 10.1016/j.jip.2017.07.003

Jussila, J., Vrezec, A., Makkonen, J., Kortet, R., and Kokko, H. (2015). "8. Invasive crayfish and their invasive diseases in Europe with the focus on the virulence evolution of the crayfish plague," in *Biological Invasions in Changing Ecosystems. Vectors, Ecological Impacts, Management and Predictions*, ed. J. Canning-Clode (Warsaw: De Gryuter), 183–211. doi: 10.1515/9783110438666-013

Kilpinen, K. (2003). *Suomen Rapu – Ravun Nousu, Tuho Ja Tulevaisuus*. Helsinki: Edita.

Kirjavainen, J., and Sipponen, M. (2004). Environmental benefit of different crayfish management strategies in Finland. *Fish. Manag. Ecol.* 11, 213–218. doi: 10.1111/j.1365-2400.2004.00388.x

Kirjavainen, J., and Westman, K. (1999). Natural history and development of the introduced signal crayfish, *Pacifastacus leniusculus*, in a small, isolated Finnish lake, from 1968 to 1993. *Aquat. Living Res.* 12, 387–401. doi: 10.1016/S0990-7440(99)00110-2

Klobučar, G., Podnar, M., Jelić, M., Franjević, D., Faller, M., Štambuk, A., et al. (2013). Role of the Dinaric Karst (western Balkans) in shaping the phylogeographic structure of the threatened crayfish *Austropotamobius torrentium*. *Freshw. Biol.* 58, 1089–1105. doi: 10.1111/fwb.12110

Kokko, H., Harlioglu, M. M., Aydin, H., Makkonen, J., Gökmen, G., Aksu, Ö, et al. (2018). Observations of crayfish plague infections in commercially important narrow-clawed crayfish populations in Turkey. *Knowl. Manag. Aquatic Ecosyst.* 419:10. doi: 10.1051/kmae/2018001

Kokko, H., Koistinen, L., Harlioğlu, M. M., Makkonen, J., AydIn, H., and Jussila, J. (2012). Recovering Turkish narrow clawed crayfish (*Astacus leptodactylus*) populations carry *Aphanomyces astaci*. *Knowl. Manag. Aquat. Ecosyst.* 404:12. doi: 10.1051/kmae/2012006

Kosunen, K. (2020). *Oral Communication*. Kuopio.

Kotovska, G., Khrystenko, D., Patoka, J., and Kouba, A. (2016). East European crayfish stocks at risk: arrival of non-indigenous crayfish species. *Knowl. Manag. Aquat. Ecosyst.* 417:37. doi: 10.1051/kmae/2016024

Kouba, A., Oficialdegui, F. J., Cuthbert, R. N., Kourantidou, M., South, J., Tricarico, E., et al. (2021). SI on economic costs of invasions – feeling the pinch: global economic costs of crayfish invasions and comparison with other aquatic crustaceans. *Res. Sq.* 13, 1488–1530. doi: 10.21203/rs.3.rs-381161/v1

Kouba, A., Petrusek, A., and Kozák, P. (2014). Continental-wide distribution of crayfish species in Europe: update and maps. *Knowl. Manag. Aquat. Ecosyst.* 413:5. doi: 10.1051/kmae/2014007

Kozubíková, E., Viljamaa-Driks, S., Heinikainen, S., and Petrusek, A. (2011). Spiny-cheek crayfish *Orconectes limosus* carry a novel genotype of the crayfish plague pathogen *Aphanomyces astaci*. *J. Invertebr. Pathol.* 108, 214–216. doi: 10.1016/j.jip.2011.08.002

Kozubíková-Balcarová, E., Koukol, A., Martín, M. P., Svoboda, J., Petrusek, A., and Diéguez-Uribeondo, J. (2013). The diversity of Oomycetes on crayfish: Morphological vs molecular identification of cultures obtained while isolating the crayfish plague pathogen. *Fungal Biol.* 117, 682–691. doi: 10.1016/j.funbio.2013.07.005

Krieg, R., and Zenker, A. (2020). A review of the use of physical barriers to stop the spread of nonindigenous crayfish species. *Rev. Fish Biol. Fish.* 30, 423–435. doi: 10.1007/s11160-020-09606-y

Krieg, R., King, A., and Zenker, A. (2020). Measures to control invasive crayfish species in Switzerland: A success story? *Front. Env. Sci.* 8:609129. doi: 10.3389/fenvs.2020.609129

Kušar, D., Vrezec, A., Ocepek, M., and Jenčič, V. (2013). *Aphanomyces astaci* in wild crayfish populations in Slovenia: first report of persistent infection in a stone crayfish *Austropotamobius torrentium* population. *Dis. Aquat. Organ.* 103, 157–169. doi: 10.3354/dao02567

Larumbe, J. (2020). *Oral Communication*. Navarra.

Lehmann, A., Overton, J. M., and Austin, M. P. (2002). Regression models for spatial prediction: their role for biodiversity and conservation. *Biodiv. Conserv.* 11, 2085–2092. doi: 10.1023/A:1021354914494

Lehtonen, J. U. E. (1975). *Kansanomainen Ravustus Ja Rapujen Hyväksikäyttö Suomessa*. Tapiola: SuomiFinland Oy Weilin+Göös Ab.

Lemmers, P., Collas, F. P. L., Gylstra, R., Crombaghs, B. H. J. M., van der Velde, G., and Leuven, R. S. E. W. (2021). Risks and management of alien freshwater crayfish species in the Rhine-Meuse river district. *Manag. Biol. Inv.* 12, 193–220. doi: 10.3391/mbi.2021.12.1.13

Litchman, E. (2010). Invisible invaders: non-pathogenic invasive microbes in aquatic and terrestrial 1298 ecosystems. *Ecol. Lett.* 13, 1560–1572. doi: 10.1111/j.1461-0248.2010.01544.x

Lodge, D. M., Taylor, C. A., Holdich, D. M., and Skurdal, J. (2000). Nonidigenous crayfish threaten North American freshwater biodiversity: lessons from Europe. *Fisheries* 25, 7–20. doi: 10.1577/15488446

Loureiro, T. G. (2020). "Non-indigenous crayfish species: a global assessment and future perspectives," in *Crayfish: Evolution, Habitat and Conservation Strategies*, ed. F. Bezerra Ribeiro (Hauppauge, NY: Nova Science Publishers), 91–119.

Lovrenčić, L., Bonassin, L., Boštjančić, L. J. L., Podnar, M., Jelić, M., Klobučar, G., et al. (2020). New insights into the genetic diversity of the stone crayfish: taxonomic and conservation implications. *BMC Evol. Biol.* 20:146. doi: 10.1186/s12862-020-01709-1

Lowe, S., Browne, M., Boudjelas, S., and De Poorter, M. (2004). *100 of the world's worst invasive alien species: A selection from the Global Invasive Species Database*. Auckland: University of Auckland.

Maciaszek, R., Bonk, M., and Strużyñski, W. (2019). New records of the invasive red swamp crayfish *Procambarus clarkii* (Girard, 1852)(Decapoda: Cambaridae) from Poland. *Knowl. Manag. Aquat. Ecosyst.* 420, 39. doi: 10.1051/kmae/2019033

Maguire, I., and Gottstein-Matočec, S. (2004). The distribution pattern of freshwater crayfish in Croatia. *Crustaceana* 77, 25–49. doi: 10.1163/156854004323037874

Maguire, I., Jelić, M., Klobučar, G., Delpy, M., Delaunay, C., and Grandjean, F. (2016). Prevalence of the pathogen *Aphanomyces astaci* in freshwater crayfish populations in Croatia. *Dis. Aquat. Org.* 118, 45–53. doi: 10.3354/dao02955

Maguire, I., Klobučar, G., Marčić, Z., and Zanella, D. (2008). The first record of *Pacifastacus leniusculus* in Croatia. *Crayfish News* 30:4.

Makkonen, J., Jussila, J., Kortet, R., Vainikka, A., and Kokko, H. (2012). Differing virulence of *Aphanomyces astaci* isolates and elevated resistance of noble crayfish *Astacus astacus* against crayfish plague. *Dis. Aquat. Org.* 102, 129–136. doi: 10.3354/dao02547

Makkonen, J., Jussila, J., Panteleit, J., Keller, N. S., Schrimpf, A., Theissinger, K., et al. (2018). MtDNA allows the sensitive detection and haplotyping of the crayfish plague disease agent *Aphanomyces astaci* showing clues about its origin and migration. *Parasitology* 145, 1210–1218. doi: 10.1017/s0031182018000227

Makkonen, J., Kokko, H., and Jussila, J. (2015). Mitochondrial cytochrome oxidase I gene analysis indicates a restricted genetic background in Finnish noble crayfish (*Astacus astacus*) stocks. *Knowl. Manag. Aquat. Ecosyst.* 416:21. doi: 10.1051/kmae/2015017

Makkonen, J., Kokko, H., Gökmen, G., Ward, J., Umek, J., Kortet, R., et al. (2019). The signal crayfish (*Pacifastacus leniusculus*) in Lake Tahoe (USA) hosts multiple *Aphanomyces species*. *J. Invertebr. Pathol.* 166:107218. doi: 10.1016/j.jip.2019.107218

Makkonen, J., Kokko, H., Henttonen, P., and Jussila, J. (2010). Crayfish plague (*Aphanomyces astaci*) can be vertically transferred during artificial incubation of crayfish eggs: preliminary results. *Freshw. Crayfish* 17, 151–153. doi: 10.5869/fc.2010.v17.151

Makkonen, J., Kokko, H., Vainikka, A., Kortet, R., and Jussila, J. (2014). Dose-dependent mortality of the noble crayfish (*Astacus astacus*) to different strains of the crayfish plague (*Aphanomyces astaci*). *J. Invertebr. Pathol.* 115, 86–91. doi: 10.1016/j.jip.2013.10.009

Manfrin, A., and Pretto, T. (2014). Aspects of health and disease prevention. RARITY Report, 123-125. RARITY project LIFE10 NAT/IT/000239. Trieste.

Marten, M., Werth, C., and Marten, D. (2004). Der Marmorkrebs (Cambaridae, Decapoda) in Deutsch- land – ein weiteres Neozoon im Einzugsgebiet des Rheins. *Lauterbornia* 50, 17–23.

Martin, P., Shen, H., Füllne, G., and Scholtz, G. (2010). The first record of the parthenogenetic Marmorkrebs (Decapoda, Astacida, Cambaridae) in the wild in Saxony (Germany) raises the question of its actual threat to European freshwater ecosystems. *Aquat. Inv.* 5, 397–403. doi: 10.3391/ai.2010.5.4.09

Martín-Torrijos, L., Campos Llach, M., Pou-Rovira, Q., and Diéguez-Uribeondo, J. (2017). Resistance to the crayfish plague, *Aphanomyces astaci* (Oomycota) in the endangered freshwater crayfish species, *Austropotamobius pallipes*. *PLoS One* 12:e01812. doi: 10.1371/journal.pone.0181226

Martín-Torrijos, L., Correa-Villalona, A., Pradillo, A., and Dieìguez-Uribeondo, J. (2021a). Coexistence of two invasive species, *Procambarus clarkii* and *Aphanomyces astaci*, in brackish waters of a Mediterranean coastal lagoon. *Front. Ecol. Evol* 8:622434. doi: 10.3389/fevo.2020.622434

Martín-Torrijos, L., Kawai, T., Makkonen, J., Jussila, J., Kokko, H., and Diéguez-Uribeondo, J. (2018). Crayfish plague in Japan: A real threat to the endemic *Cambaroides japonicus*. *PLoS One* 13:e0195353. doi: 10.1371/journal.pone.0195353

Martín-Torrijos, L., Kokko, H., Makkonen, J., Jussila, J., and Diéguez-Uribeondo, J. (2019). Mapping 15 years of crayfish plague in the Iberian Peninsula: the impact of two invasive species on the endangered native crayfish. *PLoS One* 14:e0219223. doi: 10.1371/journal.pone.0219223

Martín-Torrijos, L., Martínez-Ríos, M., Casabella-Herrero, G., Collins, J., Adams, S., and Dieìguez-Uribeondo, J. (2021b). Tracing the origin of the crayfish plague pathogen, *Aphanomyces astaci*, to the Southeastern United States. *Sci. Rep.* 11:9332. doi: 10.1038/s41598-021-88704-8

Martiny, J. B. H., Bohannan, B. J., Brown, J. H., Colwell, R. K., Fuhrman, J. A., Green, J. L., et al. (2006). Microbial biogeography: putting microorganisms on the map. *Nat. Rev. Microbiol.* 4, 102–112. doi: 10.1038/nrmicro1341

Matallanas, B., Ochando, M. D., Alonso, F., and Callejas, C. (2016). Update of genetic information for the white-clawed crayfish in Spain, with new insights into its population genetics and origin. *Org. Divers. Evol.* 16, 533–547. doi: 10.1007/s13127-016-0268-4

Matallanas, B., Ochando, M. D., Vivero, A., Beroiz, B., Alonso, F., and Callejas, C. (2011). Mitochondrial DNA variability in Spanish populations of *A. italicus* inferred from the analysis of a COI region. *Knowl. Manag. Aquat. Ecosyst.* 401:30. doi: 10.1051/kmae/2011052

Mathews, L. M., Adams, L., Anderson, E., Basile, M., Gottardi, E., and Buckholt, M. A. (2008). Genetic and morphological evidence for substantial hidden biodiversity in a freshwater crayfish species complex. *Mol. Phylogen. Evol.* 48, 126–135. doi: 10.1016/j.ympev.2008.02.006

McCarthy, J. (2019). Authoritarianism, populism, and the environment: comparative experiences, insights, and perspectives. *Ann. Am. Assoc. Geograph.* 109, 301–313. doi: 10.1080/24694452.2018.1554393

McColl, K. A., Sunarto, A., and Neave, M. J. (2018). Biocontrol of carp: more than just a herpesvirus. *Front. Microbiol.* 9:2288. doi: 10.3389/fmicb.2018.02288

Melotto, A., Manenti, R., and Ficetola, G. F. (2020). Rapid adaptation to invasive predators overwhelms natural gradients of intraspecific variation. *Nat. Commun.* 11, 1–10. doi: 10.1038/s41467-020-17406-y

Messager, M. L., and Olden, J. D. (2018). Individual-based models forecast the spread and inform the management of an emerging riverine invader. *Diver. Distrib.* 24, 1816–1829. doi: 10.1111/ddi.12829

MGOR (2020). *Invazivne Strane Vrste*. Available online at: https://gospodarstvo.gov.hr/aktualno/ministarstvo-pokrenulomobilnu-aplikaciju-za-prijavu-invazivnih-vrsta/12796. (Accessed December 30, 2020).

MMM (2020). *Vesistöt (Water bodies)*. Available online at: https://mmm.fi/vesistot (Accessed December 18, 2020)

Mojžišová, M., Mrugała, A., Kozubíková-Balcarová, E., Vlach, P., Svobodová, J., Kouba, A., et al. (2020). Crayfish plague in Czechia: Outbreaks from novel sources and testing for chronic infections. *J. Invertebr. Pathol.* 173:107390. doi: 10.1016/j.jip.2020.107390

Moorhouse, T. P., and Macdonald, D. W. (2011). The effect of removal by trapping on body condition in populations of signal crayfish. *Biol. Cons.* 144, 1826–1831. doi: 10.1016/j.biocon.2011.03.017

Mrugała, A., Kozubíková-Balcarová, E., Chucholl, C., Cabanillas Resino, S., Viljamaa-Drinks, S., Vukiæ, J., et al. (2014). Trade of ornamental crayfish in Europe as a possible introduction pathway for important crustacean diseases: crayfish plague and white spot syndrome. *Biol. Inv.* 17, 1313–1326. doi: 10.1007/s10530-014-0795-x

Naiman, R. J., and Turner, M. G. (2000). A future perspective on North America's freshwater ecosystems. *Ecol. Appl.* 10, 958–970.

Niemivuo-Lahti, J. (2012). *Kansallinen Vieraslajistrategia*. Helsinki: Maa-ja metsätalousministeriö.

Novitsky, R. A., and Son, M. O. (2016). The first records of Marmorkrebs [*Procambarus fallax* (Hagen, 1870) *f. virginalis*] (Crustacea, Decapoda, Cambaridae) in Ukraine. *Ecol.onten.* 5, 44–46. doi: 10.37828/em.2016.5.8

Nybelin, O. (1936). *Untersuchungen Uber Die Ursache Der In Schweden Gegenvartig Vorkommenden Krebspest. Report of the Institute of Freshwater Research, Drottningholm*. Drottningholm: Carl Bloms Boktr Lund Sweden, 3–29.

Oidtmann, B., Heitz, E., Rogers, D., and Hoffmann, R. W. (2002). Transmission of crayfish plague. *Dis. Aquat. Organ.* 52, 159–167. doi: 10.3354/dao052159

Pacioglu, O., Theissinger, K., Alexa, A., Samoilă, C., Sîrbu, O., Schrimpf, A., et al. (2020). Multifaceted implications of the competition between native and invasive crayfish: a glimmer of hope for the native's long-term survival. *Biol. Invasive* 22, 827–842. doi: 10.1007/s10530-019-02136-0

Paloniitty, T. (2016). The Weser case: Case C-461/13 Bund v Germany. *J. Environ. Law* 28, 151–158. doi: 10.1093/jel/eqv032

Panteleit, J., Horvath, T., Jussila, J., Makkonen, J., Perry, W., Schulz, R., et al. (2019). Invasive rusty crayfish (*Faxonius rusticus*) populations in North America are infected with the crayfish plague disease agent (*Aphanomyces astaci*). *Freshw. Sci.* 38, 425–433. doi: 10.1086/703417

Panteleit, J., Keller, N. S., Diéguez-Uribeondo, J., Makkonen, J., Martín-Torrijos, L., Patrulea, V., et al. (2018). Hidden sites in the distribution of the crayfish plague pathogen *Aphanomyces astaci* in Eastern Europe: relicts of genetic groups from older outbreaks? *J. Invertebr. Pathol.* 157, 117–124. doi: 10.1016/j.jip.2018.05.006

Panteleit, J., Keller, N. S., Kokko, H., Jussila, J., Makkonen, J., Theissinger, K., et al. (2017). Investigation of ornamental crayfish reveals new carrier species of the crayfish plague pathogen (*Aphanomyces astaci*). *Aquat. Inv.* 12, 77–83. doi: 10.3391/ai.2017.12.1.08

Pârvulescu, L., Pérez-Moreno, J. L., Panaiotu, C., Schrimpf, L., Popovici, A., Zaharia, I.-A., et al. (2019). A journey on plate tectonics sheds light on European crayfish phylogeography. *Ecol. Evol.* 9, 1957–1971. doi: 10.1002/ece3.4888

Pârvulescu, L., Schrimpf, A., Kozubíková, E., Cabanillas Resino, S., Vrålstad, T., Petrusek, A., et al. (2012). Invasive crayfish and crayfish plague on the move: first detection of the plague agent *Aphanomyces astaci* in the Romanian Danube. *Dis. Aquat. Organ.* 98, 85–94. doi: 10.3354/dao02432

Pârvulescu, L., Togor, A., Lele, S. F., Scheu, S., Şinca, D., and Panteleit, J. (2017). First established population of marbled crayfish *Procambarus fallax* (Hagen, 1870) *f. virginalis* (Decapoda, Cambaridae) in Romania. *BioInv. Rec.* 6, 357–362. doi: 10.3391/bir.2017.6.4.09

Patoka, J., Buřič, M., Kolář, V., Bláha, M., Petrtýl, M., Franta, P., et al. (2016a). Predictions of marbled crayfish establishment in conurbations fulfilled: evidences from the Czech Republic. *Biologia* 71, 1380–1385. doi: 10.1515/biolog-2016-0164

Patoka, J., Kocánová, B., and Kalous, L. (2016b). Crayfish in Czech cultural space: the longest documented relationship between humans and crayfish in Europe. *Knowl. Manag. Aquat. Ecosyst.* 417:5. doi: 10.1051/kmae/2015038

Patoka, J., Petrtýl, M., and Kalous, L. (2014). Garden ponds as potential introduction pathway of ornamental crayfish. *Knowl. Manag. Aquat. Ecosyst.* 414:8. doi: 10.1051/kmae/2014019

Pavić, D., Bielen, A., Hudina, S., Špoljarić, I., Grandjean, F., Jussila, J., et al. (2021). Monitoring of *Aphanomyces astaci*, causative agent of crayfish plague, in the Plitvice Lakes National Park. *Bioinv. Rec.* 10. (in press).

Peay, S. (2009). Invasive non-indigenous crayfish species in Europe: recommendations on managing them. *Knowl. Manag. Aquat. Ecosyst.* 394-395:3. doi: 10.1051/kmae/2010009

Peay, S., Hiley, P. D., Collen, P., and Martin, I. (2006). Biocide treatment of ponds in Scotland to eradicate signal crayfish. *Bull. Fr. Pêche Piscic.* 380-381, 1363–1379. doi: 10.1051/kmae:2006041

Peay, S., Johnsen, S. I., Bean, C. W., Dunn, A. M., Sandodden, R., and Edsman, L. (2019). Biocide treatment of invasive signal crayfish: successes, failures and lessons learned. *Diversity* 11:29. doi: 10.3390/d11030029

Perdikaris, C., Konstantinidis, E., Georgiadis, C., and Kouba, A. (2017). Freshwater crayfish distribution update and maps for Greece: combining literature and citizen-science data. *Knowl. Manag. Aquat. Ecosyst.* 418:51. doi: 10.1051/kmae/2017042

Persson, M., and Söderhäll, K. (1983). *Pacifastacus leniusculus* Dana and its resistance to the parasitic fungus *Aphanomyces astaci* Schikora. *Freshw. Crayfish* 5, 292–298. doi: 10.5869/fc.1983.v5.292

Préau, C., Nadeau, I., Sellier, Y., Isselin-Nondedeu, F., Bertrand, R., Collas, M., et al. (2020). Niche modelling to guide conservation actions in France for the endangered crayfish *Austropotamobius pallipes* in relation to the invasive *Pacifastacus leniusculus*. *Freshw. Biol.* 65, 304–315. doi: 10.1111/fwb.13422

Pyšek, P., and Richardson, D. M. (2010). Invasive species, environmental change and management, and health. *Ann. Rev. Env. Res.* 35, 25–55. doi: 10.1146/ANNUREV-ENVIRON-033009-095548

RARITY (2020). *LIFE – Rarity Project*. Available online at: http://www.ismar.cnr.it/projects/internationalprojects/copy14_of_project-001/rarity-project?set_language=en&cl=en (accessed December 28, 2020)

Reynolds, J. D. (1988). Crayfish extinctions and crayfish plague in central Ireland. *Biol. Cons.* 45, 279–285. doi: 10.1016/0006-3207(88)90059-6

Rezinciuc, S., Galindo, J., Montserrat, J., and Diéguez-Uribeondo, J. (2014). AFLP-PCR and RAPD-PCR evidences of the transmission of the pathogen *Aphanomyces astaci* (Oomycetes) to wild population of European crayfish from the invasive crayfish species, *Procambarus clarkii*. *Fungal Biol.* 118, 612–620. doi: 10.1016/j.funbio.2013.10.007

Rezinciuc, S., Sandoval-Sierra, J. V., Oidtmann, B., and Diéguez-Uribeondo, J. (2016). "The biology of crayfish plague pathogen *Aphanomyces astaci*: current answers to most frequent questions," in *Freshwater Crayfish – A Global Overview*, eds T. Kawai, Z. Faulkes, and G. Scholtz (London: Taylor and Francis Group), 182–204. doi: 10.1201/b18723-12

Richman, N. I., Böhm, M., Adams, S. B., Alvarez, F., Bergey, E. A., Bunn, J. J., et al. (2015). Multiple drivers of decline in the global status of freshwater crayfish (Decapoda: Astacidea). *Philos. Tr. R. Soc. B* 370:20140060. doi: 10.1098/rstb.2014.0060

Robbins, P. (2011). *Political Ecology: A Critical Introduction*. West Sussex: John Wiley & Sons.

Ruokonen, T. J., Sjövik, R., Erkamo, E., Tulonen, J., Ercoli, F., Kokko, H., et al. (2018). Introduced alien signal crayfish (*Pacifastacus leniusculus*) in Finland-uncontrollable expansion despite numerous crayfisheries strategies. *Knowl. Manag. Aquatic Ecosyst.* 419:27. doi: 10.1051/kmae/2018016

Sahlin, U., Edsman, L., and Bohman, P. (2017). *Riskanalys av Signalkräfta i Sverige.* CEC Rapport Nr 04. Lund: Centre for Environmental and Climate Research (CEC), Lund University.

Sahlin, U., Smith, H. G., Edsman, L., and Bengtsson, G. (2010). Time to establishment success for introduced signal crayfish in Sweden–a statistical evaluation when success is partially known. *J. Appl. Ecol* 47, 1044–1052. doi: 10.1111/j.1365-2664.2010.01849.x

Sahlin, U., Troffaes, M. C., and Edsman, L. (2021). Robust decision analysis under severe uncertainty and ambiguous tradeoffs: an invasive species case study. *Risk Anal.* doi: 10.1111/risa.13722

Sandodden, R. (2019). "Eradication of invasive alien crayfish: past experiences and further possibilities," in *Island invasives: Scaling up to Meet the Challenge, Occasional Paper SSC no. 62*, eds C. R. Veitch, M. N. Clout, A. R. Martin, J. C. Russell, and C. J. West (Gland: IUCN), 405–409.

Sandström, A., Andersson, M., Asp, A., Bohman, P., Edsman, L., Engdahl, F., et al. (2014). Population collapses in introduced non-indigenous crayfish. *Biol. Invasions* 16, 1961–1977. doi: 10.1007/s10530-014-0641-1

Saunders, G., Cooke, B., McColl, K., Shine, R., and Peacock, T. (2010). Modern approaches for the biological control of vertebrate pests: an Australian perspective. *Biol. Control* 52, 288–295. doi: 10.1016/j.biocontrol.2009.06.014

Schäperclaus, W. (1935). Die ursache der pestartigen Krebsterben. *Z. Für Fischer.* 33, 343–366.

Scheers, K., Boets, P., Abeel, T., and Van den Neucker, T. (2020). First records of alien crayfish of the *Procambarus acutus* species complex in Belgium. *BioInv. Rec.* 9, 562–569. doi: 10.3391/bir.2020.9.3.11

Scheers, K., Brys, R., Abeel, T., Halfmaerten, D., Neyrinck, S., and Adriaens, T. (2021). The invasive parthenogenetic marbled crayfish *Procambarus virginalis* Lyko, 2017 gets foothold in Belgium. *BioInv. Rec.* 10, 326–340. doi: 10.3391/BIR.2021.10.2.11

Schikora, F. (1903). Über die Krebspest und ihre Erreger. *Fischerei Zeitung* 6, 353–355.

Schikora, F. (1906). Die Krebspest. *Fischerei Zeitung* 9, 529–532,561–566,581–583.

Scoones, I., Edelman, M., Borras, S. M. Jr., Hall, R., Wolford, W., and White, B. (2018). Emancipatory rural politics: confronting authoritarian populism. *J. Peasant Stud.* 45, 1–20. doi: 10.1080/03066150.2017.1339693

Seitz, R., Vilpoux, K., Hopp, U., Harzsch, S., and Maier, G. (2005). Ontogeny of the Marmorkrebs (marbled crayfish): a parthenogenetic crayfish with unknown origin and phylogenetic position. *J. Exp. Zool. A Comp. Exp. Biol.* 303, 393–405. doi: 10.1002/jez.a.143

Simmonds, P., Aiewsakun, P., and Katzourakis, A. (2019). Prisoners of war—host adaptation and its constraints on virus evolution. *Nat. Rev. Microbiol.* 17, 321–328. doi: 10.1038/s41579-018-0120-2

Söderbäck, B. (1991). Interspecific dominance and aggressive interactions in the freshwater crayfishes *Astacus astacus* (L.) and *Pacifastacus leniusculus* (Dana). *Can. J. Zool.* 69, 1321–1325. doi: 10.1139/z91186

Söderbäck, B. (1994a). Interactions among juveniles of two freshwater crayfish species and a predatory fish. *Oecologia* 100, 229–235. doi: 10.1007/BF00316949

Söderbäck, B. (1994b). Reproductive interference between two co-occurring crayfish species, *Astacus astacus* L. and *Pacifastacus leniusculus* Dana. *Nord. J. Freshw. Res.* 69, 137–143.

Söderbäck, B. (1995). Replacement of the native crayfish *Astacus astacus* by the introduced species *Pacifastacus leniusculus* in a Swedish lake: possible causes and mechanisms. *Freshw. Biol.* 33, 291–304. doi: 10.1111/j.1365-2427.1995.tb01168.x

Söderbäck, B., and Edsman, L. (1998). *Action Plan For The Restoration Of Noble Crayfish*. Stockholm: Fiskeriverket and Naturvårdsverket.

Söderhäll, K., and Cerenius, L. (1999). The crayfish plague fungus: history and recent advances. *Freshw. Crayfish* 12, 11–35. doi: 10.1016/0044-8486(92)90133-6

Souty-Grosset, C., Holdich, D., Noel, P., Reynolds, J. D., and Haffner, P. (2006). *Atlas of Crayfish in Europe*. Paris: Museìum national d'Historie naturelle.

Spitzy, R. (1973). Crayfish in Austria, history and actual situation. *Freshw. Crayfish* 1, 9–14. doi: 10.5869/fc.1973.v1.009

Stebbing, P., Longshaw, M., Scott, A. (2014). Review of methods for the management of non-indigenous crayfish, with particular reference to Great Britain. *Ethol. Ecol. Evol.* 26, 204–231. doi: 10.1080/03949370.2014.908326

Strand, D. A., Jussila, J., Viljamaa-Drinks, S., Kokko, H., Makkonen, J., Holst-Jensen, A., et al. (2012). Monitoring the spore dynamics of *Aphanomyces astaci* in the ambient water of latent carrier crayfish. *Vet. Mic.* 160, 99–107. doi: 10.1016/j.vetmic.2012.05.008

Strayer, D. L., and Dudgeon, D. (2010). Freshwater biodiversity conservation: recent progress and future challenges. *J. North Am. Benthol. Soc.* 29, 344–358. doi: 10.1899/08-171.1

Svärdson, G. (1965). The American crayfish *Pacifastacus leniusculus* (DANA) introduced into Sweden. *Rep. Inst. Freshw. Res. Drottningholm* 46, 90–94.

Svärdson, G. (1968). *Tio år med Signalkräftan. Svenskt Fiske*. 377–379.
Svärdson, G. (1995). The early history of signal crayfish introduction into Europe. *Freshw. Crayfish* 8, 68–77. doi: 10.5869/fc.1995.v8.068
Svoboda, J., Kozubíková, E., Kozák, P., Kouba, A., Koca, S. B., Diler, Ö, et al. (2012). PCR detection of the crayfish plague pathogen in narrow-clawed crayfish inhabiting Lake Eğirdir in Turkey. *Dis. Aquatic Org* 98, 255–259. doi: 10.3354/dao02445
Svoboda, J., Kozubíková-Balcarová, E., Kouba, A., Buřič, M., Kozák, P., Dieìguez-Uribeondo, J., et al. (2013). Temporal dynamics of spore release of the crayfish plague pathogen from its natural host, American spiny-cheek crayfish (*Orconectes limosus*), evaluated by transmission experiments. *Parasitology* 140, 792–801. doi: 10.1017/S0031182012002223
Svoboda, J., Strand, D. A., Vrålstad, T., Grandjean, F., Edsman, L., Kozák, P., et al. (2014). The crayfish plague pathogen can infect freshwater−inhabiting crabs. *Freshw. Biol.* 59, 918–929. doi: 10.1111/fwb.12315
Swahn, J. Ö (2004). The cultural history of crayfish. *Bull. Fr. Pêche. Piscic* 372-73, 243–251.
Taugbøl, T. (2004). Exploitation is a prerequisite for conservation of *Astacus astacus*. *Bull. Fr. Pêche Piscic.* 372-373, 275–279. doi: 10.1051/kmae:2004002
Taugbøl, T., Edsman, L., and Souty-Grosset, C. (2004). Preface second thematic meeting of CRAYNET, Halden Norway, 1-4 September 2003. *Bull. Fr. Pêche Piscic.* 372-373, 233–239. doi: 10.1051/kmae:2004022
Teem, J. L., Alphey, L., Descamps, S., Edgington, M. P., Edwards, O., Gemmell, N., et al. (2020). Genetic biocontrol for invasive species. *Front. Bioeng. Biotech.* 8:452. doi: 10.3389/fbioe.2020.00452
Thomas, J. R., Robinson, C., Mrugaa, A., Ellison, A., Matthews, E., Griffiths, S., et al. (2020). Crayfish plague affects juvenile survival and adult behaviour of invasive signal crayfish. *Parasitol.* 147, 706–714. doi: 10.1017/s0031182020000165
Tiitinen, V. (2020). *Oral Communication*. Lappeenranta.
Todorov, M., Trichkova, T., Hubenov, Z., and Jurajda, P. (2020). *Faxonius limosus* (Rafinesque, 1817) (Decapoda: Cambaridae), a new invasive alien species of European Union concern in Bulgaria. *Acta Zool. Bulgar.* 72, 113–121.
Trožić-Borovac, S., Škrijelj, R., Vesnić, A., Đug, S., Mušović, A., Šljuka, S., et al. (2019). ""Negative effects of introducing allochthonous species *Pacifastacus leniusculus* (Dana, 1852) into aquatic ecosystems of Bosnia and Herzegovina"," in *Book of Abstracts – 3rd Symposium of Freshwater Biology*, eds M. Ivković, I. Stanković, R. Matoničkin Kepčija, and R. Gračan (Zagreb: Croatian Association of Freshwater Ecologists), 33–33.
Unestam, T. (1969a). On the adaptation of *Aphanomyces astaci* as a parasite. *Physiol. Plant.* 22, 221–235. doi: 10.1111/j.1399-3054.1969.tb07371.x
Unestam, T. (1969b). Resistance to the crayfish plague in some American, Japanese and European crayfishes. *Rep. Inst. Freshw. Res., Drott.* 49, 202–209.
Unestam, T. (1972). On the host range and origin of the crayfish plague fungus. *Rep. Inst. Freshw. Res. Drott.* 52, 192–198.
Unestam, T., and Weiss, D. W. (1970). Host parasite relationship between freshwater crayfish and crayfish disease fungus, *Aphanomyces astaci*. Responses to injection by a susceptible and a resistant species. *J. Gen. Microbiol.* 60, 77–90. doi: 10.1099/00221287-60-1-77
Ungureanu, E., Mojžišová, M., Tangerman, M., Ion, M. C., Pârvulescu, L., and Petrusek, A. (2020). The spatial distribution of *Aphanomyces astaci* genotypes across Europe: introducing the first data from Ukraine. *Freshw. Crayfish* 25, 77–87. doi: 10.5869/fc.2020.v25-1.077
Usio, N., and Townsend, C. R. (2008). Functional significance of crayfish in stream food webs: roles of omnivory, substrate heterogeneity and sex. *Oikos* 98, 512–522. doi: 10.1034/j.1600-0706.2002.980316.x
Vey, A., Söderhäll, K., and Ajaxon, A. (1983). Susceptibility of the *Orconectes limosus* Raff. to the crayfish plague, *Aphanomyces astaci* Schikora. *Freshw. Crayfish* 5, 284–291. doi: 10.5869/fc.1983.v5.284
Viljamaa-Dirks, S., Heinikainen, S., Nieminen, M., Vennerström, P., and Pelkonen, S. (2011). Persistent infection by crayfish plague *Aphanomyces astaci* in a noble crayfish population–a case report. *B. Eur. Ass. Fish Pathologists* 31, 182–188.
Vodovsky, N., Patoka, J., and Kouba, A. (2017). Ecosystem of Caspian Sea threatened by pet-traded nonindigenous crayfish. *Biol. Invasions* 19, 2207–2217. doi: 10.1007/s10530-017-1433-1
Vogt, G. (2020). "Biology, ecology, evolution, systematics and utilization of the parthenogenetic marbled crayfish, *Procambarus virginalis*," in *Crayfish: Evolution, Habitat and Conservation Strategies*, ed. F. Bezerra Riveiro (Hauppauge, NY: Nova Science Publishers), 137–227.
Vogt, G., Lukhaup, C., Williams, B. W., Pfeiffer, M., Dorn, N. J., Schulz, R., et al. (2018). Morphological characterization and genotyping of the marbled crayfish and new evidence on its origin. *Zootaxa* 4524, 329–350. doi: 10.11646/zootaxa.4524.3.3
Vojkovská, R., Horká, I., Tricarico, E., and Ďuriš, Z. (2014). New record of the parthenogenetic marbled crayfish *Procambarus fallax* f. virginalis from Italy. *Crustaceana* 87, 1386–1392. doi: 10.1163/15685403-00003365
Weinländer, M., and Füreder, L. (2016). Native and alien crayfish species: do their trophic roles differ? *Freshw. Sci.* 35, 1340–1353. doi: 10.1086/689031
Weiperth, A., Bláha, M., Szajbert, B., Seprõs, R., Bányai, Z., Patoka, J., et al. (2020). Hungary: a European hotspot of non-native crayfish biodiversity. *Know. Manag. Aquat. Ecosyst.* 421, 43. doi: 10.1051/kmae/2020035
Wells, K., Fordham, D. A., Brook, B. W., Cassey, P., Cox, T., O'Hara, R. B., et al. (2018). Disentangling synergistic disease dynamics: Implications for the viral biocontrol of rabbits. *J. Anim. Ecol.* 87, 1418–1428. doi: 10.1111/1365-2656.12871
Westman, K. (1973). The population of the crayfish, *Astacus astacus* L. in Finland and the introduction of the American crayfish *Pacifastacus leniusculus* Dana. *Freshw. Crayfish* 1, 41–55. doi: 10.5869/fc.1973.v1.041
Westman, K. (2002). "Alien crayfish in Europe: negative and positive impacts and interactions with native crayfish," in *Invasive Aquatic Species of Europe. Distribution, Impacts and Management*, eds E. Leppäkoski, S. Gollasch, and S. Olenin (Dordrecht: Springer), 76–95. doi: 10.1007/978-94-015-9956-6_9
Westman, K., and Savolainen, R. (2001). Long term study of competition between two co-occurring crayfish species, the native *Astacus astacus* L. and the introduced *Pacifastacus leniusculus* Dana, in a Finnish lake. *Bull. Franç. Pêche Piscic.* 361, 613–627. doi: 10.1051/kmae:2001008
Westman, K., Savolainen, R., and Julkunen, M. (2002). Replacement of the native crayfish *Astacus astacus* by the introduced species *Pacifastacus leniusculus* in a small, enclosed Finnish lake: a 30−year study. *Ecography* 25, 53–73. doi: 10.1034/j.1600-0587.2002.250107.x
Wu, L., Subramanian, N., Abdulrahman, M. D., Liu, C., and Pawar, K. S. (2017). Short-term versus long-term benefits: Balanced sustainability framework and research propositions. *Sust. Prod. Consumpt.* 11, 18–30. doi: 10.1016/j.spc.2016.09.003

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Ecology and Evolution

BRIEF RESEARCH REPORT
published: 11 August 2021
doi: 10.3389/fevo.2021.681026

How Raccoons Could Lead to the Disappearance of Native Crayfish in Central Italy

Elena Tricarico[1]†, Paola Ciampelli[2], Laura De Cicco[1], Sandro Aurelio Marsella[1], Lorenzo Petralia[2], Barbara Rossi[2], Antonio Zoccola[2] and Giuseppe Mazza[3†]*

[1] Department of Biology, University of Florence, Florence, Italy, [2] Reparto Carabinieri Biodiversità, Arezzo, Italy, [3] CREA – Research Centre for Plant Protection and Certification (CREA-DC), Florence, Italy

OPEN ACCESS

Edited by:
Kathrin Theissinger, LOEWE Centre for Translational Biodiversity Genomics (LOEWE-TBG), Germany

Reviewed by:
Lucian Pârvulescu, West University of Timişoara, Romania
Christoph Chucholl, Fischereiforschungsstelle Baden-Württemberg, Germany
Anne Schrimpf, University of Koblenz and Landau, Germany

***Correspondence:**
Elena Tricarico
elena.tricarico@unifi.it

†ORCID:
Elena Tricarico
orcid.org/0000-0002-6644-1429
Giuseppe Mazza
orcid.org/0000-0002-6644-1429

Specialty section:
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *15 March 2021*
Accepted: *16 July 2021*
Published: *11 August 2021*

Citation:
Tricarico E, Ciampelli P, De Cicco L, Marsella SA, Petralia L, Rossi B, Zoccola A and Mazza G (2021) How Raccoons Could Lead to the Disappearance of Native Crayfish in Central Italy. Front. Ecol. Evol. 9:681026. doi: 10.3389/fevo.2021.681026

The white-clawed crayfish *Austropotamobius pallipes* complex populations are decreasing in the Foreste Casentinesi, Monte Falterona and Campigna National Park (Central Italy), due to several factors, including illegal poaching, predatory fishes, drought, and invasive alien species. Recently, the Northern raccoon *Procyon lotor* has been reported to be present in the area of the National Park and has started to predate on the white-clawed crayfish. The aim of the study was to update the distribution and population status of *A. pallipes* in the reserves, other sites of the National Park, and surrounding areas to assess the potential effects of the raccoon. Crayfish were sampled by hand or by traps in 14 sites; sampled individuals were sexed and measured. Signs of raccoon presence (e.g., footprints and predated crayfish) were also recorded. Our study confirms the impact of raccoon on native crayfish: indeed, where the invasive mammal is present (six sites), crayfish disappeared, or their populations have been dramatically reduced in number, with a size distribution skewed towards juveniles. In two sites, close to urban settlements, fresh footprints of *P. lotor* and predated specimens of *A. pallipes* were also observed. Populations of crayfish are still abundant or even increasing as compared with samplings conducted in the past where raccoon is absent (five sites). Urgent actions (e.g., control of raccoons, and monitoring and restocking of *A. pallipes* populations if feasible and where possible) should be taken into account to guarantee the survival of this protected species.

Keywords: *Austropotamobius pallipes* complex, Foreste Casentinesi, Monte Falterona and Campigna National Park, *Procyon lotor*, predation, *Potamon fluviatile*, population decrease

INTRODUCTION

Freshwater environments are among the ecosystems most threatened by human activities, and several impacts on them lead to a crisis in freshwater biodiversity (Abell, 2002). In these ecosystems, freshwater crayfish (Crustacea: Decapoda: Astacidae) have a crucial key-role, as they act as bioindicators for water quality, keystone species in trophic webs, and ecological engineers (Reynolds et al., 2013). Recent declines in several crayfish species, caused by multiple factors, such as habitat modification, water pollution, and the invasion of alien species (e.g., Manenti et al., 2019), have highlighted the need to identify and recommend appropriate management conservation actions and policies (Richman et al., 2015).

The white-clawed crayfish *Austropotamobius pallipes* complex (hereafter referred as *A. pallipes*) is one of the most threatened crayfish species in Europe (Chucholl, 2016), characterized by declining population densities and restricted distribution (Alonso et al., 2000; Kozak et al., 2011; Mazza et al., 2011, 2017). This species usually inhabits permanent, clear, well-oxygenated, and moderately cold (with temperatures below 24°C) freshwater bodies (e.g., headwaters and streams), rich in submerged cobbles, rocks, tree roots, and detritus (Füreder et al., 2010). This K-selected species has a slow growth rate and a relatively long average life span (Aquiloni et al., 2010). Sexual maturity is reached relatively late (around 3 years), and fertility is low (50–200 eggs per female). Moreover, mating takes place only once per year in autumn and is followed by a long breeding period, until the spring/summer of the subsequent year, during which females hide in a shelter to take care first of the eggs and then of the juveniles (Aquiloni et al., 2010). All these listed features make this crayfish particularly vulnerable and, despite being listed in Annex II of the EU Directive on the Conservation of Habitats, Flora, and Fauna (92/43/EEC), in Appendix II of the Bern Convention, and being considered "endangered" on the Red List of the IUCN, its decline is still ongoing, caused by the joint action of multiple factors, such as habitat alteration, pollution, climate change, invasive alien crayfish, and disease spread (e.g., Alonso et al., 2000; Mazza et al., 2011, 2017).

Recently, among the several threats affecting this protected crayfish, the impact of the Northern raccoon *Procyon lotor* was reported in Central Italy, in the Foreste Casentinesi, Monte Falterona and Campigna National Park (Tuscany and Emilia-Romagna) (Boscherini et al., 2020; Boncompagni et al., 2021). This mammal is an opportunistic species native to Central America, the United States, and southern Canada. It was introduced to Eurasia in the 20th century for fur and as an exotic pet (Salgado, 2018). It is listed amongst the 100 worst invasive alien species in Europe due to the multiple and severe impacts (Salgado, 2018), and it is included in the list of invasive alien species of Union concern linked to the EU Regulation 1143/2014 on invasive alien species. In Italy, *P. lotor* has been recorded in eight regions, although reproductive populations only occur in Lombardy and in an area between Tuscany and Emilia-Romagna regions (e.g., Mori et al., 2015; Boscherini et al., 2020). Boncompagni et al. (2021) confirmed the omnivorous food habits of the Northern raccoon, in both the native and introduced ranges, and the evident signs of predation on *A. pallipes* in the Foreste Casentinesi National Park, already reported in Boscherini et al. (2020). In this protected area, the native crayfish is at risk due to several other factors, including illegal poaching, predatory fishes, and drought. Moreover, invasive alien species are becoming an important threat for the species. Indeed, since 2015, the presence of the invasive red swamp crayfish *Procambarus clarkii* (Mazza et al., 2011, 2017) has been reported at the border of the National Park, and it is contributing to the local sharp decline of the native clawed crayfish due to the spread of crayfish plague *Aphanomyces astaci*, already detected in the area (T. Pretto, pers. comm.). Moreover, the invasive population of the Northern raccoon is exponentially increasing in the National Park (Boscherini et al., 2020; Boncompagni et al., 2021). Thus, following the suggestions provided by Boncompagni et al. (2021), the purpose of our research was to update the distribution and population status of *A. pallipes* in the reserves, other sites of the National Park, and surrounding areas in order to assess the potential effects of the raccoon on the native crayfish and suggest management actions to limit its impacts. For the first time, this study monitored all the reserves of the National Park for the native crayfish: indeed, in the past, only some of them were sampled.

MATERIALS AND METHODS

Study Area

The National Park of the "Foreste Casentinesi, Monte Falterona e Campigna" is a ca. 36,000-ha protected area in the Tuscan–Emilian Apennines (Central Italy). This area includes one of the most high-quality forested areas in Europe and a variety of animal and plant species of great conservation value. The natural reserves of the Park protect a 53-km^2 forested area (**Figure 1**). These reserves are included within the Natura 2000 network and are completely covered by forests, with portions of old-growth forests ("Riserva Integrale di Sasso Fratino"). The main types of vegetation associations are mono-specific European beech *Fagus sylvatica* woodland on mountain tops, where residual secondary grasslands also occur. Mixed woodlands of silver fir *Abies alba* and European beech are present on warm and wet slopes; forests of oaks (*Quercus cerris*, *Quercus pubescens*, and *Quercus petraea*) and hornbeams *Ostrya carpinifolia* occur at the lowest altitudes (Petralia et al., 2019). Among carnivores, the gray wolf *Canis lupus italicus* and the European wildcat *Felis silvestris silvestris* are the apex predators. Small-sized carnivores include the red fox *Vulpes vulpes*, the European badger *Meles meles*, the stone marten *Martes foina*, the Western polecat *Mustela putorius*, and the least weasel *Mustela nivalis*. The pine marten *Martes martes* is present at low density only in the northern part of the National Park, outside the reserves (Bottacci, 2009; Ragni et al., 2015).

Crayfish Sampling

Our samplings were conducted in 14 sites (**Table 1**). We selected sites within the natural reserves and with the presence of raccoon to assess the situation of native clawed crayfish in the natural reserves of the National Park (and some areas nearby) and in the presence of raccoon. From previous samplings, the presence of *A. pallipes* was known in 11 out of the 14 sampled sites. Three sites (Fosso Abetiolo, Fosso di Campo alla Sega, and Fiume d'Isola) were monitored for the first time during the present study. All the sites (except Laghetto di Metaleto, a small lake of 1,159 m^2 located at 900 m a.s.l.) are small streams of second/third order located at an altitude range between 500 and 1,000 m a.s.l., composed of run and pools, with an average water depth of 50 cm, and a water temperature between 14 and 16°C. Crayfish and the native crab *Potamon fluviatile* also reported in the area were sampled from July to September 2020. Nighttime searching was conducted during the period of the species' maximum activity (summer, one transect per site). Crayfish (and crabs) were searched by hand for an hour (for the streams, the investigated

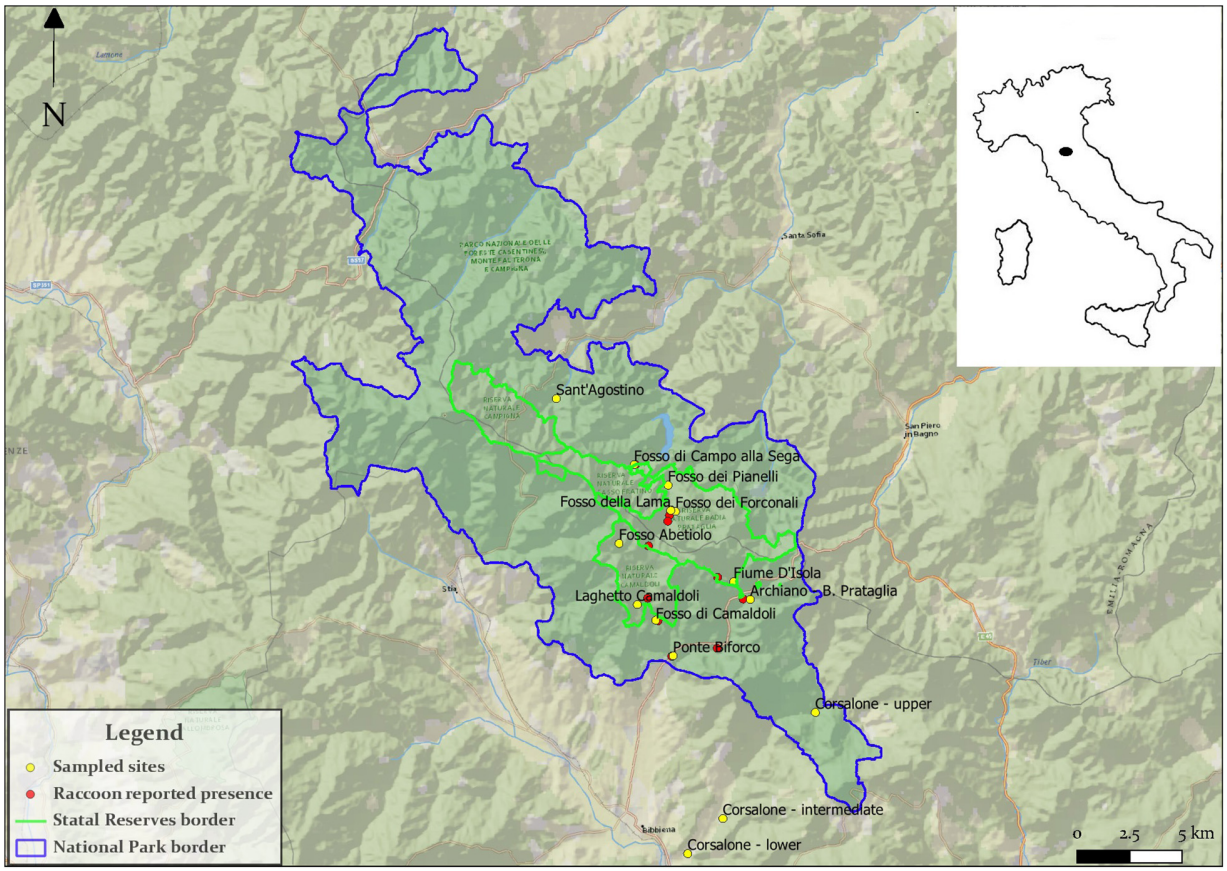

FIGURE 1 | The sampled sites in the Foreste Casentinesi, Monte Falterona, and Campigna National Park, and surroundings.

length ranged between 250 and 750 m) in all possible refuges: stones and leaf litter on the bottom of watercourses, and holes along the riverbanks. For Laghetto di Metaleto only, 10 baited crayfish traps were set for 24 h, because the hand search was not possible for its depth (2 m) and steep banks. Each captured individual was sexed and measured in the field using a digital caliper (accuracy, ±0.2 mm) [crayfish, cephalothorax length (CL) from the tip of the rostrum to the end of the cephalothorax; crab, maximum width of cephalothorax (CW)]. Crayfish with a CL $\leq$ 25 mm were considered as "juveniles," whereas specimens with CL > 25 mm were reported as "mature crayfish" (Pratten, 1980). Measurements were conducted after disinfection of equipment to avoid disease transmission; then, crustaceans were immediately released at the same place of collection. The population abundance of *A. pallipes* was estimated using the method of "catch per unit effort" (CPUE), i.e., two researchers captured all the crayfish observed within an hour of hand searching and number of sampled crayfish on the number of used traps for Laghetto di Metaleto (Mazza et al., 2012). The number of dead and predated specimens by the Northern raccoon was reported. Consumption of adult crayfish by the Northern raccoon involves mostly the abdomen and part of the thorax, with head and chelae often left unconsumed on the riverbanks or riverbeds (Boscherini et al., 2020; Boncompagni et al., 2021). This modality of predation is indeed typical of raccoon and otter (which is not, however, present in the area), but not of the other mammals present in the National Park, as reported in Boncompagni et al. (2021). Signs of raccoon presence (i.e., their footprints) were also recorded. The presence of the raccoon was confirmed also by camera trapping data (data not showed). Sex and mature/juveniles were compared within watercourse by chi-squared test (statistic: χ^2). CL of males and females within each site was compared by the non-parametric two-sample Mann–Whitney test (statistic: z). The level of significance at which the null hypothesis was rejected is $\alpha = 0.05$. Text, **Table 1** and **Figures 2**, **3** give medians and interquartile ranges (first-third quartiles).

RESULTS

Overall, CPUE was not high, excluding two sites (Laghetto di Metaleto and Bidente di Campigna). Mature and bigger individuals are more abundant when the raccoon is absent (Laghetto di Metaleto, Fosso Bidente di Campigna, Fosso di Campo alla Sega, and Torrente Corsalone, upper part; **Table 1**). Where the presence of raccoon is confirmed (six sites), crayfish were not present in two sites where they were present in the

TABLE 1 | Number, sex (M, male; F, female) and cephalothorax length (CL) in mm of sampled crayfish, the presence of raccoon signs, and the confirmed presence of raccoon per site.

Site	Reserve	Sampled crayfish									CL (mm)				Raccoon signs	
		Total	M	F	χ^2	p	Ma	J	χ^2	*p*	M	F	Z	p	Predated crayfish	Footprint
Fiume d'Isola	Badia Prataglia	0	0	0	–	–	–	–	–	–	–	–	–	–	0	No
Torrente Archiano (Badia Prataglia)	Badia Prataglia	0	0	0	–	–	–	–	–	–	–	–	–	–	1 (CL: 40 mm)	Yes
Laghetto di Metaleto	Camaldoli	114	23	90	38.55	**<0.00001**	113	1	37	**<0.00001**	49 (45–50)	42 (39–44)	−4.48	**<0.00001**	0	No
Fosso Abetiolo	Camaldoli	0	0	0	–	–	–	–	–	–	–	–	–	–	0	No
Fosso di Camaldoli	Camaldoli	0	0	0	–	–	–	–	–	–	–	–	–	–	3 (CL: 33,37,44 mm)	Yes
Fosso di Camaldoli, Ponte Biforco	–	1	1	0	–	–	–	1	–	–	24	–	–	–	0	No
Fosso Bidente di Campigna, Sant'Agostino	Campigna	109	55	54	0	1	94	15	35.6	**<0.00001**	37 (33–39.75)	34 (31.5–38)	−2.06	**0.04**	0	No
Fosso della Lama	Lama	4	2	2	0	1	1	3	5.33	**0.02**	17, 28	14.5, 24	–	–	0	No
Fosso Forconali	Lama	11	5	6	0	1	3	8	5.23	**0.02**	23 (18–27)	17 (14.75–18.5)	−1.28	0.20	0	No
Fosso di Campo alla Sega	Lama	33	17	10	2.37	0.12	12	21	8.12	**0.004**	24 (19–39)	21 (18.25–32.25)	0.95	0.34	0	No
Fosso dei Pianelli	Lama	15	7	5	0.33	0.56	6	9	4.8	**0.03**	24 (19.5–38)	32 (18–33)	0.73	0.47	2 (CL: 18,35 mm)	No
Torrente Corsalone intermediate part*	–	11	6	5	0	1	6	5	0	1	29 (25–33.75)	31 (20–34)	0.37	0.71	0	No
Torrente Corsalone lower part*	–	0	0	0	–	–	–	–	–	–	–	–	–	–	0	No
Torrente Corsalone upper part (Siregiolo)	–	55	21	33	2.67	0.10	43	12	34	**<0.00001**	32 (25–36)	32 (29–38)	0.99	0.32	0	No

Median values (+ 1st and 3rd interquartiles) are reported for the CL. Sex and mature (Ma)/juvenile (J) crayfish are compared by chi-squared test (statistic: χ^2)
CL of males and females within each site was compared by the non-parametric two-sample Mann–Whitney test (statistic: z).
Significant p-values are indicated in bold.
The asterisks indicate the sites where the native river crab Potamon fluviatile has been found. The sites where the presence of raccoon has been confirmed from previous studies and data are highlighted in bold.

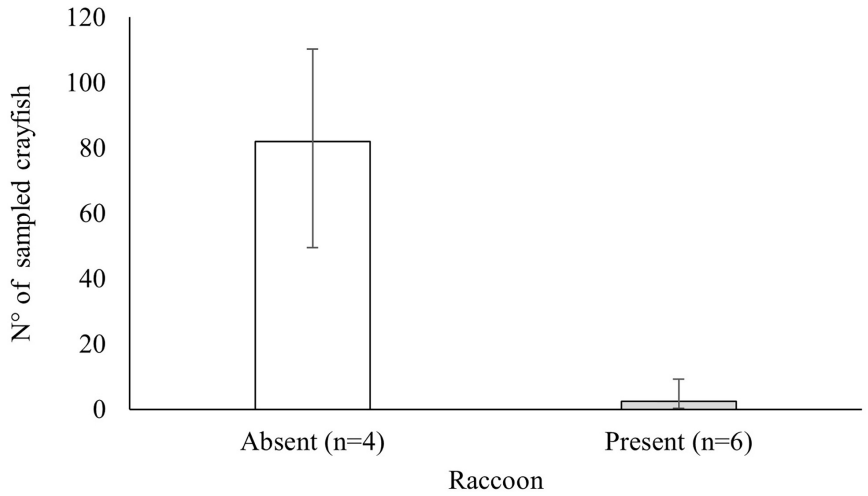

FIGURE 2 | Number of crayfish sampled in sites with or without raccoon in the Foreste Casentinesi, Monte Falterona, and Campigna National Park. The three sites sampled for the first time (Fosso Abetiolo, Fosso di Campo alla Sega, and Fiume d'Isola) were not considered in the graph. Bars represent medians + 1st and 3rd interquartiles.

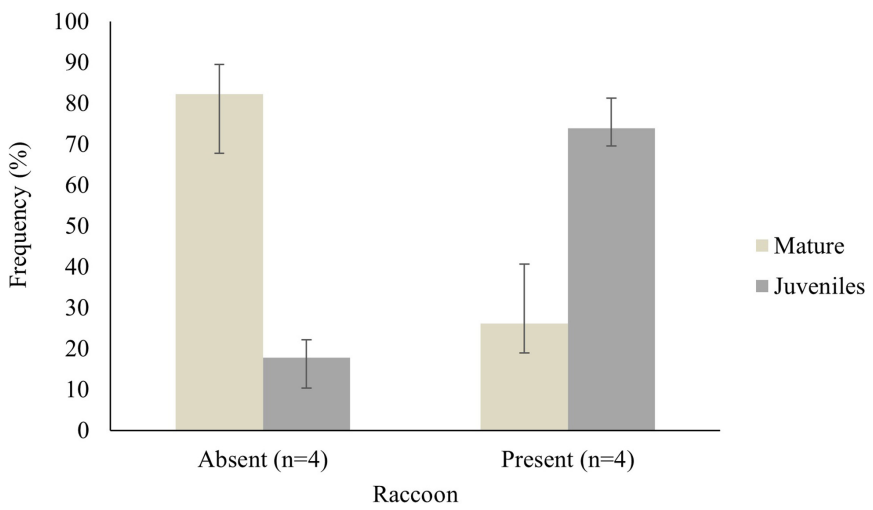

FIGURE 3 | Frequency of mature and juvenile crayfish in sites with or without raccoon in the Foreste Casentinesi, Monte Falterona, and Campigna National Park. The three sites sampled for the first time (Fosso Abetiolo, Fosso di Campo alla Sega, and Fiume d'Isola) were not considered in the graph. Bars represent medians + 1st and 3rd interquartiles.

past, while in the other four sites, CPUE was very low, individual crayfish size was very small, and crayfish were mostly juveniles (**Table 1** and **Figures 2**, **3**). Moreover, in two sites, fresh footprints and predated specimens were observed, confirming the presence of raccoon (**Figure 4** and **Table 1**). Sex ratio was balanced, except in Laghetto di Metaleto (**Table 1**). In the lower part of Corsalone, only the native crab was found: the sampled individuals were seven males and seven females with similar size: CW males = 41 (37.5–45) mm, CW females = 44 (39.5–46.5) mm ($z = 0.38$, $p = 0.70$). In the intermediate part of Corsalone, crayfish were found to co-occur with crab (one captured females of CW = 34 mm, two other individuals of similar size spotted).

DISCUSSION

The invasive population of the raccoon in Central Italy is exponentially increasing its range (Boscherini et al., 2020; Boncompagni et al., 2021). In our study area, raccoons show a wide ecological plasticity, mostly selecting the immediate surroundings of watercourses for feeding and for denning; moreover, its dispersal is associated with rivers and valleys, which may increase the risk for crustaceans (Puskas et al., 2010; Mori et al., 2015; Mazzamuto et al., 2020). The raccoon has been found to actively predate on *A. pallipes* (Boncompagni et al., 2021), and our study confirms the impact of this invasive mammal on native crayfish: indeed, where the species is present,

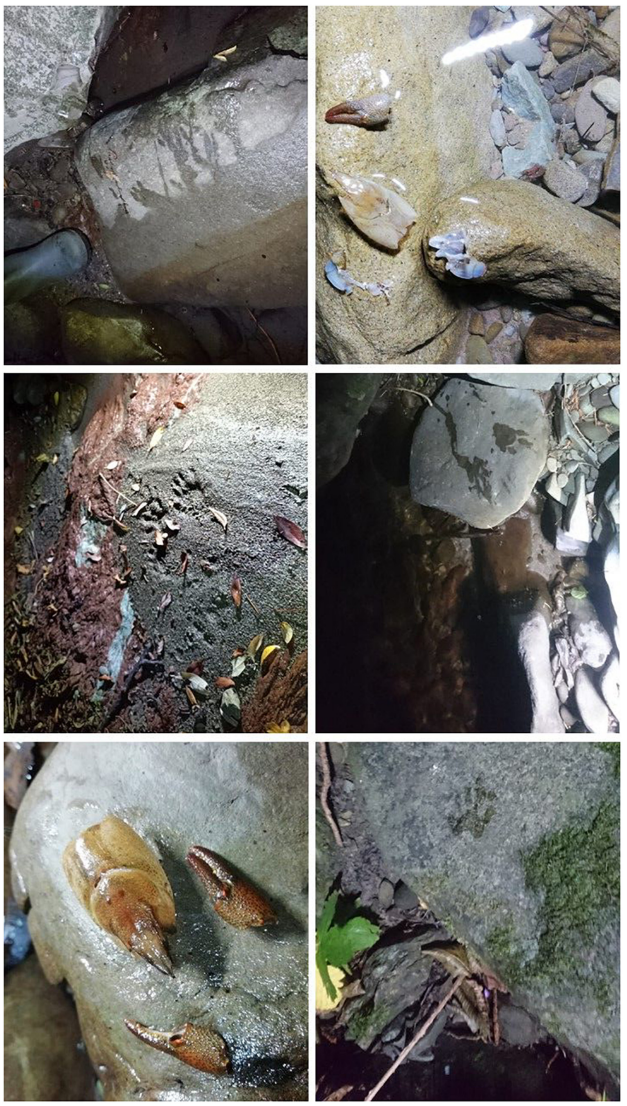

FIGURE 4 | Fresh footprints of raccoons and predated *Austropotamobius pallipes* complex specimens in the study area. Photos by© G. Mazza.

crayfish disappeared or their populations have been dramatically reduced in number, affecting their size distribution. We excluded other factors being responsible of this decline, because droughts or events of pollution were not reported, and four out of six impacted sites are also isolated and far away from human settlements (so not reachable for illegal harvesting) and with a population skewed to juveniles (that could not be the effect of a crayfish plague event). Moreover, we excluded that crayfish were preyed upon by native species (e.g., badgers, polecats, wild boars, herons, and red foxes), as this mortality rate has never been recorded before raccoon invasion (Mazza et al., 2011), when badgers and foxes were already abundant in the area. In Fosso di Camaldoli, Ponte Biforco, an abundant population of crayfish was reported in 2019 (G. Mazza, pers. comm.); and similarly, in Torrente Archiano, the presence of crayfish was detected until 2017 (A. Zoccola, pers. comm.). For these two sites, close to urban settlements, we did not consider the illegal poaching as a cause because several individuals of *P. lotor* were observed by local people and its fresh footprints and above all predated specimens of *A. pallipes* were reported during our survey (**Figure 3** and **Table 1**). An episode of crayfish plague could not be completely discarded, even if only one event of crayfish plague was reported and far from these sites (T. Pretto, pers. comm.). Thus, the presence of raccoon weighs in favor of its impact (but the mammal could have transported the plague as well; see the conclusions). As showed by Boncompagni et al. (2021) and in our study (**Table 1**), the raccoon predates on medium-large individuals, which are the reproductive ones: indeed, in Fosso Forconali, Fosso dei Pianelli, and Fosso della Lama where in 2019 many predated adult crayfish were found (Boncompagni et al., 2021), only few individuals, mostly juveniles, have been sampled. Indeed, in these water courses, the CPUE sharply decreased through years (48 crayfish per hour in 1999: Cenni, 2001; 45 crayfish per hour in 2008–2009: Mazza et al., 2011; 22 crayfish per hour in 2019: Boncompagni et al., 2021). Considering that the native crayfish starts reproducing after 3 years, it is evident that these affected populations could disappear if management measures (i.e., controlling the raccoon) to protect them would not be considered. Studies conducted in central Spain (García et al., 2012) and in the United States (Byrne and Chamberlain, 2012) detected raccoon eating crayfish as well as predated crayfish and raccoon tracks, confirming that this crustacean is an important food source for the species and supporting the findings of the present study. Crayfish are also absent from Fiume d'Isola and Fosso Abetiolo, where the raccoon is not present: this could be due to these sites' environmental characteristics, because they are very narrow and not deep streams (water depth is less than 10 cm) without pools, but no previous surveys have been conducted to confirm this hypothesis.

Populations of crayfish are still abundant in Bidente di Campigna (CPUE in 2008–2009: 122 crayfish per hour; Mazza et al., 2011) and even increased in Laghetto di Metaleto (CPUE in 2012: 26 crayfish; Mazza et al., 2012; CPUE in 2016 and 2017: 35 and 38 crayfish, respectively, G. Mazza, unpublished data) and Torrente Corsalone upper part, Siregiolo (CPUE in 2015: 22 crayfish per hour; Mazza et al., 2017). In these sites, the raccoon is absent; moreover, Laghetto di Metaleto, being fenced and deep, permits the survival of this endangered crayfish. Concerning Fosso di Campo alla Sega, we do not have previous data, on crayfish as it was the first time that the species was sampled.

Our study confirms the co-occurrence of native crayfish and crabs in Corsalone stream found by Mazza et al. (2017) with similar distribution, CPUE, and size of sampled animals, highlighting that this co-occurrence is stable through time and that when the two species are present in the same watercourse, crabs and crayfish tend to occupy the lower and upper parts, respectively.

No reliable data on the population size of raccoon are available, although Boscherini et al. (2020) presented several records in this area. Additionally, data on impacts are scattered and concern predation on pets, livestock, and recently the indigenous crayfish. It could be interesting to evaluate its

feeding activity on native crab present in the National Park. Thus, monitoring of established raccoon populations and early detection of new nuclei require particular attention and should be constantly carried out. Unfortunately, since 2015, few management actions (e.g., capture of few individuals) have been conducted to control the raccoon. Thus, urgent actions (e.g., control of raccoons, monitoring of *A. pallipes* populations, and a recovery through restocking activities where possible) should be taken into account to guarantee the survival of this crayfish species, which is protected according to national and international laws. Moreover, considering the presence of the red swamp crayfish and fast spread of the raccoon, the possibility that this invasive mammal can carry the crayfish plague should be assessed. It would be also relevant to analyze raccoon's scats and stomach content (if dead specimens are available) to definitively support our findings. In conclusion, the introduction of raccoons appears to have caused a decrease in the native crayfish populations: these invasive mammals currently constitute a major risk factor for the survival of the crayfish species in this area, already threatened by several other factors (Mazza et al., 2011, 2017). Management actions are mandatory; otherwise, as narrated in the Indian Legend "Raccoon and the Crawfish" (Holmgren, 1990)[1], the destiny of the crayfish is already written, and the risk of losing several populations of this crustacean will become a reality.

[1] https://www.terrain.org/fiction/5/raccooncrayfish.htm

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

ET conceived the study and collected and analyzed the field data. GM conceived the study and collected the field data. ET and GM led the manuscript writing. LD collected and inserted the field data. SM, LP, BR, and AZ collected the field data. PC planned the sampling and provided funding. All authors contributed critically to the draft and gave final approval for submission.

FUNDING

This study has been financed by Reparto Carabinieri Biodiversità (Stia, Arezzo, Italy).

ACKNOWLEDGMENTS

We warmly thank operaio OTD Andrea Ciani and Alessandro Tondo for their help during the sampling sessions. This paper is dedicated to our friend Alessandro Elio Agnelli who recently passed away and loved this National Park and Corsalone stream.

REFERENCES

Abell, R. (2002). Conservation biology for the biodiversity crisis: a freshwater follow-up. *Conserv. Biol.* 16, 1435–1437. doi: 10.1046/j.1523-1739.2002.01532.x

Alonso, F., Temiño, C., and Diéguez-Uribeondo, J. (2000). Status of the white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858), in Spain: distribution and legislation. *Bull. Fr. Pêche Piscic.* 356, 31–53. doi: 10.1051/kmae:2000003

Aquiloni, L., Tricarico, E., and Gherardi, F. (2010). Crayfish in Italy: distribution, threats and management. *Int. Aquat. Res.* 2, 1–14.

Boncompagni, L., Molfini, M., Ciampelli, P., Fazzi, P., Lucchesi, M., Mori, E., et al. (2021). No country for native crayfish: importance of crustaceans in the diet of native and alien Northern raccoons. *Ethol. Ecol. Evol.* doi: 10.1080/03949370.2021.1872710

Boscherini, A., Mazza, G., Menchetti, M., Laurenzi, A., and Mori, E. (2020). Time is running out! Rapid range expansion of the invasive northern raccoon in central Italy. *Mammalia* 84, 98–101. doi: 10.1515/mammalia-2018-0151

Bottacci, A. (2009). *La Riserva Naturale Integrale di Sasso Fratino: 1959-2009. 50 Anni di Conservazione Della Biodiversità [The Natural Reserve of Sasso Fratino: 1959-2009. 50 Years of Biodiversity Conservation]*. Arezzo: CFS/UTB Pratovecchio Editions.

Byrne, M., and Chamberlain, M. J. (2012). Using first-passage time to link behaviour and habitat in foraging paths of a terrestrial predator, the racoon. *Anim. Behav.* 84, 593–601. doi: 10.1016/j.anbehav.2012.06.012

Cenni, F. (2001). *Austropotamobius Pallipes nel Parco Nazionale Delle Foreste Casentinesi, Monte Falterona e Campigna: Struttura di Popolazione e Tasso di Infestazione da Branchiobdellidi [Austropotamobius Pallipes in the Foreste Casentinesi, Monte Falterona and Campigna National Park: Population Structure and Infestation Rate by Branchiobdellids]*. Ph.D. thesis. Florence: Università degli Studi di Firenze.

Chucholl, C. (2016). The bad and the super-bad: prioritising the threat of six invasive alien to three imperilled native crayfishes. *Biol. Invasions* 18, 1967–1988. doi: 10.1007/s10530-016-1141-2

Füreder, L., Gherardi, F., Holdich, D. M., Reynolds, J. D., Sibley, P., and Souty-Grosset, C. (2010). *Austropotamobius pallipes. The IUCN Red List of Threatened Species. Version 2014.3*. Available online at: https://www.iucnredlist.org (accessed July 1, 2021).

García, J. T., García, F. J., Alda, F., Gonzalez, J. L., Aramburù, M. J., Cortès, Y., et al. (2012). Recent invasion and status of the raccoon (*Procyon lotor*) in Spain. *Biol. Invasions* 14, 1305–1310. doi: 10.1007/s10530-011-0157-x

Holmgren, V. C. (1990). *Raccoons: In folklore, History & Today's Backyards*. Santa Barbara, CA: Capra Press, 157.

Kozak, P., Fureder, L., Kouba, A., Reynolds, J., and Souty-Grosset, C. (2011). Current conservation strategies for European crayfish. *Knowl. Manag. Aquat. Ec.* 401:1. doi: 10.1051/kmae/2011018

Manenti, R., Ghia, D., Fea, G., Ficetola, G. F., Padoa-Schioppa, E., and Canedoli, C. (2019). Causes and consequences of crayfish extinction: stream connectivity, habitat changes, alien species and ecosystem services. *Freshw. Biol.* 64, 284–293. doi: 10.1111/fwb.13215

Mazza, G., Agostini, N., Aquiloni, L., Carano, G., Inghilesi, A. F., Tricarico, E., et al. (2011). The indigenous crayfish *Austropotamobius pallipes* complex in a national park of central Italy. *Knowl. Manag. Aquat. Ec.* 401:24. doi: 10.1051/kmae/2011041

Mazza, G., Bottacci, A., Cianferoni, F., Rocchi, S., Rossi, B., Terzani, F., et al. (2012). "Il gambero di fiume (*Austropotamobius pallipes* complex) nella Riserva naturale biogenetica di Camaldoli," in *La Riserva Naturale Biogenetica di Camaldoli. 1012-2012. Mille Anni di Rapporto Uomo-Foresta*, ed. A. Bottacci (Arezzo: CFS/UTB Pratovecchio), 317–321. doi: 10.4129/ifm.2012.4.01

Mazza, G., Tricarico, E., Cianferoni, F., Stasolla, G., Inghilesi, A. F., Zoccola, A., et al. (2017). Native crab and crayfish cooccurrence: first evidence in Europe. *Biologia* 72, 790–795. doi: 10.1515/biolog-2017-0086

Mazzamuto, M. V., Panzeri, M., Bisi, F., Wauters, L. A., Preatoni, D., and Martinoli, A. (2020). When management meets science: adaptive analysis for the optimization of the eradication of the Northern raccoon (*Procyon lotor*). *Biol. Invasions* 22, 3119–3130. doi: 10.1007/s10530-020-02313-6

Mori, E., Mazza, G., Menchetti, M., Panzeri, M., Gager, Y., Bertolino, S., et al. (2015). The masked invader strikes again: the conquest of Italy by the Northern raccoon. *Hystrix* 26, 47–51.

Petralia, L., Lucchesi, M., and Zoccola, A. (2019). Segnalazioni faunistiche, 181 – *Potamon fluviatile* fluviatile Herbst, 1785 (Crustacea: Decapoda: Potamidae) [Faunistic records, 181 – *Potamon fluviatile* fluviatile Herbst, 1785 (Crustacea: Decapoda: Potamidae)]. *Quad. Studi. Nat. Romagna* 50, 202–203.

Pratten, D. J. (1980). Growth in the crayfish *Austropotamobius pallipes* (Crustacea: Astacidae). *Freshw. Biol.* 10, 401–402. doi: 10.1111/j.1365-2427.1980.tb01215.x

Puskas, R. B., Fischer, J. W., Swope, C. B., Dunbar, M. R., McLean, R. G., and Root, J. J. (2010). Raccoon (*Procyon lotor*) movements and dispersal associated with ridges and valleys of Pennsylvania: implications for rabies management. *Vector Borne Zoonotic Dis.* 10, 1043–1048. doi: 10.1089/vbz.2009.0079

Ragni, B., Vercillo, F., and Grelli, D. (2015). *Studio su Carnivori di Interesse Conservazionistico e Gestionale Nell'Area del Parco [Study on Carnivores of Conservation and Management Concern Within the Park Area]. [Technical Report]*. Arezzo: Ente Parco Nazionale delle Foreste Casentinesi.

Reynolds, J., Souty-Grosset, C., and Richardson, A. (2013). Ecological roles of crayfish in freshwater and terrestrial habitats. *Freshw. Crayfish* 19, 197–218.

Richman, N. I., Boehm, M., Adams, S. B., Alvarez, F., Bergey, E. A., Bunn, J. J. S., et al. (2015). Multiple drivers of decline in the global status of freshwater crayfish (Decapoda: Astacidea). *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370:20140060. doi: 10.1098/rstb.2014.0060

Salgado, I. (2018). Is the raccoon (*Procyon lotor*) out of control in Europe? *Biodiv. Conserv.* 27, 2243–2256. doi: 10.1007/s10531-018-1535-9

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 27 August 2021
doi: 10.3389/fevo.2021.647037

Controlled Infection Experiment With *Aphanomyces astaci* Provides Additional Evidence for Latent Infections and Resistance in Freshwater Crayfish

Caterina Francesconi[1], Jenny Makkonen[2†], Anne Schrimpf[1], Japo Jussila[2], Harri Kokko[2] and Kathrin Theissinger[1,3]*

[1] Institute for Environmental Sciences, University of Koblenz and Landau, Landau, Germany, [2] Department of Environmental and Biological Sciences, University of Eastern Finland, Kuopio, Finland, [3] LOEWE Centre for Translational Biodiversity Genomics, Senckenberg Biodiversity and Climate Research Centre, Frankfurt, Germany

OPEN ACCESS

Edited by:
Adam Petrusek,
Charles University, Czechia

Reviewed by:
Frederic Grandjean,
University of Poitiers, France
Agata Mrugala,
Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Germany

***Correspondence:**
Caterina Francesconi
francesconi@uni-landau.de

†Present address:
Jenny Makkonen,
BioSafe – Biological Safety Solutions Ltd./Oy, Kuopio, Finland

Specialty section:
This article was submitted to Population, Community, and Ecosystem Dynamics, a section of the journal Frontiers in Ecology and Evolution

Received: 28 December 2020
Accepted: 05 August 2021
Published: 27 August 2021

Citation:
Francesconi C, Makkonen J, Schrimpf A, Jussila J, Kokko H and Theissinger K (2021) Controlled Infection Experiment With Aphanomyces astaci Provides Additional Evidence for Latent Infections and Resistance in Freshwater Crayfish. Front. Ecol. Evol. 9:647037. doi: 10.3389/fevo.2021.647037

For 150 years the crayfish plague disease agent *Aphanomyces astaci* has been the cause of mass mortalities among native European crayfish populations. However, recently several studies have highlighted the great variability of *A. astaci* virulence and crayfish resistance toward the disease. The main aim of this study was to compare the response of two crayfish species, the European native noble crayfish (*Astacus astacus*) and the invasive alien marbled crayfish (*Procambarus virginalis*), to an *A. astaci* challenge with a highly virulent strain from haplogroup B and a lowly virulent strain from haplogroup A. In a controlled infection experiment we showed a high resistance of marbled crayfish against an *A. astaci* infection, with zoospores from the highly virulent haplogroup B strain being able to infect the crayfish, but unable to cause signs of disease. Furthermore, we demonstrated a reduced virulence in the *A. astaci* strain belonging to haplogroup A, as shown by the light symptoms and the lack of mortality in the generally susceptible noble crayfish. Interestingly, in both marbled crayfish and noble crayfish challenged with this strain, we observed a significant decrease of the detected amount of pathogen's DNA during the experiment, suggesting that this *A. astaci* haplogroup A strain has a decreased ability of penetrating into the cuticle of the crayfish. Our results provide additional evidence of how drastically strains belonging to *A. astaci* haplogroup B and haplogroup A differ in their virulence. This study confirmed the adaptation of one specific *A. astaci* haplogroup A strain to their novel European hosts, supposedly due to reduced virulence. This feature might be the consequence of *A. astaci*'s reduced ability to penetrate into the crayfish. Finally, we experimentally showed that marbled crayfish are remarkably resistant against the crayfish plague disease and could potentially be latently infected, acting as carriers of highly virulent *A. astaci* strains.

Keywords: marbled crayfish, noble crayfish, host-pathogen co-evolution, crayfish plague, experimental infection

INTRODUCTION

The causative agent of crayfish plague, *Aphanomyces astaci*, has been introduced in southern Europe in the 19th century, and quickly spread across the native crayfish stocks of most of the continent (Alderman, 1996). The colonization of Europe by the pathogen took place through two different waves (Alderman, 1996). During the first wave in the 19th century, strains belonging to haplogroup A spread throughout the continent (Huang et al., 1994), presumably without their original host (Alderman, 1996). The second wave was caused by multiple introductions of different species of North American crayfish (Alderman, 1996). It is believed that each of them carried its own specific haplogroup of *A. astaci*, resulting in the introduction into Europe of three new haplogroups: B, D, and E (Huang et al., 1994; Diéguez-Uribeondo et al., 1995; Kozubíková et al., 2011; Makkonen et al., 2018; Jussila et al., 2021). North American crayfish are resistant against *A. astaci* and can act as reservoirs for the pathogen (Unestam and Weiss, 1970; Unestam and Nylund, 1972; Alderman, 1996). Such resistance is presumably the result of a shared coevolution history in their original habitat that allowed for the establishment of a fine-tuned balance between host and parasite (Unestam, 1969). The susceptible European crayfish, however, when challenged with the new pathogen, faced disastrous crayfish plague epizootics, often resulting in the eradication of entire populations (Alderman, 1996).

In recent years, infection experiments aimed to evaluate the virulence of the different *A. astaci* strains have highlighted a considerable variance in the ability of the different haplogroups to cause the insurgence of the disease. Generally, *A. astaci* haplogroup B is classified as highly virulent, with the disease caused by this strain usually culminating in the death of all the challenged noble crayfish (Makkonen et al., 2012a, 2014; Jussila et al., 2013, 2015; Gruber et al., 2014; Becking et al., 2015). On the other hand, *A. astaci* haplogroup A has been shown to be less virulent in general, and its strains have a much more variable virulence (Makkonen et al., 2012a, 2014; Becking et al., 2015; Jussila et al., 2015). Furthermore, increased resistance during infection experiments in some populations of noble crayfish has been reported (Makkonen et al., 2014), and several reports of latently infected European crayfish populations have emerged (Jussila et al., 2011a, 2017; Viljamaa-Dirks et al., 2011; Schrimpf et al., 2012; Kusar et al., 2013; Maguire et al., 2016). In addition, the decline of some wild American crayfish populations due to crayfish plague epizootics has been observed (Jussila et al., 2014; Sandström et al., 2014) and laboratory experiments have shown that North American crayfish can be susceptible to *A. astaci* when under stressful conditions (Thörnqvist and Söderhäll, 1993; Aydin et al., 2014).

Few decades ago, yet another invasive crayfish species, the parthenogenetic marbled crayfish *Procambarus virginalis* Lyko, 2017, appeared in Europe (Chucholl et al., 2012; Lyko, 2017). It has first been spotted in 1995 in the German pet trade and has since then established numerous populations on the continent (Vogt, 2018). It evolved from *Procambarus fallax*, an American species native of Florida, after triploidization (Vogt et al., 2018). As no known primary population is present in America, it is thought that the species may have evolved in captivity in the pet trade environment (Vogt et al., 2018). *Procambarus virginalis* can act as *A. astaci* carrier, and both wild and captive specimens have been found infected with *A. astaci* (Keller et al., 2014; Mrugała et al., 2015; Makkonen et al., 2018). In two instances it was possible to genotype the strains infecting *P. virginalis* specimens, and they were identified as haplogroup D (Keller et al., 2014; Mrugała et al., 2015), characterized by elevated virulence (Martín-Torrijos et al., 2017). Therefore, we expect the marbled crayfish to be rather resistant to the crayfish plague.

With this study we aim to shed some light on the adaptation process between *A. astaci* and its new European crayfish hosts. The increasing number of reports of latently infected European crayfish populations indicates that the continuous interaction between host and pathogen might be leading to new equilibria, balanced by an increased resistance of the crayfish and/or a decreased virulence of the pathogen (Jussila et al., 2014). We tested and compared the susceptibility of noble crayfish and marbled crayfish against a highly virulent (haplogroup B; Makkonen et al., 2019) and a lowly virulent (haplogroup A) *A. astaci* strain. The *A. astaci* haplogroup A strain has been isolated from the Finnish noble crayfish population from Lake Venesjärvi. In the last 50 years, this population has survived at least three different crayfish plague epizootics, last of which took place around the year 2000 (Jussila et al., unpublished data). Since then, the population has been slowly recovering, and the noble crayfish are now asymptomatic carriers of the pathogen. By using this *A. astaci* strain, we aim to provide additional evidence of the existence of latently infected wild noble crayfish populations. We hypothesized no mortality in both species of crayfish infected with haplogroup A. Furthermore, we hypothesized the highly virulent haplogroup B to cause the death of the noble crayfish, but no or less intense symptoms in the marbled crayfish. Finally, we expected different onset of the symptoms, with the marbled crayfish showing delayed signs of the disease compared to the noble crayfish.

MATERIALS AND METHODS

Crayfish Species

The noble crayfish were collected from a wild population in Lake Rytky, Kuopio, Finland (62°51′22″N, 27°25′06″E), while the marbled crayfish were obtained from lake Singliser See, Hessen, Germany (51°3′35″N, 9°18′18″E; import license to Finland, ID: ESAVI/15535/04.10.12/2019, date 10.5.2019; ID: Diaari nro 842/5719/2019, date 16.4.2019). Both populations had been tested for *A. astaci* presence on previous occasions and no infections were detected (Keller et al., 2014; Jussila et al., 2017). After collection and transport from the airport in Helsinki to the University of Eastern Finland, the marbled crayfish have been placed for 3 weeks in holding tanks with no food. In the first 2 weeks the crayfish were kept at 6°C, while the third week the temperature was raised to 18°C. The water was changed once a week. The noble crayfish were kept in holding tanks at 18°C for 1 week. All holding tanks were equipped with one aeration pump to ensure adequate level of dissolved oxygen in the water.

Twenty days prior to the challenge experiment, the crayfish were transferred to the individual tanks of the experimental infection system for acclimatization following a randomized system (e.g., Makkonen et al., 2019). For every crayfish, carapace length and sex were determined, and notes made on any specific features, e.g., missing limbs or injuries. Marbled crayfish produced eggs throughout both the acclimatization period and the challenge experiment. The eggs were systematically removed prior to the challenge experiment. After the challenge, the eggs were not removed to avoid additional stress to the crayfish. During acclimatization period and challenge experiment the crayfish were given preboiled frozen sweet corn every second day. Eventual leftover corn was removed before the next feeding.

A. astaci Isolates and Zoospores Production

Two *A. astaci* strains were used for the experiment. The highly virulent *A. astaci* isolate UEF_T16B, isolated from a signal crayfish (*Pacifastacus leniusculus*) from Lake Tahoe, United States (39°05′30″N, 120°02′30″E), in 2013. The strain belongs to haplogroup B based on mitochondrial markers (Makkonen et al., 2019). Haplogroup B corresponds to RAPD-PCR group B (Makkonen et al., 2018). The second strain was VEN5/14 a), isolated from a noble crayfish from Lake Venesjärvi, Kankaanpää, Finland (61°4′41″N, 22°10′26″E), in 2014. The isolation of the *A. astaci* culture was successful despite the fact that the qPCR did not detect *A. astaci* DNA in the tissues of the crayfish population (Jussila et al., unpublished data). This strain belongs to haplogroup A which includes RAPD-PCR groups A and C (Makkonen et al., 2018), and likely also additional *A. astaci* strains, considering its wide geographic distribution (Martín-Torrijos et al., 2021). While no further genetic analysis of this strain has been conducted, it can be assumed it belongs to RAPD-PCR group A, as only strains belonging to RAPD-PCR groups A and B have been isolated from crayfish populations present in Finland (Viljamaa-Dirks et al., 2016).

The production of zoospores followed the method used in Makkonen et al. (2012a) with some modifications. Three pieces of agar (4 mm^2 each) were cut from solid PG1 medium containing *A. astaci* hyphae and incubated in 150 mL of liquid PG1 medium at 20°C for 1 week. Subsequently the hyphae have been finely cut with a sterile scalpel and incubated in new liquid PG1 medium at 20°C for 3 days. At the end of the 3 days, to stimulate zoospores production, the hyphae have been washed four times with autoclaved water, and then incubated in the same water on a horizontal shaker at 18°C for one night. For each strain twelve replicates have been produced. The density of the zoospore solution was estimated with an optical microscope (total magnification of 100x) using a Bürker chamber.

Experimental System

The experimental infection system (RapuLatorio) consisted of individual interconnected 2 L tanks with recirculating filtered water from lake Kallavesi (Jussila et al., 2011b). The water filtration was ensured by a biological filter and a set of three 5 μm filters (Spunflow QN, Domnick Hunter Technologies Ltd., England) and two 5 μm absolute filters (Pleatflow II, Prosep Filter Systems Ltd., England). This system ensures that all *A. astaci* zoospores are eliminated from the circulating water (Jussila et al., 2011b). During the experiment, water pressure before the absolute filters was regularly monitored. Filters were substituted when water pressure exceeded 2×10^5 Pa. Water temperature was maintained stable by air conditioning at 18.8 ± 1.1°C. A day-night rhythm was mimicked through artificial lights, with 8 h of light and 16 h of dark. Water quality parameters (oxygen levels, temperature, conductivity, and pH) were monitored once a day. The dissolved oxygen was 93 ± 12.5% (min-max, 37–100%). The minimum value of 37% was registered on day 1 of the challenge, after the interruption of the water circulation prior to the addition of the zoospores to the tanks. The conductivity was 222 ± 8.1 μS/cm (min-max, 212–256 μS/cm), pH was 7.8 ± 0.2 (min-max, 7.2–8). The pH value was artificially lowered 24 h before the start of the challenge by three additions of 1 mL of HNO_3 to the circulating water to maintain the pH value 7.8, considered adequate for the infection process (Unestam, 1966).

Experiment Setup and Infection

The treatment groups (*A. astaci* haplogroup A-challenged crayfish, *A. astaci* haplogroup B-challenged crayfish and controls) consisted of 20 crayfish each, for a total of 120 crayfish (60 noble crayfish and 60 marbled crayfish). During day 0 of the infection the zoospore suspension was added to the individual tanks to reach a concentration of 1000 zoospores/mL in tank water. Controls have been treated similarly by adding autoclaved water from lake Kallavesi. Prior to the addition of the zoospores, the water circulation was interrupted to maintain the concentration of zoospores constant during the infection process. Water circulation was resumed After 16 h. During the experiment, the crayfish were monitored for symptoms, either gross signs of infection (scratching, loss of balance, aimless movements of the appendages, and loss of appendages) or death, multiple times per day. Moribund crayfish were removed from the system and stored at −20°C. During the challenge, crayfish were removed from the experimental system to sample their tissues (hemolymph, hepatopancreas, and gills) as part of an overlapping experiment where tissues from alive crayfish were needed for RNA isolation and subsequent gene expression analysis (**Table 1**; Boštjančić et al., 2021). The crayfish were removed on two different dates. The first sampling took place on day 3 of the challenge. All the haplogroup B-challenged noble crayfish showing gross signs of infection were sampled (n = 19), as they were likely to die in the following days (Makkonen et al., 2012a). Five individuals were sampled for each of the other experimental groups on the same day. During the second sampling, carried out on day 21 of the experiment, five crayfish per infection group were removed from the system. As a result, ten crayfish per group were left until the end of the experiment, except the noble crayfish challenged with the strain from haplogroup B, where all crayfish were sampled on day 3 or died on day 7. The experiment lasted 45 days, after which all remaining crayfish were considered successful survivors. We expected eventual symptoms of infection to manifest themselves within this timeframe, as other comparable infection experiments have shown (Makkonen et al., 2012a, 2014).

TABLE 1 | Study design and sampling time point.

Species	Treatment group	*N*	Sampling time point		
			Day 3	Day 21	Day 45
Noble crayfish	*A. astaci* of haplogroup A	20	**5**	**5**	10
	A. astaci of haplogroup B	20	**19***	–	–
	Control	20	**5**	**5**	10
Marbled crayfish	*A. astaci* of haplogroup A	20	**5**	**5**	10
	A. astaci of haplogroup B	20	**5**	**5**	10
	Control	20	**5**	**5**	10
Total		120	44	25	50

*The number of specimens (N) belonging to each experimental group is reported. During each time point specimens belonging to each treatment group were sampled and removed from the experiment. *19 crayfish were sampled on day 3, as they were showing symptoms of crayfish plague and were expected to die in the next days. The remaining crayfish of this group did not show symptoms before the sampling date, and was therefore left in the experiment and it died on day 7.*
Bold numbers refer to the number of samples used for RNA sequencing for gene expression analysis (see Boštjančić et al., 2021).

DNA Extraction, qPCR and *A. astaci* DNA Quantification

To test for the presence of *A. astaci* DNA in the crayfish tissues, qPCR of the samples was conducted. Tissue samples were taken from uropods, walking legs, and abdominal cuticle. DNA extraction was conducted following a modified protocol described in Vrålstad et al. (2009). The qPCR was conducted using the assay, primers and probe developed and shared from work in progress at the Norwegian Veterinary Institute (David A. Strand, unpublished). The new and more specific assay has been used in the light of possible cross-reaction of the Vrålstad assay with another *Aphanomyces species* (Viljamaa-Dirks and Heinikainen, 2019). As for the assay described in Vrålstad et al. (2009), the primers of the Strand et al. assay target the ITS region and the two assays are, therefore, comparable. The details of the new assay are: forward primer 5′-AAC TAT CCA CGT GAA TGT ATT CTT TAT-3′, reverse primer 5′-CGG CTA AGT TTA TCA GTA TGT TAT TTA-3′, and probe 5′-6-FAM-AAG AAC ATC CCA GCA CAA-MGBNFQ-3′. For each reaction, the qPCR analysis was performed in 20 μL reaction volume consisting of 10 μL of TaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific, Waltham, MA, United States), 500 nM of each primer, 200 nM of probe, nuclease free water, and 5 μl of DNA sample. The amplification protocol consisted of an initial warming at 95°C for 10 min and 50 cycles of denaturation phase (95°C for 15 s) and annealing phase (62°C for 60 s). PCR forming units (PFUs) were calculated following Vrålstad et al. (2009). Only samples with PFU $\geq$ 5 were considered positive. PFU = 5 is the limit of detection of the assay and it represents the lowest concentration that yields a probability of false negatives <5% (Vrålstad et al., 2009).

Statistical Analysis

The Shapiro–Wilk test was used to assess if the PFU values in each group were normally distributed. The Levene's test was used to test the equality of variance of the PFUs values in the different groups. As for most groups the PFU values didn't follow a normal distribution and the variances among groups were not equal, the significance of the differences of the PFU values among the different experimental groups was tested with the Kruskal–Wallis test. Finally, the pairwise Wilcoxon rank-sum test was used to evaluate pairwise differences among all the experimental groups, including control groups, and across the different time points. The Benjamini–Hochberg (BH) method was used for *p*-value adjustment (Benjamini and Hochberg, 1995). Only biologically relevant comparisons were taken into account (e.g., comparisons between different time points of the same experimental group and comparisons between noble crayfish and marbled crayfish challenged with the same *A. astaci* strain at the same time point).

RESULTS

Signs of Infection

Noble Crayfish

Among the haplogroup B-challenged noble crayfish group, all individuals showed signs of infection between day 1 and day 5 in the form of scratching of the eyes, walking legs and abdomen (**Figure 1**). The scratching of the eyes generally lasted for several seconds. Of these crayfish, 19 were removed from the experimental system on day 3, as they were considered moribund. The last crayfish of this group started showing signs of infection on day 5, and died 2 days later. Under microscopic examination, this crayfish showed a heavy presence of hyphae in its abdominal cuticle. Subsequently, the soft cuticle of this single crayfish has been used for re-cultivation of *A. astaci*. Because of this, the tissues commonly used for the qPCR were not available, and thus for this crayfish the analysis was not conducted. In the noble crayfish group challenged with haplogroup A, 11 out of 20 crayfish showed signs of infection in the form of light scratching of eyes, abdomen and walking legs, or slow, aimless movements of the walking legs with the appendages fluctuating back and forth. These signs of infection were observed between day 5 and day 31 (**Figure 1**). All crayfish belonging to this group survived until the end of the experiment. No crayfish belonging to the control group showed signs of infection or died during the experiment.

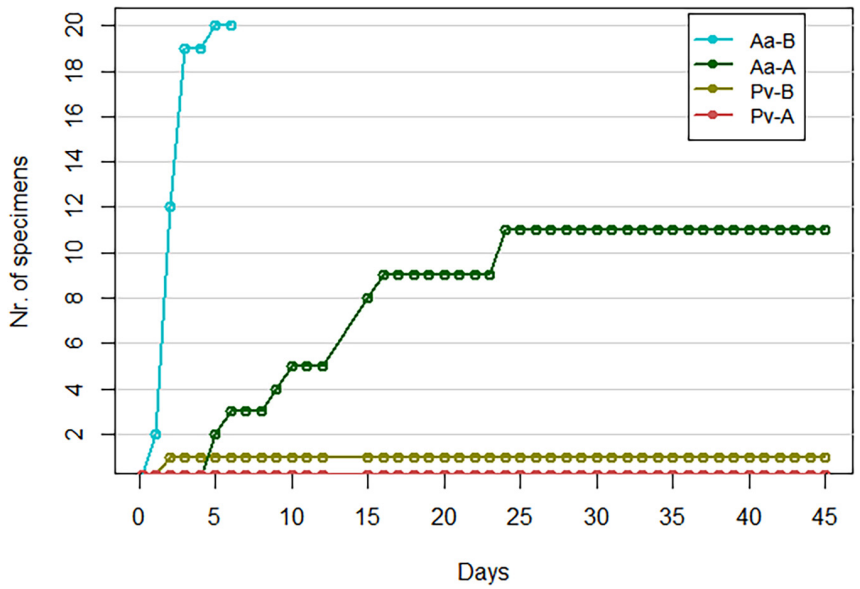

FIGURE 1 | Cumulative number of crayfish showing gross signs of infection. Pv, marbled crayfish; Aa, noble crayfish; A, *A. astaci* of haplogroup A-challenged crayfish; B, *A. astaci* of haplogroup B-challenged crayfish.

Marbled Crayfish

Only one marbled crayfish belonging to the haplogroup B-challenged group showed signs of infection by scratching the eyes on day 2 (**Figure 1**). This particular behavior was only observed once. However, follow up observations were not possible as the crayfish was sampled the following day. As none of the control marbled crayfish were observed with similar behaviors, the scratching was considered a sign of infection. None of the crayfish belonging to the haplogroup A-challenged marbled crayfish and to the control group showed signs of infection. All marbled crayfish belonging to the three groups survived until the end of the experiment; out of these, one individual belonging to the control group molted.

qPCR

Noble Crayfish

In the haplogroup B-challenged noble crayfish, 15 out of 19 tested crayfish were positive for *A. astaci* DNA with the PFU values between 9 and 12949. In the haplogroup-A challenged group, *A. astaci* DNA was detected in 4 out of 20 noble crayfish (20%), with the positive samples detected only in the first time point. The PFU values of the positive samples ranged between 8 and 129. There was no significant difference in terms of *A. astaci* load between haplogroup A-challenged and haplogroup B-challenged groups during the first sampling point (Pairwise Wilcoxon rank-sum test, $n_1 = 5$, $n_2 = 19$, $p = 0.18$, **Supplementary Tables 1**, **2**). *Aphanomyces astaci* DNA was not detected in any of the crayfish in the control groups (**Figure 2** and **Table 2**).

Marbled Crayfish

In total, *A. astaci* DNA was detected via qPCR in 12 out of 20 (60%) haplogroup B-challenged marbled crayfish, even though only one of them showed apparent behavioral signs of infection. The positive samples were detected in all time points, with two positive crayfish in the first time point, four in the second, and six in the third (**Table 2**). The PFU values of the positive crayfish ranged between 7 and 289. The only symptomatic marbled crayfish in this group tested negative in the qPCR. *Aphanomyces astaci* DNA was detected in two of the 20 marbled crayfish (10%) from the haplogroup A-challenged group, with PFU values of 6 and 11 (**Figure 2**). Both crayfish were sampled at the first time point (3 days after exposure to *A. astaci* spores). The comparison of the PFU values between haplogroup A-challenged and haplogroup B-challenged groups showed no significant difference in the first sampling point for marbled crayfish (Pairwise Wilcoxon rank-sum test, $n_1 = 5$, $n_2 = 5$, $p = 0.27$, **Supplementary Tables 1**, **2**). All crayfish from the control group were negative.

All the remaining relevant comparisons between treatment groups resulted non-significant (**Supplementary Tables 1**, **2**).

DISCUSSION

The main aim of this study was to compare the response of two crayfish species, the European native noble crayfish and the invasive marbled crayfish, to an *A. astaci* challenge with a highly virulent strain from the haplogroup B and a lowly virulent strain from the haplogroup A. We showed a high resistance of marbled crayfish against an *A. astaci* infection, with zoospores from the highly virulent haplogroup B strain being able to infect the host, but unable to cause the disease. Furthermore, we demonstrated a reduced virulence in the *A. astaci* Venesjärvi strain belonging to haplogroup A, as shown by the light symptoms and the lack of mortality in the noble crayfish. Interestingly, in both

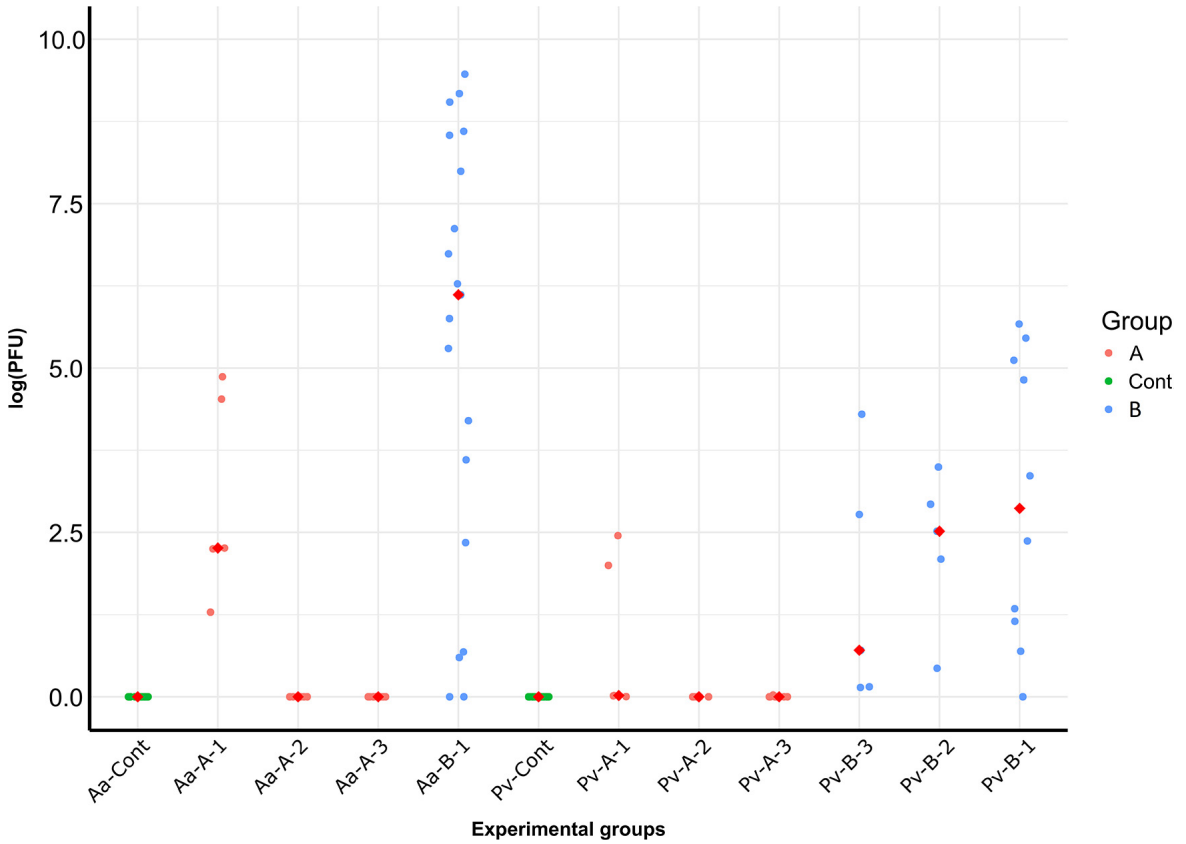

FIGURE 2 | PFU values of *A. astaci* DNA detected in the crayfish of the different experimental groups, divided per sampling date. Medians are represented as red diamonds. Pv, marbled crayfish; Aa, noble crayfish; A, *A. astaci* of haplogroup A-challenged; B, *A. astaci* of haplogroup-B challenged; 1, first sampling point, day 3; 2, second sampling point, day 21; 3, third sampling point, day 45.

TABLE 2 | Median of the PCR forming units (PFU) value and the range of the PFU values of each experimental group by sampling points.

Species	Group	Sampling time point	Number of samples	Positive samples	PFUs (median)	PFUs range
Noble crayfish	*A. astaci* of haplogroup A	Day 3	5	4	50.06	8.49–129
		Day 21	5	0	–	–
		Day 45	10	0	–	–
	A. astaci of haplogroup B	Day 3	19	15	842	9.43–12949
	Control	Day 3	5	0	–	–
		Day 21	5	0	–	–
		Day 45	10	0	–	–
Marbled crayfish	*A. astaci* of haplogroup A	Day 3	5	2	–	6.38–10.6
		Day 21	5	0	–	–
		Day 45	10	0	–	–
	A. astaci of haplogroup B	Day 3	5	2	–	15–72.50
		Day 21	5	4	15.55	7.11–31.9
		Day 45	10	6	144.50	9.7–289
	Control	Day 3	5	0	–	–
		Day 21	5	0	–	–
		Day 45	10	0	–	–

marbled crayfish and noble crayfish challenged with this strain, the pathogen DNA was only detected in the tissues of the crayfish sampled on day 3 (**Figure 2**), suggesting that this *A. astaci* haplogroup A strain has a decreased ability of penetrating into the cuticle of the crayfish and infecting the crayfish. Finally, our results prove once more how drastically the strains

belonging to *A. astaci* haplogroup B and haplogroup A differ in their virulence.

Elevated Resistance in Marbled Crayfish

Our experiment showed a strong resistance of marbled crayfish against *A. astaci.* The crayfish were not affected by the two *A. astaci* strains used in this study, which both failed to cause symptoms in the marbled crayfish, with only a single exception (**Figure 1**). This only symptomatic crayfish, belonging to the *A. astaci* haplogroup B-challenged group, showed a mild version of the gross signs of infection, with only briefly scratching its eyes. Although the marbled crayfish in the haplogroup B-challenged group mainly did not show gross signs of an *A. astaci* infection, the amount of *A. astaci* DNA detected in their tissues clearly showed that some of these crayfish were indeed infected (**Table 2**). This might indicate that *A. astaci* was able to germinate and penetrate into the cuticle of the crayfish, however, the marbled crayfish's immune system was capable of preventing the manifestation of the disease. This shows that the marbled crayfish has the potential to be latently infected with highly virulent strains and to act as their carrier. Interestingly, the amount of *A. astaci* DNA detected in the tissues of the marbled crayfish infected with the haplogroup B strain indicates a possible increase of the pathogen DNA over time (**Table 2** and **Figure 2**), although this increment was not statistically significant. The increase of pathogen DNA might be the result of spores attached to the cuticle of the crayfish without germinating and causing infection. However, our experimental system allowed for a constant exchange of water, with spore-free water flowing into the tanks, and contaminated water being directed from the tanks to the filters. For this reason, it is more likely that the detected increment in PFU values is the result of an active *A. astaci* infection. From the few studies where it was possible to confirm the presence of *A. astaci* in marbled crayfish specimens, it is clear that this species can withstand higher levels of infection than the one observed in this study (Keller et al., 2014; Mrugała et al., 2015). Quantitative PCR conducted on seemingly healthy specimens from a laboratory-cultured population revealed high amount of pathogen DNA in the sampled tissues ($10^4 \leq$ PFU $\leq 10^5$, Keller et al., 2014). It might be interesting in future studies to perform an even longer lasting experiment to assess the progression of the infection, and to evaluate if it would result in the manifestation of the disease.

When marbled crayfish was first discovered in open waters in Germany in 2003 (Marten et al., 2004) it was assumed to have a big invasion potential because of its high fecundity and parthenogenetic reproduction (Scholtz et al., 2003). However, since then single individuals unable to produce established populations have often been observed (Vogt, 2020). Although it is known that marbled crayfish can survive winters in Central Europe (Veselỳ et al., 2015), their optimal temperature for reproduction is between 20 and 25°C (Seitz et al., 2005), similar to the water temperature from Florida, where *P. fallax*, their closest relative, lives. Martin et al. (2010) already speculated that marbled crayfish might establish more successful populations in the newly invaded Madagascar, where the climate is milder. Few years later this predicted scenario has proven true, and it has been shown that in warmer climates the marbled crayfish is able to increase its range dramatically (Andriantsoa et al., 2019). In Europe, however, this species does not spread as fast and the population growth is limited, probably because of the colder temperatures (Günter et al., 2019). It has been observed that when new populations become established in Europe, it is usually not due to the invasion potential of marbled crayfish, as is the case for red swamp crayfish (*Procambarus clarkii)* or calico crayfish (*Faxonius immunis*), but because of human mediated releases. This might change when more populations will be released in watercourses as it was observed in Slovakia and Hungary (Lipták et al., 2016; Weiperth et al., 2020). Marbled crayfish is a popular pet in the aquarium trade worldwide, and it has now established populations in at least 16 countries (Vogt, 2020), including those with warmer climate where the invasion will be more successful.

While it has been confirmed that marbled crayfish can act as *A. astaci* carrier, analysis of specimens of this species from the wild and from the aquarium trade have shown that the presence of *A. astaci* in their tissues often cannot be verified (Lipták et al., 2016, 2017; Patoka et al., 2016; Pârvulescu et al., 2017; Andriantsoa et al., 2019; Ercoli et al., 2019; Lenich, 2019; own unpublished data). No infection could be confirmed in 100 tested crayfish from Madagascar (Andriantsoa et al., 2019), 67 specimens from Slovakia (Lipták et al., 2016, 2017), four specimens from Czechia (Patoka et al., 2016), nine specimens from Romania (Pârvulescu et al., 2017), six specimens from Estonia (Ercoli et al., 2019), and 20 specimens from Germany (Lenich, 2019). On the other hand, when marbled crayfish co-exists with North American crayfish species, it is usually found to be infected. This was the case for marbled crayfish held with other North American crayfish species in common aquaria (Keller et al., 2014; Mrugała et al., 2015), or for marbled crayfish co-existing with *Faxonius limosus* in the wild (Keller et al., 2014; own unpublished data). Therefore, it can be speculated that marbled crayfish was not infected when it developed in the aquarium environment, and only becomes carrier of *A. astaci* when in contact with North American crayfish species. Because of its invasive potential and its *A. astaci* carrier status the trade with marbled crayfish is now officially forbidden by the EU Regulation 1143/2014 on Invasive Alien Species in EU countries.

It is generally expected that North American crayfish are comparatively more resistant to *A. astaci* infections than European crayfish (Svoboda et al., 2017). As shown in our study, the marbled crayfish appeared to be highly resistant to *A. astaci* infections. This resistance might be a consequence of the shared coevolution history of *A. astaci* and North American crayfish, of which marbled crayfish is a recent descendant. While marbled crayfish and its closest relative *P. fallax* share similar morphological characters, coloration and some ecological features, the triploidization in the marbled crayfish genome (a third identical copy of the *P. fallax* chromosome set, without any additional or changed DNA sequences) has led to an enhanced body size, fecundity and longevity, which contribute to its invasive capabilities (Vogt et al., 2019). More information about the resistance or eventual susceptibility of American crayfish in their natural habitat is needed to better understand the consequences of the host-pathogen coevolution process.

Latent Infections Due to Reduced Virulence

The tested *A. astaci* Venesjärvi strain did not cause any mortality among the challenged noble crayfish. While light gross signs of infection were observed (**Figure 1**), those appeared to be less pronounced than the ones caused by the haplogroup B strain, and did not lead to the death of any crayfish. Only four out of 20 crayfish belonging to this group were shown to be infected by *A. astaci* in our experiment. However, the qPCR assay cannot demonstrate the absence of infection, as the amount of pathogen present in the crayfish tissue might be below the detection level and the infection might be localized in different tissues to the sampled ones. It has been shown on different occasions, that haplogroup A includes strains that differ greatly in their virulence (Makkonen et al., 2012a, 2014; Becking et al., 2015; Mrugała et al., 2016; Jussila et al., 2017). For example, in Makkonen et al. (2012a) crayfish belonging to the same population and exposed to the same experimental conditions were infected with two different strains both belonging to haplogroup A. While one strain caused 100% of mortality within 19 days, the second strain failed to cause any significant increase in the death rate of the experimental crayfish. Strains belonging to haplogroup A arrived to Europe presumably without their original host (Alderman, 1996). This might have worked as selective pressure toward reduced virulence (Makkonen et al., 2012b; Jussila et al., 2015). On the other hand, a host-parasite equilibrium could be reached not only by lowered virulence of the parasite but also by increased resistance of the host. However, the noble crayfish population from Lake Rytky used in this study has already been shown, in comparable experiments, to be susceptible to *A. astaci* strains belonging to haplogroup A (Makkonen et al., 2012a). This, together with the haplogroup A strain being isolated from a latently infected population, suggests a decreased virulence of the respective *A. astaci* strain.

The lack of mortality and severe gross signs in the crayfish challenged with *A. astaci* of haplogroup A could be taken as experimental proof of the existence of wild noble crayfish populations latently infected with *A. astaci*, which then may act as carriers of the pathogen (Jussila et al., 2011a; Viljamaa-Dirks et al., 2011). A similar case of latent infection has been reported by Jussila et al. (2011a) for a noble crayfish population in Lake Mikitänjärvi. No population decline or increased mortality was observed, and the population was considered healthy until the qPCR analysis revealed that some of the specimens were infected with *A. astaci* (Jussila et al., 2011a). Unfortunately, in that instance *A. astaci* itself was not isolated, nor was it possible to identify its haplogroup (Jussila et al., 2011a). The impossibility of conducting tests on the virulence of the strain on other noble crayfish populations makes it difficult to speculate on the effective virulence of the *A. astaci* strain in this case. However, subsequent experiments showed a higher resistance of the Lake Mikitänjärvi population to both haplogroups A and B when compared to other noble crayfish populations (Makkonen et al., 2014). The two cases of latent infections of wild noble crayfish populations from Lake Mikitänjärvi and Lake Venesjärvi are just two examples of the occasional status of equilibrium tentatively reached by European crayfish populations and *A. astaci*. In the past 20 years, cases of populations latently infected with *A. astaci* haplogroup A have been reported not only in noble crayfish, but also in other European crayfish species such as white-clawed crayfish (*Austropotamobius papilles*), stone crayfish (*Austropotamobius torrentium*), and narrow-clawed crayfish (*Pontastacus leptodactylus*) (Ungureanu et al., 2020). Interestingly, it has been observed that latent infections are not only caused by the lowly virulent *A. astaci* haplogroup A, but also by the more virulent haplogroup B (Ungureanu et al., 2020). In Europe, the situation concerning invasive crayfish species is constantly changing and new species are expected to start spreading across the continent. It might soon be the case for *Faxonius rusticus*, which is already present in the European pet trade and known for its invasive potential (Chucholl, 2012). *Faxonius rusticus* in North America carries a distinct strain of *A. astaci* belonging to haplogroup A (Panteleit et al., 2019). The introduction of additional North American crayfish species could bring new *A. astaci* strains to Europe, as demonstrated, e.g., by *F. rusticus*. It remains to be seen if the coevolution between European crayfish and specific *A. astaci* strains might eventually lead to European crayfish populations being better equipped to face infections from novel *A. astaci* strains.

Interestingly, for both species of crayfish challenged with haplogroup A strain, only the specimens sampled during the first time point tested positive in the qPCR (**Table 2** and **Figure 2**). This pattern could be explained by the detection of spores merely attached to the cuticle of the crayfish during the first sampling point. Spores unable to germinate would then detach themselves without causing an infection and get filtered away through the system, leading to negative results in the qPCR in the second and third sampling points. However, the observed pattern might also indicate a decreased capacity of this particular *A. astaci* strain of haplogroup A to penetrate the cuticle of the host. The colonization of the host by *A. astaci* starts when the spores, covered by sticky substances, attach themselves to the host surface (Cerenius et al., 2009). The germination process of the spores begins, followed by the penetration of the newly germinated hyphae into the cuticle of the crayfish (Cerenius et al., 2009). With the penetration of the hyphae, the infected host's immune system and the pathogen start interacting (Hauton, 2012). North American crayfish can, to a different level, resist the penetration of the hyphae, while native European crayfish are normally susceptible to the *A. astaci* infection. These moments of germination and penetration are crucial for the fate of the *A. astaci* infection process. The results of the transcriptome analysis of the crayfish sampled during the experiment revealed that this haplogroup A was able to trigger the immune response in the marbled crayfish 3 days after challenge (own unpublished data). This suggests that, while the spores were able to germinate, their ability to penetrate the crayfish cuticle was very limited. It is likely that the detection of the pathogen DNA in the first sampling point derives from *A. astaci* spores attached to the cuticle of the crayfish. Most of them might have detached themselves from the host, and even those germinating failed to establish in the host, which

would explain the negative qPCR results at the second and third sampling points. Further infection experiments might shed some light on the mechanisms that resulted in this speculated reduced ability of this *A. astaci* strain to penetrate into the cuticle of crayfish.

Drastically different was the response of the noble crayfish challenged with the haplogroup B strain (**Table 2** and **Figure 2**) compared to those of haplogroup A-challenged noble crayfish. The difference in PFU value between the two groups is not significant, as the variation within the groups were very high, with some of the crayfish in the haplogroup B-challenged group resulting negative despite the clear symptoms of infection. This might be the reflection of several aspects, both biological and methodological. The high within group variations might be the result of real biological differences in the resistance or sensitivity of the crayfish in the same experimental group. On the other hand, the qPCR assay is semi-quantitative. It is not possible to use the entire soft cuticle of the specimens for the qPCR. Because of this, different parts of the soft cuticles are sampled to maximize the chance of sampling cuticles containing hyphae. As a result, the amount of *A. astaci* DNA detected in the tissues is influenced by the sampling. However, in this case, the difference in the effects of *A. astaci* haplogroup A and haplogroup B on the noble crayfish are clear when considering the observed symptoms. The isolate from Lake Tahoe is highly virulent, and it caused clear symptoms of morbidity in the challenged noble crayfish, indicating a likely death of the noble crayfish in 1 or 2 days after the onset of the gross signs, as recently shown in Makkonen et al. (2019). While the *A. astaci* strain from haplogroup A used in this study was probably adapted to its new European hosts due to the absence of the original carrier (Makkonen et al., 2012b, 2018), the situation is very different for *A. astaci* belonging to haplogroup B. This haplogroup was introduced to Europe with its original host (*P. leniusculus*) which then has established numerous populations on the European continent (Alderman, 1996; Kouba et al., 2014). In this situation, scenarios where this strain wipes out the susceptible European crayfish populations would not have significant repercussion on *A. astaci*, which would still be free to circulate in the usually more resistant crayfish populations of North American origin (Jussila et al., 2015). Nonetheless, in the past decades there have been reports of populations of the native European *P. leptodactylus* latently infected with *A. astaci* strains belonging to haplogroup B (Ungureanu et al., 2020). *Pontastacus leptodactylus* is the most resistant among the native European species, but still it is considered susceptible to the crayfish plague (Svoboda et al., 2017; Jussila et al., 2020) and has been shown to suffer from mass mortalities caused by the disease (Rahe and Soylu, 1989; Timur, 1990). However, latently infected populations have been observed in Croatia (Maguire et al., 2016), Romania and Moldova (Panteleit et al., 2018), Turkey (Svoboda et al., 2014; Kokko et al., 2018), and Ukraine (Ungureanu et al., 2020). Until now, there have been no reports of noble crayfish populations resistant to *A. astaci* haplogroup B, although less susceptible populations have been detected (Makkonen et al., 2012a). In future, it will be interesting to see if the selection of European crayfish populations resistant to *A. astaci* haplogroup A would lead to the development of resistance also toward more virulent or new haplogroups.

CONCLUSION

This study demonstrated the adaptation of *A. astaci* haplogroup A strain isolated from Lake Venesjärvi to their novel European hosts, supposedly due to reduced virulence. Our results indicate that this feature might be the consequence of *A. astaci*'s reduced ability to penetrate into the cuticle of crayfish. Our observations support the growing number of reports of latent infections among native European crayfish stocks, providing additional evidence that the relationship between European crayfish and *A. astaci* might be slowly heading toward an equilibrium. Finally, we empirically demonstrated that marbled crayfish are highly resistant against *A. astaci* and add evidence to the ability of this species to become latently infected and act as a carrier of highly virulent *A. astaci* strains.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

CF set up the experiment, controlled the experiment, analyzed the data, and wrote the manuscript. JM set up the experiment and supported the study design. AS provided the expertise on the handling of the samples. JJ and HK supported the study design and control of the experiment. KT acquired the funding, conceived the study, and the experimental design. All authors edited and finalized the manuscript.

FUNDING

This work was financed by the Struktur- und Genehmigungsdirektion Süd (Rheinland-Pfalz, Germany).

ACKNOWLEDGMENTS

We thank David A. Strand (Norwegian Veterinary Institute) for kindly allowing us to use and publish his new assay, and Christoph Dümpelmann for catching the marbled crayfish from Singliser See and Vidhi Mathur (University of Koblenz and Landau) for helping with the preparation of the experimental infection system and monitoring the crayfish during the experiment. We also thank crayfisher person's from Kankaanpää,

Finland, for valuable information on lake Venesjärvi noble crayfish and crayfish plague. Finally, we thank AP (Charles University) for his invaluable insights during the editorial process and the three referees for their constructive comments on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.647037/full#supplementary-material

REFERENCES

Alderman, D. J. (1996). Geographical spread of bacterial and fungal diseases of crustaceans. *Rev. Sci. Tech.* 15, 603–632. doi: 10.20506/rst.15.2.943

Andriantsoa, R., Tönges, S., Panteleit, J., Theissinger, K., Carneiro, V. C., Rasamy, J., et al. (2019). Ecological plasticity and commercial impact of invasive marbled crayfish populations in Madagascar. *BMC Ecol.*19:1–10. doi: 10.1186/s12898-019-0224-1

Aydin, H., Kokko, H., Makkonen, J., Kortet, R., Kukkonen, H., and Jussila, J. (2014). The signal crayfish is vulnerable to both the as and the PsI-isolates of the crayfish plague. *Knowl. Managt. Aquatic Ecosyst.* 413:03. doi: 10.1051/kmae/2014004

Becking, T., Mrugała, A., Delaunay, C., Svoboda, J., Raimond, M., Viljamaa-Dirks, S., et al. (2015). Effect of experimental exposure to differently virulent Aphanomyces astaci strains on the immune response of the noble crayfish *Astacus astacus*. *J. Invertebr. Pathol.* 132, 115–124. doi: 10.1016/j.jip.2015.08.007

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. R. Stat. Soc. Series B Stat. Methodol.* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

Boštjančić, L. L., Francesconi, C., Rutz, C., Hoffbeck, L., Poidevin, L., Kress, A., et al. (2021). Comparative transcriptomics analysis of noble crayfish and marbled crayfish immune response to crayfish plague disease. *bioRxiv* [Preprint]. doi: 10.1101/2021.05.25.44516

Cerenius, L., Andersson, M. G., and Söderhäll, K. (2009). "*Aphanomyces astaci* and crustaceans," in *Oomycete Genetics and Genomics: Diversity, Interactions, and Research Tools*, eds K. Lamour and S. Kamoun (Hoboken, NJ: John Wiley & Sons, Inc).

Chucholl, C. (2012). Invaders for sale: trade and determinants of introduction of ornamental freshwater crayfish. *Biol. Invas.* 15, 125–141. doi: 10.1007/s10530-012-0273-2

Chucholl, C., Morawetz, K., and Groß, H. (2012). The clones are coming – strong increase in Marmorkrebs [Procambarus fallax (Hagen, 1870) f. virginalis] records from Europe. *Aquat. Invas.* 7, 511–519. doi: 10.3391/ai.2012.7.4.008

Diéguez-Uribeondo, J., Huang, T. H., Cerenius, L., and Söderhäll, K. (1995). Physiological adaptation of an *Aphanomyces astaci* strain isolated from the freshwater crayfish *Procambarus clarkii*. *Mycol. Res.* 99, 574–578. doi: 10.1016/S0953-7562(09)80716-8

Ercoli, F., Kaldre, K., Paaver, T., and Gross, R. (2019). First record of an established marbled crayfish *Procambarus virginalis* (Lyko, 2017) population in Estonia. *Bioinvas. Rec.* 8, 675–683. doi: 10.3391/bir.2019.8.3.25

Gruber, C., Kortet, R., Vainikka, A., Hyvaerinen, P., Rantala, M., Pikkarainen, A., et al. (2014). Variation in resistance to the invasive crayfish plague and immune defence in the native noble crayfish. *Ann. Zool. Fenn.* 51, 371–389. doi: 10.5735/086.051.0403

Günter, C., Lehninger, M., Wolf, W., Buri, D., Pfeiffer, M., and Chucholl, C. (2019). Umweltinduzierte Plastizität eines pathenogenetischen Flusskrebses: Populationsökologie des Marmorkrebses *Procambarus viriginalis* (LYKO 2017) in Südwestdeutschland. *Verein. Forum Flussk.* 30, 25–30.

Hauton, C. (2012). The scope of the crustacean immune system for disease control. *J. Invertebr. Pathol.* 110, 251–260. doi: 10.1016/j.jip.2012.03.005

Huang, T. S., Cerenius, L., and Söderhäll, K. (1994). Analysis of genetic diversity in the crayfish plague fungus, *Aphanomyces astaci*, by random amplification of polymorphic DNA. *Aquaculture* 126, 1–9. doi: 10.1016/0044-8486(94)90243-7

Jussila, J., Edsman, L., Maguire, I., Dieguez-Uribeondo, J., and Theissinger, K. (2021). Money kills native ecosystems: european crayfish as an example. *Front. Ecol. Evol.* 9:648495. doi: 10.3389/fevo.2021.648495

Jussila, J., Kokko, H., Kortet, R., and Makkonen, J. (2013). Aphanomyces astaci PsI-genotype isolates from different Finnish signal crayfish stocks show variation in their virulence but still kill fast. *Knowl. Managt. Aquatic Ecosyst.* 126:10. doi: 10.1051/kmae/2013077

Jussila, J., Maguire, I., Kokko, H., Tiitinen, V., and Makkonen, J. (2020). Narrow-clawed crayfish in Finland: *Aphanomyces astaci* resistance and genetic relationship to other selected European and Asian populations. *Knowl. Managt. Aquatic Ecosyst.* 421:30. doi: 10.1051/kmae/2020022

Jussila, J., Makkonen, J., Vainikka, A., Kortet, R., and Kokko, H. (2011a). Latent crayfish plague (*Aphanomyces astaci*) infection in a robust wild noble crayfish (Astacus astacus) population. *Aquaculture* 321, 17–20. doi: 10.1016/j.aquaculture.2011.08.026

Jussila, J., Makkonen, J., and Kokko, H. (2011b). Peracetic acid (PAA) treatment is an effective disinfectant against crayfish plague (*Aphanomyces astaci*) spores in aquaculture. *Aquaculture* 320, 37–42. doi: 10.1016/j.aquaculture.2011.08.008

Jussila, J., Makkonen, J., Vainikka, A., Kortet, R., and Kokko, H. (2014). Crayfish plague dilemma: how to be a courteous killer? *Bor. Env. Res.* 19, 235--244.

Jussila, J., Vrezec, A., Jaklič, T., Kukkonen, H., Makkonen, J., and Kokko, H. (2017). *Aphanomyces astaci* isolate from latently infected stone crayfish (*Austropotamobius torrentium*) population is virulent. *J. Invertebr. Pathol.* 149, 15–20. doi: 10.1016/j.jip.2017.07.003

Jussila, J., Vrezec, A., Makkonen, J., Kortet, R., and Kokko, H. (2015). "Invasive crayfish and their invasive diseases in Europe with the focus on the virulence evolution of the crayfish plague," in *Biological Invasions in Changing Ecosystems*, ed. J. Canning-Clode (Warsaw, Poland: De Gruyter Open).

Keller, N. S., Pfeiffer, M., Roessink, I., Schulz, R., and Schrimpf, A. (2014). First evidence of crayfish plague agent in populations of the marbled crayfish (*Procambarus fallax forma virginalis*). *Knowl. Managt. Aquatic Ecosyst.* 414, 15. doi: 10.1051/kmae/2014032

Kokko, H., Harlioglu, M. M., Aydin, H., Makkonen, J., Gökmen, G., Aksu, Ö, et al. (2018). Observations of crayfish plague infections in commercially important narrow-clawed crayfish populations in Turkey. *Knowl. Manag. Aquat. Ecosyst.* 419:10. doi: 10.1051/kmae/2018001

Kouba, A., Petrusek, A., and Kozák, P. (2014). Continental-wide distribution of crayfish species in Europe: update and maps. *Knowl. Manag. Aquat. Ecosyst.* 413:05. doi: 10.1051/kmae/2014007

Kozubíková, E., Viljamaa-Dirks, S., Heinikainen, S., and Petrusek, A. (2011). Spiny-cheek crayfish *Orconectes limosus* carry a novel genotype of the crayfish plague pathogen *Aphanomyces astaci*. *J. Invertebr. Pathol* 108, 214–216. doi: 10.1016/j.jip.2011.08.002

Kusar, D., Vrezec, A., Ocepek, M., and Jencic, V. (2013). *Aphanomyces astaci* in wild crayfish populations in Slovenia: first report of persistent infection in a stone crayfish *Austropotamobius torrentium* population. *Dis. Aquat. Org.* 103, 157–169. doi: 10.3354/dao02567

Lenich, F. (2019). Feldforschung extrem – Krebse beobachten und erforschen in ihrem eigenen Lebensraum unter Wasser. *Verein. Florum Flussk.* 31, 30–38.

Lipták, B., Mojžišová, M., Grul'a, D., Christophoryová, J., Jablonski, D., Bláha, M., et al. (2017). Slovak section of the Danube has its well-established breeding ground of marbled crayfish *Procambarus fallax F. virginalis*. *Knowl. Manag. Aquat. Ecosyst.* 418:40. doi: 10.1051/kmae/2017029

Lipták, B., Mrugała, A., Pekárik, L., Mutkoviè, A., Grul'a, D., Petrusek, A., et al. (2016). Expansion of the marbled crayfish in Slovakia: beginning of an invasion in the Danube catchment? *J. Limnol.* 75:2. doi: 10.4081/jlimnol.2016.1313

Lyko, F. (2017). The marbled crayfish (*Decapoda*: Cambaridae) represents an independent new species. *Zootaxa* 4363, 544–552. doi: 10.11646/zootaxa.4363.4.6

Maguire, I., Jelič, M., Klobuèar, G., Delpy, M., Delaunay, C., and Grandjean, F. (2016). Prevalence of the pathogen Aphanomyces astaci in freshwater crayfish populations in Croatia. *Dis. Aquat. Org.* 118, 45–53. doi: 10.3354/dao02955

Makkonen, J., Jussila, J., and Kokko, H. (2012b). The diversity of the pathogenic Oomycete (*Aphanomyces astaci*) chitinase genes within the genotypes indicate adaptation to its hosts. *Fungal. Genet. Biol.* 49, 635–642. doi: 10.1016/j.fgb.2012.05.014

Makkonen, J., Jussila, J., Kortet, R., Vainikka, A., and Kokko, H. (2012a). Differing virulence of *Aphanomyces astaci* isolates and elevated resistance of noble crayfish *Astacus astacus* against crayfish plague. *Dis. Aquat. Org.* 102, 129–136. doi: 10.3354/dao02547

Makkonen, J., Jussila, J., Panteleit, J., Keller, N. S., Schrimpf, A., Theissinger, K., et al. (2018). MtDNA allows the sensitive detection and haplotyping of the crayfish plague disease agent *Aphanomyces astaci* showing clues about its origin and migration. *Parasitology* 145, 1210–1218. doi: 10.1017/S0031182018000227

Makkonen, J., Kokko, H., Gökmen, G., Ward, J., Umek, J., Kortet, R., et al. (2019). The signal crayfish (*Pacifastacus leniusculus*) in Lake Tahoe (USA) hosts multiple Aphanomyces species. *J. Invertebr. Pathol.* 166:107218. doi: 10.1016/j.jip.2019.107218

Makkonen, J., Kokko, H., Vainikka, A., Kortet, R., and Jussila, J. (2014). Dose-dependent mortality of the noble crayfish (*Astacus astacus*) to different strains of the crayfish plague (*Aphanomyces astaci*). *J. Invertebr. Pathol.* 115, 86–91. doi: 10.1016/j.jip.2013.10.009

Marten, M., Werth, C., and Marten, D. (2004). Der Marmorkrebs (Cambaridae, *Decapoda*) in Deutsch - land - ein weiteres Neozoon im Einzugsgebiet des Rheins. *Lauterbornia* 50, 17–23.

Martin, P., Shen, H., Füllne, G., and Scholtz, G. (2010). The first record of the parthenogenetic Marmorkrebs (*Decapoda, Astacida*, Cambaridae) in the wild in Saxony (Germany) raises the question of its actual threat to European freshwater ecosystems. *Aquat. Invas.* 5, 397–403. doi: 10.3391/ai.2010.5.4.09

Martín-Torrijos, L., Campos Llach, M., Pou-Rovira, Q., and Diéguez-Uribeondo, J. (2017). Resistance to the crayfish plague, *Aphanomyces astaci* (Oomycota) in the endangered freshwater crayfish species, *Austropotamobius pallipes*. *PLoS One* 12:e0181226. doi: 10.1371/journal.pone.0181226

Martín-Torrijos, L., Martínez-Ríos, M., Casabella-Herrero, G., Collins, J., Adams, S., and Dieìguez-Uribeondo, J. (2021). Tracing the origin of the crayfish plague pathogen, *Aphanomyces astaci*, to the Southeastern United States. *Sci. Rep.* 11:9332. doi: 10.1038/s41598-021-88704-8

Mrugała, A., Kozubíková-Balcarová, E., Chucholl, C., Resino, S. C., Viljamaa-Dirks, S., Vukič, J., et al. (2015). Trade of ornamental crayfish in Europe as a possible introduction pathway for important crustacean diseases: crayfish plague and white spot syndrome. *Biol. Invas.* 17, 1313–1326. doi: 10.1007/s10530-014-0795-x

Mrugała, A., Veselỳ, L., Petrusek, A., Viljamaa-Dirks, S., and Kouba, A. (2016). May Cherax destructor contribute to *Aphanomyces astaci* spread in Central Europe? *Aquat. Invas.* 11, 459–468. doi: 10.3391/ai.2016.11.4.10

Panteleit, J., Horvath, T., Jussila, J., Makkonen, J., Perry, W., Schulz, R., et al. (2019). Invasive rusty crayfish (*Faxonius rusticus*) populations in North America are infected with the crayfish plague disease agent (*Aphanomyces astaci*). *Freshw. Sci.* 38, 425–433. doi: 10.1086/703417

Panteleit, J., Keller, N. S., Diéguez-Uribeondo, J., Makkonen, J., Martín-Torrijos, L., Patrulea, V., et al. (2018). Hidden sites in the distribution of the crayfish plague pathogen *Aphanomyces astaci* in Eastern Europe: relicts of genetic groups from older outbreaks? *J. Invertebr. Pathol.* 157, 117–124. doi: 10.1016/j.jip.2018.05.006

Pârvulescu, L., Togor, A., Lele, S. F., Scheu, S., Sinca, D., and Panteleit, J. (2017). First established population of marbled crayfish *Procambarus fallax* (Hagen, 1870) f. virginalis (*Decapoda*, Cambaridae) in Romania. *BioInvasions Rec* 6, 357–362. doi: 10.3391/bir.2017.6.4.09

Patoka, J., Buøiè, M., Koláø, V., Bláha, M., Petrtıl, M., Franta, P., et al. (2016). Predictions of marbled crayfish establishment in conurbations fulfilled: evidences from the Czech republic. *Biologia* 71, 1380–1385. doi: 10.1515/biolog-2016-0164

Rahe, R., and Soylu, E. (1989). Identification of the pathogenic fungus causing destruction to Turkish crayfish stocks (*Astacus leptodactylus*). *J. Invertebr. Pathol.* 54, 10–15. doi: 10.1016/0022-2011(89)90132-8

Sandström, A., Andersson, M., Asp, A., Bohman, P., Edsman, L., Engdahl, F., et al. (2014). Population collapses in introduced non-indigenous crayfish. *Biol. Invasions* 16, 1961–1977. doi: 10.1007/s10530-014-0641-1

Scholtz, G., Braband, A., Tolley, L., Reimann, A., Mittmann, B., Lukhaup, C., et al. (2003). Parthenogenesis in an outsider crayfish. *Nature* 421, 806. doi: 10.1038/421806a

Schrimpf, A., Pârvulescu, L., Copilaş-Ciocianu, D., Petrusek, A., and Schulz, R. (2012). Crayfish plague pathogen detected in the Danube Delta – a potential threat to freshwater biodiversity in southeastern Europe. *Aquat. Invasions* 7, 503–510. doi: 10.3391/ai.2012.7.4.007

Seitz, R., Vilpoux, K., Hopp, U., Harzsch, S., and Maier, G. (2005). Ontogeny of the Marmorkrebs (marbled crayfish): a parthenogenetic crayfish with unknown origin and phylogenetic position. *J. Exp. Zool. A Comp. Exp. Biol.* 303, 393–405. doi: 10.1002/jez.a.143

Svoboda, J., Mrugała, A., Kozubíková—Balcarová, E., and Petrusek, A. (2017). Hosts and transmission of the crayfish plague pathogen *Aphanomyces astaci*: a review. *J. Fish Dis.* 40, 127–140. doi: 10.1111/jfd.12472

Svoboda, J., Strand, D. A., Vrålstad, T., Grandjean, F., Edsman, L., Kozák, P., et al. (2014). The crayfish plague pathogen can infect freshwater-inhabiting crabs. *Freshw. Biol.* 59, 918–929. doi: 10.1111/fwb.12315

Thörnqvist, P.-O., and Söderhäll, K. (1993). *Psorospermium haeckeli* and its interaction with the crayfish defence system. *Aquaculture* 117, 205–213. doi: 10.1016/0044-8486(93)90320-x

Timur, G. (1990). Crayfish plague in some lakes of Turkey. *Bull. Eur. Ass. Fish Path.* 10, 100–103.

Unestam, T. (1966). Studies on the crayfish plague fungus *Aphanomyces astaci*. II. Factors affecting zoospores and zoospore production. *Physiol. Plant.* 19, 1110–1119. doi: 10.1111/j.1399-3054.1966.tb07104.x

Unestam, T. (1969). On the adaptation of *Aphanomyces astaci* as a parasite. *Physiol. Plant* 22, 221–235. doi: 10.1111/j.1399-3054.1969.tb07371.x

Unestam, T., and Nylund, J.-E. (1972). Blood reactions in vitro in crayfish against a fungal parasite, *Aphanomyces astaci*. *J. Invertebr. Pathol.* 19, 94–106. doi: 10.1016/0022-2011(72)90194-2

Unestam, T., and Weiss, D. W. (1970). The host-parasite relationship between freshwater crayfish and the crayfish disease fungus *Aphanomyces astaci*: responses to infection by a susceptible and a resistant species. *J. Gen. Microbiol.* 60, 77–90. doi: 10.1099/00221287-60-1-77

Ungureanu, E., Mojžišová, M., Tangerman, M., Ion, M. C., Pârvulescu, L., and Petrusek, A. (2020). The spatial distribution of *Aphanomyces astaci* genotypes across Europe: introducing the first data from Ukraine. *Freshw. Crayfish* 25, 77–87. doi: 10.5869/fc.2020.v25-1.077

Veselỳ, L., Buøiè, M., and Kouba, A. (2015). Hardy exotics species in temperate zone: can "warm water" crayfish invaders establish regardless of low temperatures? *Sci. Rep.* 5:16340. doi: 10.1038/srep16340

Viljamaa-Dirks, S., and Heinikainen, S. (2019). A tentative new species *Aphanomyces fennicus* sp. nov. interferes with molecular diagnostic methods for crayfish plague. *J. Fish Dis.* 42, 413–422. doi: 10.1111/jfd.12955

Viljamaa-Dirks, S., Heinikainen, S., Nieminen, M., Vennerström, P., and Pelkonen, S. (2011). Persistent infection by crayfish plague *Aphanomyces astaci* in a noble crayfish population – A case report. *Bull. Eur. Ass. Fish. Pathol.* 31, 182--188.

Viljamaa-Dirks, S., Heinikainen, S., Virtala, A. M., Torssonen, H., and Pelkonen, S. (2016). Variation in the hyphal growth rate and the virulence of two genotypes of the crayfish plague organism *Aphanomyces astaci*. *J. Fish Dis.* 39, 753–764. doi: 10.1111/jfd.12407

Vogt, G. (2018). Investigating the genetic and epigenetic basis of big biological questions with the parthenogenetic marbled crayfish: a review and perspectives. *J. Biosci.* 43, 189–223. doi: 10.1007/s12038-018-9741-x

Vogt, G. (2020). "Biology, ecology, evolution, systematics and utilization of the parthenogenetic marbled crayfish, *Procambarus virginalis*," in *Crayfish: Evolution, Habitat and Conservation Strategies*, ed. F. B. Ribeiro (Hauppauge, NY: Nova Publishers).

Vogt, G., Dorn, N. J., Pfeiffer, M., Lukhaup, C., Williams, B. W., Schulz, R., et al. (2019). The dimension of biological change caused by autotriploidy: a meta-analysis with triploid crayfish *Procambarus virginalis* and its diploid parent *Procambarus fallax*. *Zool. Anzeiger* 281, 53–67. doi: 10.1016/j.jcz.2019.06.006

Vogt, G., Lukhaup, C., Williams, B. W., Pfeiffer, M., Dorn, N. J., Schulz, R., et al. (2018). Morphological characterization and genotyping of the marbled crayfish and new evidence on its origin. *Zootaxa* 4524, 329–350. doi: 10.11646/zootaxa.4524.3.3

Vrålstad, T., Knutsen, A. K., Tengs, T., and Holst-Jensen, A. (2009). A quantitative TaqMan MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. *Vet. Microbiol.* 137, 146–155. doi: 10.1016/j.vetmic.2008.12.022

Weiperth, A., Bláha, M., Szajbert, B., Seprõs, R., Bányai, Z., Patoka, J., et al. (2020). Hungary: a European hotspot of non-native crayfish biodiversity. *Knowl. Manag. Aquat. Ecosyst.* 421:43. doi: 10.1051/kmae/2020035

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past collaboration with several of the authors JM, HK, JJ, and AS.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 21 September 2021
doi: 10.3389/fevo.2021.669292

Unraveling the Hidden Diversity of the Native White Claw Crayfish in the Iberian Peninsula

Laura Martín-Torrijos[1], David Buckley[2,3], Ignacio Doadrio[4], Annie Machordom[4] and Javier Diéguez-Uribeondo[1]*

[1] Department of Mycology, Real Jardín Botánico-CSIC (RJB-CSIC), Madrid, Spain, [2] Department of Biology (Genetics), Universidad Autónoma de Madrid (UAM), Madrid, Spain, [3] Centro de Investigación en Biodiversidad y Cambio Global (CIBC-UAM), Universidad Autónoma de Madrid (UAM), Madrid, Spain, [4] Department of Biodiversity and Evolutionary Biology, Museo Nacional de Ciencias Naturales-CSIC (MNCN-CSIC), Madrid, Spain

Several European freshwater crayfish species are currently included in one of the IUCN Red list categories. In the Iberian Peninsula, the native *Austropotamobius pallipes* species complex (the white clawed crayfish, WCC) has experienced a drastic decline since 1973. Implementing conservation management strategies for this species requires a better understanding of the patterns and structure of its genetic diversity. In this study, we assessed the levels and patterns of genetic variation in 71 populations along the whole distributional range of the WCC in the Iberian Peninsula. The two mitochondrial markers analyzed (*Cytochrome Oxidase subunit I* and *16S rRNA* genes) indicated high levels of genetic diversity, which are significantly geographically structured in three main genetic groups, two corresponding to Northern and one to Central-Eastern and the westernmost Iberian Peninsula. The diversity found included new private haplotypes, and revealed the potential effect of paleogeographic barriers and last glaciations in the population structure observed. Current conservation and management programs for the WCC in the Iberian Peninsula should consider these three phylogeographic groups as essential management units in order to preserve the remaining genetic diversity in the species.

Keywords: *Austropotamobius pallipes*, mitochondrial *16S rRNA*, mitochondrial *COI*, genetic diversity, phylogeographic structure, conservation

OPEN ACCESS

Edited by:
Leopold Füreder, University of Innsbruck, Austria

Reviewed by:
Lucian Pârvulescu, West University of Timişoara, Romania
Elena Tricarico, University of Florence, Italy

****Correspondence:***
Laura Martín-Torrijos
lmtorrijos@rjb.csic.es

Specialty section:
This article was submitted to Conservation and Restoration Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *18 February 2021*
Accepted: *26 August 2021*
Published: *21 September 2021*

Citation:
Martín-Torrijos L, Buckley D, Doadrio I, Machordom A and Diéguez-Uribeondo J (2021) Unraveling the Hidden Diversity of the Native White Claw Crayfish in the Iberian Peninsula. Front. Ecol. Evol. 9:669292. doi: 10.3389/fevo.2021.669292

INTRODUCTION

The status of the European crayfish represents a paradigmatic example of the worldwide freshwater biodiversity crisis. Currently, there are six native European crayfish species: the white-clawed crayfish (WCC) (*Austropotamobius pallipes* species complex, see below), the stone crayfish (SC) (*Austropotamobius torrentium*), the noble crayfish (NC) (*Astacus astacus*), the narrow-clawed crayfish (NCC) (*Pontastacus leptodactylus*), the thick-clawed crayfish (TCC) (*Pontastacus pachypus*), and the recently described *Austropotamobius bihariensis* (Pârvulescu, 2019). These crustaceans have experienced a rapid decimation due to overexploitation, water pollution, flow modification, habitat destruction, habitat and populations fragmentation, invasive species, and especially the emerging crayfish plague disease caused by the pathogen *Aphanomyces astaci* (Jussila et al., 2021 for review).

As a consequence of this decline, several crayfish species are included in one of the IUCN Red list categories (e.g., WCC and NC are listed as endangered and vulnerable, respectively) with a declining population trend (IUCN, 2021). Thus, there has been a recent interest in studying the patterns of genetic diversity in the European crayfish species to establish sound management and conservation plans (Schubart and Huber, 2006; Akhan et al., 2014; Jelić et al., 2016; Bláha et al., 2017; Schrimpf et al., 2017; Lovrenčić et al., 2020). This is especially true for the WCC in the Iberian Peninsula since this species has experienced a drastic decline for the last 45 years (Füreder et al., 2010). The taxonomic status of WCC, furthermore, is complex. Initial analyses based on nuclear DNA supported the existence of one species (Chiesa et al., 2011; Scalici and Bravi, 2012) while analyses based on mitochondrial DNA (mtDNA) and allozymes suggested that *Austrapotamobius pallipes* actually represents a species complex. This complex was proposed to comprise two species, *A. pallipes sensu stricto* and *A. italicus*, which in turn includes four subspecies: *A. i. carsicus, A. i. meridionalis, A. i. italicus* and *A. i. carinthiacus* (Fratini et al., 2005).

The WCC populations of the Iberian Peninsula have been referred as the westernmost part of the *A. i. italicus* lineage. They were thought as an introduction from the Italian Peninsula (Vedia and Miranda, 2013; Clavero et al., 2016), although they are now considered of autochthonous/natural origin (e.g., Beroiz et al., 2008; Diéguez-Uribeondo et al., 2008; Pedraza-Lara et al., 2010; Matallanas et al., 2011, 2016). Historically, its distributional range in the Iberian territory covered most part of this area until the introduction of two North American invasive crayfish, *Procambarus clarkii* and *Pacifastacus leniusculus*, which are chronic carriers of the crayfish plague pathogen (Alonso et al., 2000). Since the introduction of this pathogen, at least 80% of the original European populations have disappeared (Füreder et al., 2010), and the trend of the remaining Iberian populations is still decreasing (Aldabe et al., 1991; Temiño and Sáez-Royuela, 1998). The remaining Iberian WCC populations, and their genetic diversity, thrive in isolated mountainous creeks or in inaccessible brooks. Initial studies analyzing the genetic diversity of the Iberian WCC were based on mitochondrial regions, e.g., *16S rRNA* and *Cytochrome Oxidase Subunit I* (*COI*). These investigations did not find significant levels of genetic diversity among the populations studied (Santucci et al., 1997; Grandjean et al., 2000, 2001, 2002; Trontelj et al., 2005). However, subsequent studies evidenced higher levels of genetic diversity and a perceptible genetic structure by increasing the number of populations and sample size (Beroiz et al., 2008; Diéguez-Uribeondo et al., 2008), especially when new markers were designed. These studies indicated a strong geographical structure and confirmed the existence of high genetic diversity, similar to the levels found in other European crayfish populations (Pedraza-Lara et al., 2010; Matallanas et al., 2011, 2016). In these studies, however, the distributional range of WCC in the Iberian Peninsula was not fully covered. Although this species does not appear to be naturally distributed in some areas of the Western Iberia peninsula (is absent or very rare in the acid rock areas of Portugal, Galicia, Extremadura and West of Andalucía) (Alonso et al., 2000), there was an overlooked gap in the Central-Western area, still under-sampled and unstudied. The fact that WCC has not been studied within its whole range of distribution, could bias the estimates for overall genetic patterns of diversity for the species in the Iberian Peninsula. Therefore, a study considering the whole range of this species in the Iberian Peninsula is in need, and will provide new insights that will also help clarifying previous claims of a non-native origin of this species in the Iberian Peninsula (Vedia and Miranda, 2013; Clavero et al., 2016).

Thus, the aim of this study was to unravel the potential hidden genetic diversity within the unexplored Iberian WCC populations and to identify patterns of the genetic variation and structure. For this purpose, we have significantly increased the number of sampled populations from previous studies along a wider distributional range in the Iberian Peninsula, applying two highly informative mitochondrial DNA regions, the *16S rRNA* and *COI* genes. Approaching the true diversity and identifying patterns of genetic diversity of the native WCC in the Iberian Peninsula is crucial for maintaining the genetic pool of this endangered species. Moreover, these results will improve the design of conservation programs for the species.

MATERIALS AND METHODS

Crayfish Sampling

A total of 265 specimens of WCC were collected from 47 populations throughout the geographical distribution of the WCC in the Iberian Peninsula (**Table 1**). Due to the conservation status of some populations, and variability in populations size, the number of specimens per population included in the study was unequal. Crayfish were captured using nets and by hand in collaboration with the environmental officers of each of the localities. In addition, and to cover the geographical range of the WCC distribution, we included specimens from the "Crayfish Collection" of the RJB-CSIC in Madrid (set of historical samples collected and preserved since 1998). A walking leg from each individual was excised and preserved in 96% ethanol in a 2 ml tube until the molecular analysis. All crayfish were returned to their habitat alive.

DNA Extraction, Amplification and Sequencing

Samples were first rinsed with TE buffer (Tris 10 mM/EDTA 1 mM, pH 8) to remove the preserving ethanol. Each walking leg was cleaned up to three times with TE and left overnight in the buffer. Each sample was then transferred to a 2 ml tube, which was frozen at −80°C and afterward lyophilized in a freeze dryer VirTis BenchTop K for 24 h (≤−50°C; ≤ 20 mTorr) to facilitate the grinding of the genetic material by mechanical rupture using a TissueLyser (QIAGEN).

Genomic DNA was extracted with an E.Z.N.A. ®Insect DNA Kit (Omega bio-tek, Norcross, Atlanta, United States). The election of the mitochondrial markers used in this study was made trying to maximized the information obtained from the sequences as well as the compatibility with the information available in GenBank. Therefore, we selected the mitochondrial *16S rRNA* and *COI* genes. The primers pair used to amplify the

TABLE 1 | Populations and locations of the white-clawed crayfish analyzed in the present work (**Figure 3**).

Population	Location	Catchment	SAMOVA	*n*	S	H	Hd	π	D	Fs	Collection
AL1	Altube/Álava	Ebro	G3	10	0	1 (H24)	0.000	0.0000	n/c	n/c	#21# Matallanas et al., 2016
AS1	Cangas de Onis/Asturias	Sella	G2	2	0	1 (H30)	0.000	0.0000	n/c	n/c	This study
AS2	Cangas de Onis/Asturias	Sella	G2	10	0	1 (H30)	0.000	0.0000	n/c	n/c	#3# Matallanas et al., 2016
AS3	Cangas de Onis/Asturias	Sella	G2	10	0	1 (H30)	0.000	0.0000	n/c	n/c	#15# Matallanas et al., 2016
AV1	Sanchorreja/Ávila	Duero	G1	9	0	1 (H1)	0.000	0.0000	n/c	n/c	This study
AV2	Santa María del Cubillo/Ávila	Duero	G1	8	0	1 (H1)	0.000	0.0000	n/c	n/c	This study
AV3	Sanchorreja/Ávila	Duero	G1	9	0	1 (H1)	0.000	0.0000	n/c	n/c	This study
BU4	Rebolledo de la Torre/Burgos	Duero	G2	10	1	2 (H30, H32)	0.356	0.0001	0.015	0.417	This study
BU7	Santa María del Campo/Burgos	Duero	G3	3	0	1 (H24)	0.000	0.0000	n/c	n/c	This study
BU22	Fuentenebro/Burgos	Duero	G1	9	3	4 (H1, H9, H13, and H15)	0.833	0.0005	0.794	−0.450	This study
BU34	Hontoria de Valdearados/Burgos	Duero	G1	6	0	1 (H16)	0.000	0.0000	n/c	n/c	This study
BU53	Santo Domingo de Silos//Burgos	Duero	G3	7	4	4 (H24, H26, H27, and H28)	0.810	0.0006	−0.319	−0.655	This study
BU58	Santo Domingo de Silos/Burgos	Duero	G2	10	1	2 (H24, H30)	0.533	0.0002	1.303	1.029	This study
BU64	Arauzo de Miel/Burgos	Ebro	G1	9	1	2 (H1, H13)	0.500	0.0002	0.986	0.849	This study
BU82	San Zadornil/Burgos	Ebro	G2	2	1	2 (H29, H30)	1.000	0.0004	0.000	0.000	This study
BU83	San Zadornil/Burgos	Ebro	G2	4	0	1 (H30)	0.000	0.0000	n/c	n/c	This study
BU84	San Zadornil/Burgos	Ebro	G2	5	0	1 (H30)	0.000	0.0000	n/c	n/c	This study
BU85	San Zadornil/Burgos	Ebro	G2	3	0	1 (H30)	0.000	0.0000	n/c	n/c	This study
BU86	San Zadornil/Burgos	Ebro	G2	5	2	3 (H30, H34, and H35)	0.700	0.0004	0.243	−0.475	This study
BU98	Padrones de Burela/Burgos	Ebro	G2	10	1	2 (H30, H31)	0.200	0.0001	−1.112	−0.339	#11# Matallanas et al., 2016
BU99	Rebolledo Traspeña/Burgos	Duero	G2	10	6	2 (H21, H30)	0.200	0.0005	−1.796	2.607	#16# Matallanas et al., 2016
CAS1	Lucena del Cid/Castellon	Júcar	G1	10	0	1 (H1)	0.000	0.0000	0.000	n/c	#18# Matallanas et al., 2016
CAS2	La Pobla de Benifassa/Castellon	Júcar	G1	10	2	3 (H1, H5, and H16)	0.644	0.0004	1.743	0.643	#23# Matallanas et al., 2016
CR2	Pozuelo de Calatrava/Ciudad Real	Guadiana	G1	4	1	2 (H1, H16)	0.667	0.0003	1.633	0.540	This study
CU1	Almagraro/Cuenca	Júcar	G1	2	1	2 (H1, H16)	1.000	0.0004	0.000	0.000	This study
CU2	Las Truchas/Cuenca	Tajo	G1	3	0	1 (H16)	0.000	0.0000	n/c	n/c	This study
CU3	Pedregoso/Cuenca	Júcar	G1	3	1	2 (H16, H19)	0.667	0.0003	0.000	0.201	This study
CU4	Pozuelo/Cuenca	Tajo	G1	3	3	3 (H1, H16, and H24)	1.000	0.0008	0.000	−0.693	This study
CU5	Valmelero/Cuenca	Tajo	G1	4	0	1 (H16)	0.000	0.0000	n/c	n/c	This study
CU6	Vaquerizas/Cuenca	Tajo	G1	3	0	1 (H16)	0.000	0.0000	n/c	n/c	This study
CU7	Huerta de Obispalia/Cuenca	Guadiana	G1	10	1	2 (H16, H17)	0.200	0.0001	−1.112	−0.339	#5# Matallanas et al., 2016
CU8	Pozuelo/Cuenca	Tajo	G1	10	0	1 (H16)	0.000	0.0000	0.000	n/c	#20# Matallanas et al., 2016
CU9	Valdemoro/Cuenca	Júcar	G1	10	2	3 (H1, H4, and H16)	0.600	0.0003	0.120	−0.101	#24# Matallanas et al., 2016
GIR1	Escaramat/Gerona	Cataluña	G1	3	3	2 (H14, H16)	0.667	0.0008	0.000	1.609	This study
GIR2	Falgars/Gerona	Cataluña	G1	11	1	2 (H1, H2)	0.327	0.0001	−0.100	0.356	This study

(Continued)

TABLE 1 | (Continued)

Population	Location	Catchment	SAMOVA	*n*	S	H	Hd	π	D	Fs	Collection
GIR3	La Fabrega/Gerona	Cataluña	G1	12	1	2 (H1, H16)	0.485	0.0002	1.066	1.003	This study
GIR4	La Plana/Gerona	Cataluña	G1	11	3	3 (H1, H10, and H20)	0.346	0.0002	−1.600	0.885	This study
GIR6	Santa Llucía//Gerona	Cataluña	G1	11	2	3 (H1, H2, and H16)	0.564	0.0003	0.036	−0.113	This study
GIR7	Olot/Gerona	Cataluña	G1	10	1	2 (H1, H6)	0.200	0.0001	−1.112	−0.339	This study
GRA1	Albuñuelas/Granada	Guadalquivir	G1	10	0	1 (H1)	0.000	0.0000	n/c	n/c	#9# Matallanas et al., 2016
GU1	Chaparrillo/Guadalajara	Tajo	G1	5	1	2 (H1, H16)	0.600	0.0002	1.225	0.626	This study
GU2	Río Gallo/Guadalajara	Tajo	G1	10	1	2 (H1, H16)	0.556	0.0002	1.464	1.096	#22# Matallanas et al., 2016
HU1	Barranco Villano/Huesca	Ebro	G1	5	0	1 (H16)	0.000	0.0000	n/c	n/c	This study
HU2	Formiga/Huesca	Ebro	G1	2	0	1 (H1)	0.000	0.0000	n/c	n/c	This study
HU3	Casbas/Huesca	Ebro	G1	10	7	4 (H1, H3, H12, and H24)	0.711	0.0008	−0.926	0.517	#2# Matallanas et al., 2016
JA1	Cazorla/Jaen	Guadalquivir	G1	2	0	1 (H1)	0.000	0.0000	0.000	0.000	This study
LE1	Lugán/León	Duero	G3	10	3	3 (H1, H24, and H30)	0.600	0.0004	−0.658	0.206	#14# Matallanas et al., 2016
LE3	Garrafe de Torios/León	Duero	G1	11	1	2 (H1, H13)	0.436	0.0002	0.671	0.779	This study
LER1	Pont de Suert/Lérida	Ebro	G1	10	2	3 (H16, H22, and H23)	0.622	0.0003	0.019	−0.156	#1# Matallanas et al., 2016
LU1	Pol/Lugo	Miño	G1	10	1	2 (H1, H8)	0.200	0.0001	−1.112	−0.339	#4# Matallanas et al., 2016
LU2	Castro de Rei/Lugo	Miño	G1	10	0	1 (H1)	0.000	0.0000	n/c	n/c	#13# Matallanas et al., 2016
NA2	Doneztebe/Navarra	Bidasoa	G3	3	1	2 (H24, H30)	0.667	0.0003	0.000	0.201	This study
NA3	Aoiz51/Artanga/Navarra	Ebro	G3	3	3	2 (H16, H24)	0.667	0.0008	0.000	1.609	This study
NA4	Bidaurreta/Ultzama-Araquil/Navarra	Ebro	G3	4	0	1 (H24)	0.000	0.0000	n/c	n/c	This study
NA5	Leurtza/Navarra	Bidasoa	G3	4	1	2 (H24, H30)	0.500	0.0002	−0.612	0.172	This study
NA7	Sunbilla/Navarra	Bidasoa	G2	2	1	2 (H24, H30)	1.000	0.0004	0.000	0.000	This study
NA8	Estella/Navarra	Ebro	G3	10	3	2 (H11, H24)	0.200	0.0002	−1.562	1.225	#19# Matallanas et al., 2016
PA1	Herrera del Pisuerga/Palencia	Duero	G1	4	4	2 (H16, H30)	0.667	0.0011	2.080	2.719	This study
SO1	Navaceno/Soria	Duero	G1	10	1	2 (H1, H16)	0.356	0.0001	0.015	0.417	This study
SO2	Navaceno/Soria	Duero	G1	9	1	2 (H1, H16)	0.500	0.0002	0.983	0.849	This study
SO3	Mont Vicarias/Soria	Ebro	G1	2	1	2 (H1, H16)	1.000	0.0004	0.000	0.000	This study
SO8	Almarza/Soria	Duero	G1	9	2	3 (H1, H13, and H16)	0.556	0.0003	−0.583	−0.532	This study
SO15	Devanos/Soria	Ebro	G3	9	0	1 (H24)	0.000	0.0000	n/c	n/c	This study
TE1	Valderrobles/Teruel	Ebro	G1	2	1	2 (H1, H7)	1.000	0.0004	0.000	0.000	This study
TE2	Beceite/Teruel	Ebro	G1	10	0	1 (H1)	0.000	0.0000	n/c	n/c	#6# Matallanas et al., 2016
TE3	Castellote/Teruel	Ebro	G1	10	1	2 (H1, H16)	0.467	0.0002	0.819	0.818	#10# Matallanas et al., 2016
TE5	Cucalon/Teruel	Ebro	G1	10	7	5 (H1, H8, H16, H18, and H33)	0.800	0.0008	−1.002	−0.733	#12# Matallanas et al., 2016
VA1	Utiel/Valencia	Júcar	G1	10	4	2 (H16, H25)	0.356	0.0006	0.023	3.025	#17# Matallanas et al., 2016
VALL1	Adalia/Valladolid	Duero	G1	6	0	1 (H1)	0.000	0.0000	n/c	n/c	This study
VALL2	Adalia/Valladolid	Duero	G1	2	0	1 (H1)	0.000	0.0000	n/c	n/c	This study
ZA1	Santa Eulalia de Gállego/Zaragoza	Ebro	G1	10	3	3 (H1, H16, and H24)	0.689	0.0005	0.775	0.985	#7# Matallanas et al., 2016

Catchment, hydrogeographic catchment; SAMOVA, grouping structure assigned by SAMOVA; n, sample size; S, number of polymorphic sites; H, number and haplotypes found and their reference code between brackets; Hd, haplotype diversity; π, nucleotide diversity; D, Tajima's D; Fs, Fu's Fs.
The "n/c" means not calculated.

mitochondrial *16S rRNA* gene, 1472 (Crandall and Fitzpatrick, 1996) and Tor12sc (Largiadèr et al., 2000) amplified a fragment that included partial sequences of the *12S rRNA*, the *16S rRNA* and the *val-tRNA*. From hereafter, the combination of the *12S rRNA*, *val-tRNA* and *16S rRNA* regions will be referred as *16S*. The primers pair used to amplify the mitochondrial *COI* gene was C/N 2769 (Gopurenko et al., 1999) and LCO1490 (Folmer et al., 1994). Both were used in a single round PCR following the protocols in Matallanas et al. (2016). Negative controls containing no DNA were included in all single round PCR for both primer pairs.

We checked for positive amplicons by running an electrophoresis with 3-μl aliquots of the amplification product in 1% agarose TAE gels stained with SBYR-Safe (Thermo Fisher Scientific). Amplified products were purified using a QIAquick PCR Purification Kit (Qiagen, Germany). Double strand PCR positive products were sequenced using an automated sequencer (Applied Biosystems 3730xl DNA, Macrogen, Netherlands).

Sequence Data

Both mtDNA sequence strands were assembled and analyzed using the program Geneious v10.0.2 (Kearse et al., 2012). We ran BLAST searches to check the nature of the generated sequences. We revised the sequences chromatograms for double-peaks and performed the alignments using the MAFFT algorithm (Katoh et al., 2002). The final alignments included sequences of 1,317 base pairs (bp) for *16S* gene and 1,151 bp for *COI* gene.

Additionally, we downloaded a total of 748 sequences for the *16S* gene and 669 sequences for the *COI* gene from GenBank from previous studies (**Supplementary Appendix 1**). Moreover, the sequences for the *16S* and *COI* genes from the genome of *Austropotamobius torrentium* were also downloaded from GenBank (accession numbers NC_033504), as well as the sequences for the *16S* and *COI* for *A. italicus carsicus* (accession numbers KX370126 and KX369706, respectively) and were used as outgroups in the phylogenetic analyses (**Supplementary Appendix 1**).

Data Sets

We designed three different data sets to take advantage of the genetic information in previous studies. Data Set 1 was designed to frame the samples from this study within the last phylogenetic scenario proposed by Jelić et al. (2016). Data Set 2 was designed to reconstruct the phylogenetic relationships of lineages within the Iberian Peninsula. Data Set 3 was designed to estimate the genetic diversity and the population structure of the existing populations in the Iberian Peninsula.

Data Set 1 comprised all the sequences from the range of distribution of WCC in Europe, including three subsets: (i) *16S* with a total of 1,013 sequences, (ii) *COI* with a total of 934 sequences, and (iii) concatenated *16S* and *COI* genes from the specimens that had both genes sequenced with a total of 934 specimens. *Austropotamobius torrentium* was used as an outgroup in all the subsets.

The Data Set 2 comprised all the sequences from the distributional range of the WCC in the Iberian Peninsula, including three subsets: (i) *16S* with a total of 706 sequences, (ii) *COI* with a total of 706, and (iii) concatenated *16S* and *COI* genes from the specimens that had both genes sequenced with a total of 706 specimens. One specimen from the sister clade of the Iberian WCC populations (*A. italicus carsicus*) was used as an outgroup in all the subsets.

The Data Set 3 comprised a total of 505 sequences with the largest base pair length for the three defined subsets: (i) *16S*, (ii) *COI* and (iii) concatenated *16S* and *COI* genes, of which 265 sequences were obtained from the 47 populations of this study (GenBank accession numbers MW317197-MW317461 for *16S* and MW325345-MW325609 for *COI*). The remaining 240 sequences belong to 24 populations spanning the distributional range of the *A. pallipes* complex in the Iberian Peninsula (Matallanas et al., 2016) (**Table 1**). This data set was used to determine both genetic diversity and genetic structure of the existing populations in the Iberian Peninsula.

Phylogenetic Relationships

Phylogenetic relationships were analyzed for the Data Set 1 and Data Set 2. We identified the best model of nucleotide substitution and best partition schemes for the *16S* and the *COI* genes for each of the data sets in Partition Finder v2.1.1 (Lanfear et al., 2016), using the Bayesian Information criterion (BIC). The base frequencies were estimated using maximum likelihood (+X) rather than empirically (+F) for the implemented models.

Phylogenetic analyses for the *16S*, the *COI* and the concatenated *16S* and *COI* genes were run under Bayesian inference (BI) and maximum likelihood (ML). The BI analysis was performed in MrBayes v.3.2.6 software (Ronquist et al., 2012) using the default MCMCMC search algorithm with 100,000,000 generations, three runs (eight chains per run) with a burn-in of 25% generations. Nodes with posterior probability (pp) values $\geq$ 0.95 were considered as supported. Tracer v1.6.0 (Rambaut et al., 2014) was used to check for convergence and stationarity of the three runs. The ML analysis was performed in RAxML v.8 (Stamatakis, 2014) as implemented in raxmlGUI v1.5b1 (Silvestro and Michalak, 2012), with 100 independent replicates and 1,000 rapid bootstraps. Nodes with bootstrap values $\geq$ 75 were considered as supported. The resulting trees from the BI and ML analyses were visualized in FigTree v1.4.2 (Rambaut, 2012).

Genetic Diversity and Genetic Structure

We examined the genetic structure of the WCC in the Iberian Peninsula using the Data Set 3 with a Spatial Analysis of the Molecular Variance (SAMOVA v2.0) (Dupanloup et al., 2002). This method defines groups of populations (k) that are genetically and geographically homogeneous and maximally differentiated from each other (it maximizes the proportion of total genetic variance, F_{CT}) to identify genetic barriers. Moreover, it also defines groups of populations that are maximally differentiated from each other, without constraint for the geographic composition of the groups. We run SAMOVA v2.0 from $k = 2$ to $k = 20$ and each run was with 1,000 simulated annealing processes.

We used TCS v.1.21 (Clement et al., 2000) to represent the mutational changes between the sequences throughout

the most parsimonious haplotype network and the genealogical relationships were visualized using PopArt v1.7.2 (Leigh and Bryant, 2015).

To further dissect the patterns of genetic diversity and the genetic structure of the WCC populations in the Iberian Peninsula, we defined three grouping strategies of the Data Set 3: (I) populations as independent units, (II) populations grouped in hydrogeographic areas, and (III) populations grouped in the phylogeographic areas determined by SAMOVA (**Table 1**). Due to the unequal number of the samples conforming each sampled population, we carried out the rarefaction of the data in the populations grouped in hydrogeographic areas and in the populations grouped by phylogeographic areas. For this, we selected randomly n* individuals, subsampled without replacement from the larger of the original samples, and equaled the size of the smaller original sample ($n = n^* = 4$) (Magurran and McGill, 2011).

We performed two independent analyses for the two grouping structures (hydrogeographic areas and the phylogeographic areas), first for the raw data and then for the rarefied data. We estimated the number of polymorphic (segregating) sites (S), the number of haplotypes (H), the haplotype diversity (Hd), the average number of nucleotide differences (k) and the nucleotide diversity (π) using the program DNAsp v.5.10.01 (Librado and Rozas, 2009). We estimated the haplotypes frequencies and the genetic diversity indices (Tajima's D and Fu's Fs) with the software Arlequin v3.5.2.2 (Excoffier et al., 2005). The patterns of genetic variation with the analysis of the molecular variance (AMOVA) were analyzed in Arlequin v3.5.2.2 (Excoffier et al., 2005). Significance values ($p < 0.05$) were assessed by using 10,000 permutations.

RESULTS

Phylogenetic Relationships

The phylogenetic analyses of the independent *16S* and *COI* genes subsets (Data Set 1) provided congruent trees. The concatenated mtDNA fragments conforming Data Set 1 were divided in three partitions. First partition included the *12S rRNA* and *val-tRNA* genes (155 pb) with a JC substitution model (Jukes and Cantor, 1969), a second partition included the *16S rRNA* gene (1,162 pb) with a HKY + I + X substitution model (Hasegawa et al., 1985) and a third partition included the *COI* gene (1,151 pb) with a HKY + G + X substitution model (Hasegawa et al., 1985). The analyses (BI and ML) of the concatenated data set showed a clear differentiation between *A. pallipes* and *A. italicus* (**Figure 1**). Within *A. italicus*, sequences attributed to *A. i. italicus*, *A. i. carinthiacus*, and *A. i. carsicus* formed a well-supported Clade (Clade I). The remaining samples corresponding to *A. i. meridionalis* did not form a monophyletic group, as defined in Fratini et al. (2005) (**Figure 1**). The samples of *A. i. italicus* and *A. i. carinthiacus* from Austria, Italy, France, and the Iberian Peninsula grouped in a well-supported sub-clade, sister to the samples of *A. i. carsicus* (**Figure 1**).

The analyses of the Data Set 2 showed that the independent analysis of the two subsets of *16S* and *COI* genes provided congruent trees. PartitionFinder subdivided the concatenated Data Set 2 into three partitions. The first partition included *12S rRNA* and *val-tRNA* genes (155 pb) with a JC substitution model (Jukes and Cantor, 1969), a second partition included the *16S rRNA* gene (1,162 pb) with a HKY + G substitution model (Hasegawa et al., 1985), and a third partition included the *COI* gene (1,151 pb) with a HKY + G substitution model (Hasegawa et al., 1985).

The phylogenetic analyses (BI and ML) resulting from this concatenated dataset showed no clear relationships among Iberian *A. i. italicus* populations. Some samples grouped together with high support but sometimes with no clear geographic correspondence (**Figure 2**).

Genetic Diversity and Genetic Structure

The SAMOVA analysis resolved three main groups ($K = 3$) out of the 71 populations of WCC in the Iberian Peninsula: the Central-Eastern (Group 1), the North-Western Group (Group 2) and the North-Central Group (Group 3) (**Figure 3**), representing the Iberian populations with and without a geographical constraint (equal values for complete data and rarefied samples, $F_{CT} = F_{CT}^* = 0.73$). Group 1 included 48 populations, Group 2 included 13, and Group 3 included 10 populations (**Table 1**).

The most parsimonious haplotype network showed 35 haplotypes in the Iberian Peninsula (**Figure 4**). Four of them (H1, H16, H24, and H30) were the most represented in the area. The two haplotypes H1 and H16 covered the Center, South, East and the westernmost populations of the Iberian Peninsula (**Figure 3**) (Group1 defined by SAMOVA), haplotype H30 covered the North-Western of the Iberian Peninsula (Group2 defined by SAMOVA), and the haplotype H24 covered the North-Center of the Iberian Peninsula (Group3 defined by SAMOVA). We recovered six shared haplotypes (H1, H2, H13, H16, H24, and H30) among different populations, presenting medium-high frequencies, while the 29 remaining haplotypes appeared as unique from one specific population, conforming private haplotypes with low-medium frequencies (**Table 2**).

The 71 Iberian WCC populations hosted 35 haplotypes, representing a noteworthy mean haplotype diversity (Hd = 0.775), but low nucleotide diversity ($\pi = 0.00073$). We found that 27 out of the 71 populations were monomorphic for one of four different haplotypes (H1, H16, H24, or H30). The highest number of haplotypes per population was found in TE5 with five haplotypes (H1, H8, H16, H18, and H33), followed by other three populations hosting four different haplotypes each: BU22 (H1, H9, H13, and H15), BU53 (H24, H26, H27, and H28), and HU3 (H1, H3, H12, and H24) (**Table 1**). These four populations hosted a high haplotype diversity ($Hd_{TE5} = 0.800$; $Hd_{BU22} = 0.833$; $Hd_{BU53} = 0.810$, and $Hd_{HU3} = 0.711$), and medium-high nucleotide diversity ($\pi_{TE5} = 0.0008$, $\pi_{BU22} = 0.0005$, $\pi_{BU53} = 0.0006$, and $\pi_{HU3} = 0.0008$) (**Table 1**).

Following the Iberian hydrogeographic river basins, we defined 10 hydrogeographic areas (**Table 1**). The results for the raw data set showed differences between the hydrogeographic areas, with the Ebro area standing out by hosting the largest number of haplotypes (18 out of 35 haplotypes). The Ebro river basin also presented the highest haplotype

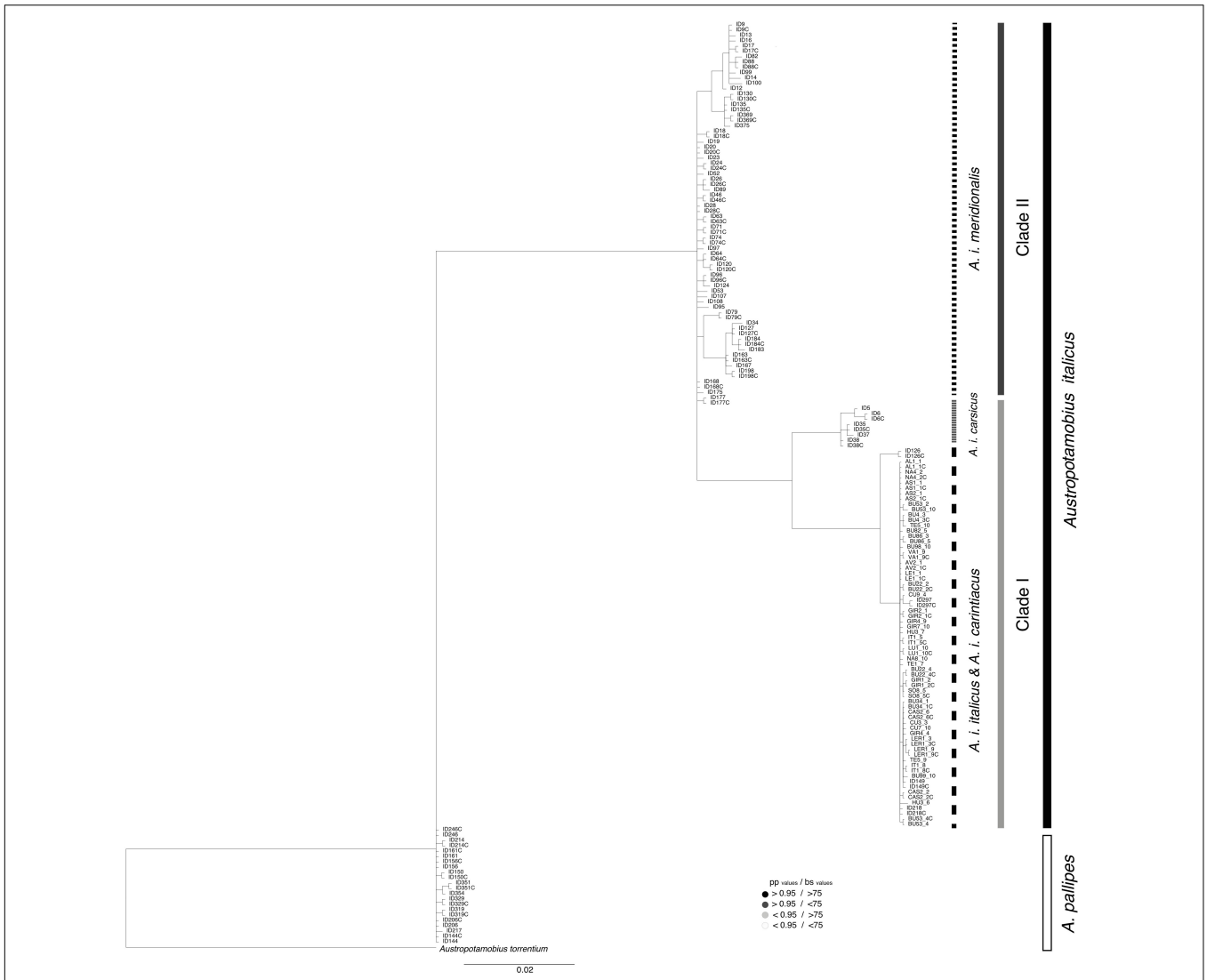

FIGURE 1 | Bayesian inference analyses based on concatenated mitochondrial *16S rRNA* and *COI* genes sequences from Data Set 1. The posterior probability values (pp values) are from Bayesian inference analysis and the bootstrap support values (bs values) from Maximum Likelihood. Nodes with black circles have pp values higher than 0.95 and bs higher than 75, nodes with dark gray circles have pp higher than 0.95 and bs lower than 75, nodes with light gray circles have pp lower than 0.95 and bs higher than 75, and nodes with white circles have pp lower than 0.95 and bs lower than 75. Scales bar for phylogenetic analysis indicates substitutions per site.

diversity (Hd = 0.803) and nucleotide diversity (π = 0.00082) (**Table 3**). Only two hydrogeographic areas (Guadalquivir and Sella) were monomorphic for two different haplotypes (H1 and H30, respectively) (**Table 3**). The rarefied data set for the 10 hydrogeographic areas included nine individuals per area (**Table 3**). There were again differences among the hydrogeographic areas, with the Ebro and Duero areas hosting five different haplotypes. Rarefied samples from the Ebro area hosted haplotypes H1, H30, H11, H16, and H22, and Duero hosted haplotypes H1, H9, H16, H24, and H30. Both presented the highest haplotype (Hd_{Ebro} = 0.861 and Hd_{Duero} = 0.806) and nucleotide (π_{Ebro} = 0.001 and π_{Duero} = 0.0007) diversities. Three hydrogeographic areas were monomorphic for two different haplotypes H1 for Guadalquivir and Miño areas, and H30 for Sella area (**Table 3**). Tajima's D and Fu's Fs were non-significant ($p > 0.05$) for the 10 hydrogeographic areas in both raw and rarefied data, indicating no evidence of recent demographic expansion within these grouping structures (**Table 3**).

Results of AMOVA analysis using the 10 hydrogeographic areas with the raw data suggested more genetic differentiation among populations within hydrogeographic areas (52.58% of variation, $p < 0.0001$) than between the hydrogeographic areas (20.46% of variation, $p < 0.0001$). The rarefied data suggested on the other hand more genetic differentiation between hydrogeographic areas (51.96% of variation, $p < 0.0001$), although they also showed high genetic differentiation among populations within hydrogeographic areas (31.08% of variation, $p < 0.03$). Fixation indices F_{ST} and F_{SC} presented medium/high

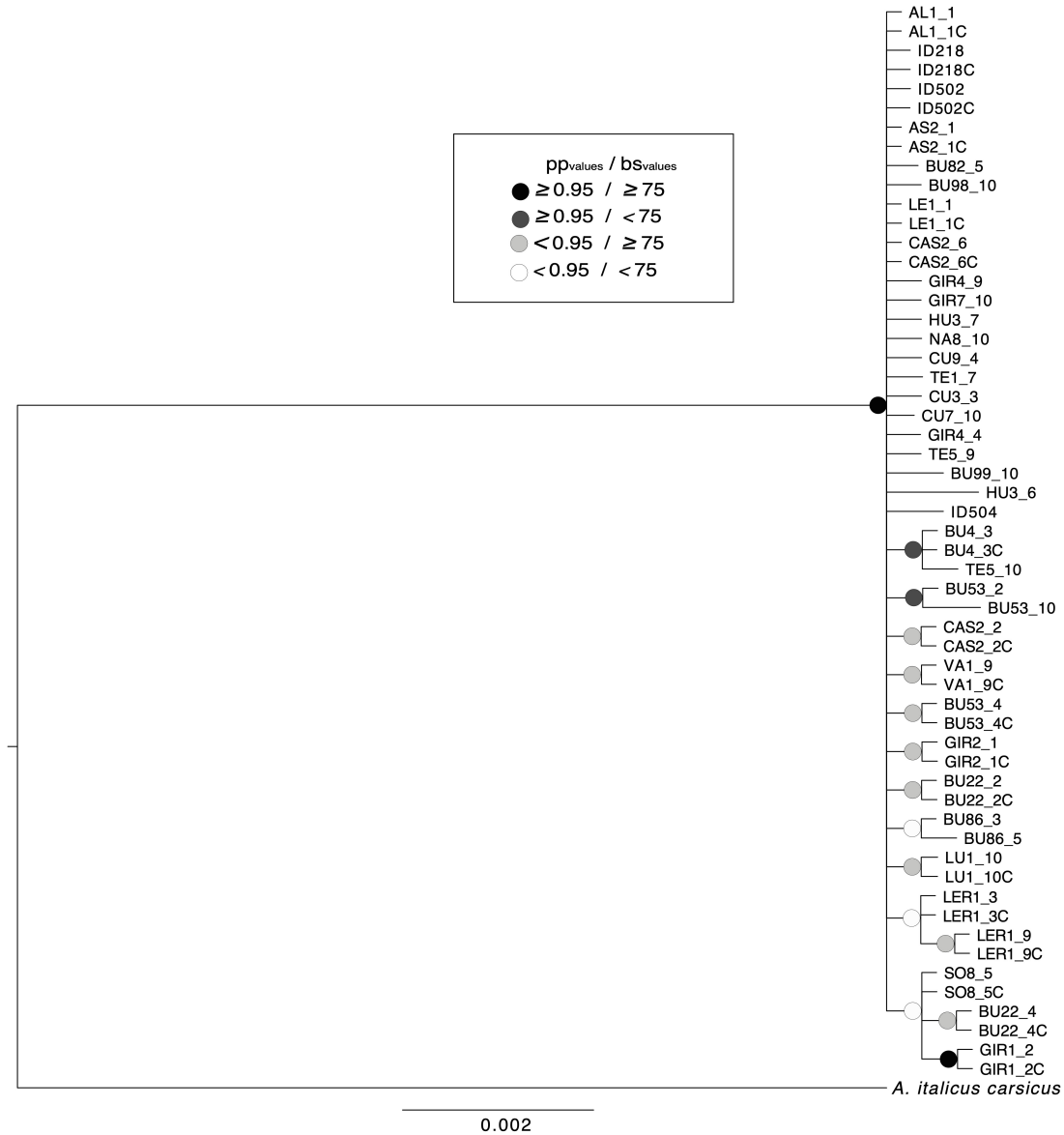

FIGURE 2 | Bayesian inference analyses based on concatenated mitochondrial *16S rRNA* and *COI* genes sequences from Data Set 2. The posterior probability values (pp values) are from Bayesian inference analysis and the bootstrap support values (bs values) from Maximum Likelihood. Nodes with black circles have pp values higher than 0.95 and bs higher than 75, nodes with dark gray circles have pp higher than 0.95 and bs lower than 75, nodes with light gray circles have pp lower than 0.95 and bs higher than 75, and nodes with white circles have pp lower than 0.95 and bs lower than 75. Scales bar for phylogenetic analysis indicates substitutions per site.

scores for the raw data (F_{ST} = 0.73032 and F_{SC} = 0.66096) and rarefied data (F_{ST} = 0.83034 and F_{SC} = 0.64684) showing visible genetic differentiation within populations and among populations within hydrogeographic areas. On the other hand, the fixation indices found no noteworthy differentiation among hydrogeographic areas, being bigger for the rarefied than for the raw data (F_{CT-RAW} = 0.20456 and $F_{CT-RAREFIED}$ = 0.51959).

According to the third grouping structure, we defined three phylogeographic areas, one grouping most of the localities from the Center and East of the Iberian Peninsula (Group 1), and two in the North (Group 2 and Group 3) (**Figure 3**). The results for the raw data set showed differences among three phylogeographic areas, with the Group 1 hosting 28 out of 35 haplotypes [haplotype diversity (Hd) = 0.636, and nucleotide diversity (π) = 0.00038, and no monomorphic groups for any of the locations] (**Table 4**). The rarefied data set for each of the three phylogeographic areas included 62 individuals per basin (**Table 4**). None of the groups were monomorphic, and there were differences among them, with the Group 1 also hosting the largest set of haplotypes (9 out of a total of 18), with the highest haplotype (Hd = 0.646) and nucleotide (π = 0.00039) diversities. Values for Tajima's D and Fu's Fs were significant ($p < 0.05$) for

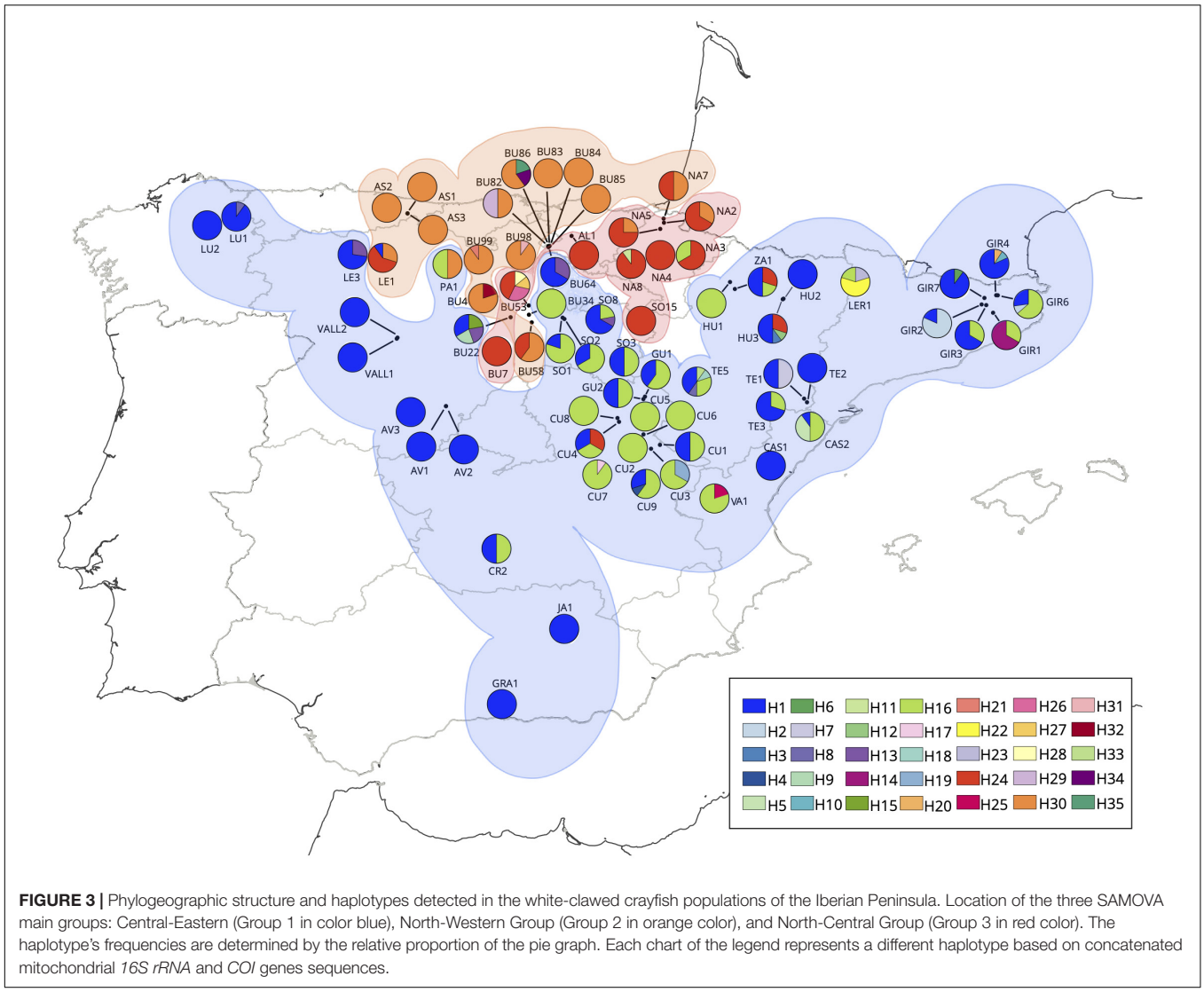

FIGURE 3 | Phylogeographic structure and haplotypes detected in the white-clawed crayfish populations of the Iberian Peninsula. Location of the three SAMOVA main groups: Central-Eastern (Group 1 in color blue), North-Western Group (Group 2 in orange color), and North-Central Group (Group 3 in red color). The haplotype's frequencies are determined by the relative proportion of the pie graph. Each chart of the legend represents a different haplotype based on concatenated mitochondrial *16S rRNA* and *COI* genes sequences.

the three phylogeographic areas for both raw and rarefied data, with the exception of Tajima's D on rarefied data from Group 1. These significances indicated an evidence of recent demographic expansions within each of these three groups.

Results of the AMOVA analysis using the three phylogeographic areas suggested more genetic differentiation among phylogeographic areas for both raw and rarefied data (73.01% of variation, $p < 0.0001$ for raw data, and 73.80% of variation, $p < 0.0001$ for rarefied data). Moreover, the variation within populations was noticeable (16.91% of variation, $p < 0.0001$ for raw data and 18.23% of variation, $p < 0.0001$ for rarefied data). Fixation indices differed, presenting F_{ST} high values for the raw data ($F_{ST} = 0.83091$) and rarefied data ($F_{ST} = 0.81768$), but lower F_{SC} values for the raw data ($F_{SC} = 0.37358$) and rarefied data ($F_{SC} = 0.30425$), showing a visible genetic differentiation within populations but not among populations within three phylogeographic areas. On the other hand, the fixation indices found a statistically significant differentiation ($p < 0.05$) among the three phylogeographic areas for both raw and rarefied data ($F_{CT-RAW} = 0.73007$ and $F_{CT-RAREFIED} = 0.73796$).

DISCUSSION

As far as we know, this work represents the most complete and updated approximation of the WCC genetic diversity in the Iberian Peninsula. The results obtained complete and confirm the historical scenario proposed by previous studies on the genetic diversity of the WCC (Pedraza-Lara et al., 2010; Jelić et al., 2016; Matallanas et al., 2016), but also reveal new patterns of genetic diversity and phylogeographic structure. The phylogeographic approach used here, which incorporates samples from previously unexplored areas, has allowed us to find a higher genetic variation in the WCC than previously reported and also identifies new private haplotypes in the Iberian Peninsula. Moreover, the results regarding the origin of the genetic diversity and its phylogeographic structure do not support the hypothesis of an

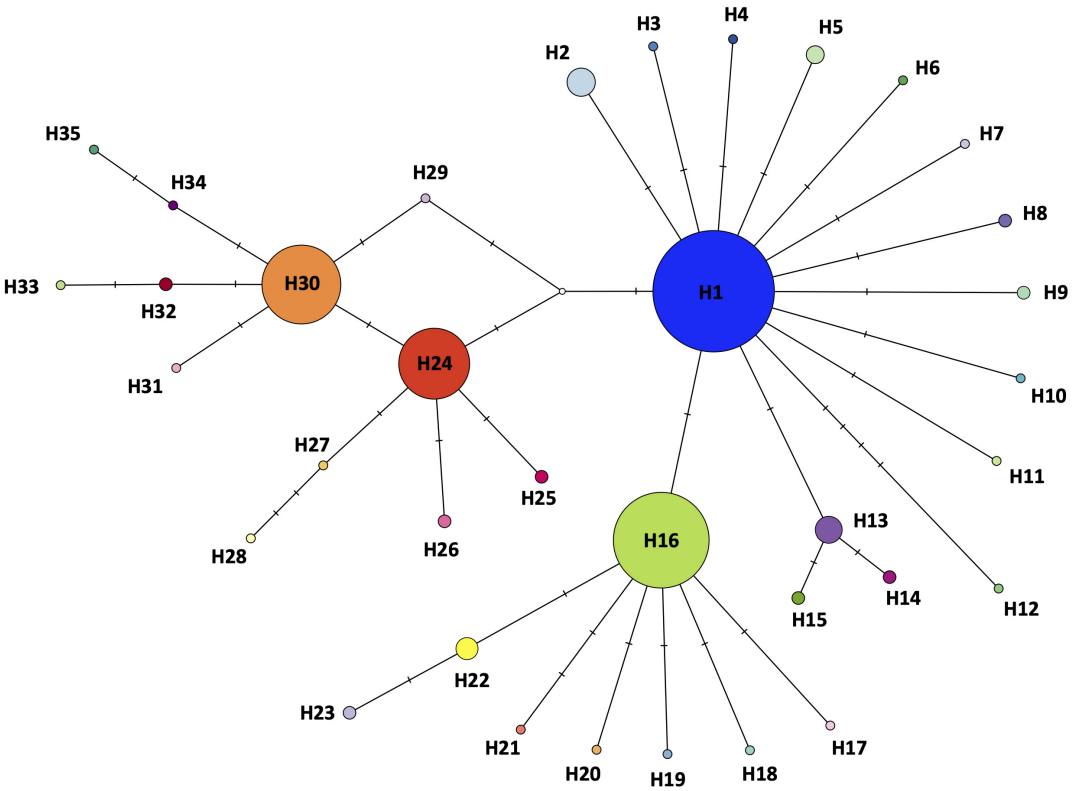

FIGURE 4 | Haplotype network based on concatenated mitochondrial *16S rRNA* and *COI* genes sequences, generated by statistical parsimony. The area of the circles is proportional to the number of specimens sharing haplotype. Mutation steps between haplotypes are shown as hatch marks (**Supplementary Appendix 2**).

introduction from Italy in the 17th century (Vedia and Miranda, 2013; Clavero et al., 2016), and, instead, strongly suggest a native origin of the WCC in the Iberian Peninsula.

Genetic variation found within Iberian WCC populations is strongly structured geographically. The results from this phylogenetic approach, also supports the scenario proposed by Pedraza-Lara et al. (2010) and Jelić et al. (2016) for the Iberian *A. i. italicus* populations within the European area. In these scenarios, the Iberian lineages grouped together with populations from Austria, Italy and South France. This group is closely related to the *A. i. carsicus*, which occurs in Croatia and Italy, and phylogenetically separated from the Central European WCC populations. Following these previous studies and the results in here, the existing genetic differentiation between Central and Southern Europe WCC populations would be of Pleistocenic/Holocenic origin, and might be related to the climate oscillations and glaciations, including the phylogeographic and demographic effects of the ice sheet presence during and after the Last Glacial Maximum (LGM) (Hewitt, 2000, 2004). The fact that Central regions of Europe were glaciated during the LGM led many species to remain isolated in the Southern glacial refugia, i.e., the Iberian, Italian, and Balkan Peninsulas. Besides the presence of the ice itself, the LGM also entailed drastic changes in temperature, droughts, desertification, and large drops in the sea levels changes, that might have shaped the range area in WCC. The distribution of *A. i. italicus* follows a Circum-Mediterranean distribution. This distribution is also found in other freshwater species (Perea et al., 2010) associated with the isolation in glacial refugia during the LGM, and/or with ancient paleogeography events. For instance, the Alps orogenesis during the late Miocene until the Pleistocene had isolated the Iberian Peninsula from Central Europe and prevented most Mediterranean freshwater species, such as the European cyprinids, to move northward (Zardoya and Doadrio, 1999; Perea et al., 2010).

The phylogenetic analyses for the Iberian WCC populations showed a basal polytomy, indicating non-solved phylogenetic relationships among them. Although some well-supported grouping are presented, more molecular markers would be necessary to identify evolutionary lineages and their relationships. These results agree with the previous assignation of Pedraza-Lara et al. (2010) and Matallanas et al. (2016) for the Iberian populations. On the other hand, we confirmed the utility of these mitochondrial markers that revealed variability patterns not found in previous studies (Toon et al., 2009). The combination of a greater sampling effort and the use of these two regions revealed a total of 35 haplotypes, 16 of which were new for the Iberian Peninsula, while Matallanas et al. (2016) found 19 haplotypes using the same *16S* and *COI* genes.

In addition, we found a strong phylogeographic structure in the Iberian populations. Previous studies designated two phylogeographic areas, while we found three genetically and geographically differentiated areas. Thus, we found two areas in

TABLE 2 | Frequency of the haplotypes found in the 71 analyzed populations (the population's code is detailed in **Table 1**).

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26	H27	H28	H29	H30	H31	H32	H33	H34	H35
AL1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·
AS1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·
AS2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·
AS3	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·
AV1	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
AV2	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
AV3	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
BU22	0.33	·	·	·	·	·	·	·	0.22	·	·	·	0.22	·	0.22	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
BU34	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
BU4	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.80	·	0.20	·	·	·
BU53	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.43	·	0.29	0.14	0.14	·	·	·	·	·	·	·
BU58	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.40	·	·	·	·	·	0.60	·	·	·	·	·
BU64	0.67	·	·	·	·	·	·	·	·	·	·	·	0.33	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
BU7	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·
BU82	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.50	0.50	·	·	·	·	·
BU83	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·
BU84	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·
BU85	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·
BU86	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.60	·	·	·	0.20	0.20
BU98	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.90	0.10	·	·	·	·
BU99	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.10	·	·	·	·	·	·	·	·	0.90	·	·	·	·	·
CAS1	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CAS2	0.10	·	·	·	0.40	·	·	·	·	·	·	·	·	·	·	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CR2	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CU1	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CU2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CU3	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.67	·	·	0.33	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CU4	0.33	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.33	·	·	·	·	·	·	·	0.33	·	·	·	·	·	·	·	·	·	·	·
CU5	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CU6	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CU7	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.90	0.10	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CU8	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CU9	0.30	·	·	0.10	·	·	·	·	·	·	·	·	·	·	·	0.60	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GIR1	·	·	·	·	·	·	·	·	·	·	·	·	·	0.67	·	0.33	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GIR2	0.18	·	·	0.82	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GIR3	0.67	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.33	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·

(Continued)

TABLE 2 | (Continued)

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26	H27	H28	H29	H30	H31	H32	H33	H34	H35
GIR4	0.82	·	·	·	·	·	·	·	·	0.09	·	·	·	·	·	·	·	·	·	0.09	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GIR6	0.27	0.09	·	·	·	·	·	·	·	·	·	·	·	·	·	0.64	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GIR7	0.90	·	·	·	·	0.10	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GRA1	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GU1	0.40	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.60	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
GU2	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
HU1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
HU2	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
HU3	0.50	·	0.10	·	·	·	·	·	·	·	·	0.10	·	·	·	·	·	·	·	·	·	·	·	0.30	·	·	·	·	·	·	·	·	·	·	·
JA1	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
LE1	0.10	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.60	·	·	·	·	·	0.30	·	·	·	·	·
LE3	0.73	·	·	·	·	·	·	·	·	·	·	·	0.27	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
LER1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.20	·	·	·	·	·	0.60	0.20	·	·	·	·	·	·	·	·	·	·	·	·
LU1	0.90	·	·	·	·	·	·	0.10	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
LU2	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
NA2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.67	·	·	·	·	·	0.33	·	·	·	·	·
NA3	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.33	·	·	·	·	·	·	·	0.67	·	·	·	·	·	·	·	·	·	·	·
NA4	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·
NA5	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.75	·	·	·	·	·	0.25	·	·	·	·	·
NA7	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.50	·	·	·	·	·	0.50	·	·	·	·	·
NA8	·	·	·	·	·	·	·	·	·	·	0.10	·	·	·	·	·	·	·	·	·	·	·	·	0.90	·	·	·	·	·	·	·	·	·	·	·
PA1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	0.50	·	·	·	·	·
SO1	0.20	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.80	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
SO15	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	1.00	·	·	·	·	·	·	·	·	·	·	·
SO2	0.33	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.67	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
SO3	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
SO8	0.67	·	·	·	·	·	·	·	·	·	·	·	0.11	·	·	0.22	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
TE1	0.50	·	·	·	·	·	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
TE2	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
TE3	0.70	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.30	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
TE5	0.40	·	·	·	·	·	·	0.10	·	·	·	·	·	·	·	0.30	·	0.10	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.10	·	·
VA1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.80	·	·	·	·	·	·	·	·	0.20	·	·	·	·	·	·	·	·	·	·
VALL1	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
VALL2	1.00	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
ZA1	0.50	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.20	·	·	·	·	·	·	·	0.30	·	·	·	·	·	·	·	·	·	·	·

The symbol "·" means zero frequency.

TABLE 3 | Genetic diversity within white-clawed crayfish hydrogeographic catchments in the Iberian Peninsula for the raw and rarefied data.

	n	S	H	Hd	π	D	Fs
Raw data							
Bidasoa	9	1	2	0.5	0.0002	0.98627	0.849
Ebro	145	20	18	0.80316	0.00082	−1.22384	−6.389
Cataluña	58	7	7	0.65094	0.00036	−1.06969	−2.232
Júcar	45	7	6	0.65354	0.00042	−0.96976	−1.075
Guadalquivir	12	0	1	0	0	n/c	n/c
Guadiana	14	2	3	0.38462	0.00016	−0.95919	−0.855
Tajo	38	3	3	0.38265	0.00019	−0.73449	0.115
Duero	142	14	12	0.76136	0.00075	−0.70395	−1.964
Miño	20	1	2	0.1	0.00004	−1.16439	−0.879
Sella	22	0	1	0	0	n/c	n/c
Rarefied data							
Bidasoa	9	1	2	0.5	0.0002	0.98627	0.849
Ebro	9	6	5	0.861	0.00101	0.57782	−0.354
Cataluña	9	2	3	0.667	0.00032	0.1959	−0.108
Júcar	9	1	2	0.556	0.00023	1.40117	1.015
Guadalquivir	9	0	1	0	0	n/c	n/c
Guadiana	9	2	3	0.417	0.00018	−1.3624	−1.081
Tajo	9	1	2	0.389	0.00016	0.15647	0.477
Duero	9	5	5	0.806	0.00068	−0.39837	−1.26
Miño	9	0	1	0	0	n/c	n/c
Sella	9	0	1	0	0	n/c	n/c

n, sample size; S, number of polymorphic sites; H, number of haplotypes found; Hd, haplotype diversity; π, nucleotide diversity; D, Tajima's D; Fs, Fu's Fs. The "n/c" means not calculated.

TABLE 4 | Genetic diversity within white-clawed crayfish SAMOVA groups in the Iberian Peninsula for the raw and rarefied data.

	n	S	H	Hd	π	D	Fs
Raw Data							
G1	360	28	25	0.636	0.00038	**−2.08288***	**−23.426***
G2	83	10	8	0.267	0.00017	**−2.07311***	**−6.285***
G3	62	8	7	0.32	0.0002	**−1.85126***	**−1.85126***
Rarefied data							
G1	62	12	9	0.646	0.00039	−1.77358	**−3.902***
G2	62	9	6	0.268	0.00018	**−2.07514***	**−3.479***
G3	62	8	7	0.32	0.0002	**−1.85126***	**−1.85126***

*n, sample size, S, number of polymorphic sites; H, number of haplotypes found; Hd, haplotype diversity; π, nucleotide diversity; D, Tajima's D; Fs, Fu's Fs; *p < 0.05.*

the North (North-Central and North-Western) represented by two main haplotypes, H24 and H30, respectively. This matches with the differentiation and structure found in other terrestrial and aquatic species, such as *Salamandra salamandra* (García-París et al., 2003), *Ichthyosaura alpestris* (Recuero et al., 2014) or *Lissotriton helveticus* (Recuero and García-París, 2011). During the LGM, strong range shifts and bottlenecks occurred and they probably played a key role in Iberian WCC populations. Although the Northern regions of the Iberian Peninsula were mostly covered with ice, glaciers remained confined within the mountain systems and did not reach the surrounding lowlands (Oliva et al., 2019). As a consequence, a number of unglaciated habitats are believed to have acted as refugia for several species (Gómez and Lunt, 2007). The identification of private haplotypes in this area could indicate that these WCC populations were geographically limited and isolated in these regions, as it occurred to other species (Hewitt, 2000). A significant postglacial expansion of these endemic haplotypes might have occurred from several populations during favorable climate periods (Hewitt, 2004). Moreover, the genetic results and the shape of the haplotype network indicated an evidence of recent demographic expansions within these two groups. These results are consistent with those of Matallanas et al. (2016), in which molecular estimations dated last WCC population expansion back to Pleistocene.

The third phylogeographic area that comprises Central-Eastern and the westernmost Iberian populations, suggested another expansion event. The evidences from the significant results obtained in the genetic diversity analysis, as well as the star-like haplotype network showed another demographic

expansion, and also reflect the possibility of another LGM-refugium. The Central-Eastern area is also represented by two main haplotypes, H1 and H16. During the LGM, these populations could have persisted in Southern areas of the Iberian Peninsula in absence of geographical or climate barriers. As evidenced by the analyses and the samples studied, these two main haplotypes could represent ancestral haplotypes: H1 (the most frequent), and H16 (only separated by one mutational step from H1). Besides, the H1 has been found in other European populations (Jelić et al., 2016; Matallanas et al., 2016). The Central-Eastern area could have had an earlier expansion than that of the Northern areas, since this area was not covered by ice during the LGM and thus, in absence of barriers, crayfish could have expanded over long distances to some extent (Robinson et al., 2000), and a better climate would have favored it. However, the unequal sampling of the specimens within the studied populations seem to have favored higher frequencies of the H1 and H16 haplotypes, so this statement must be taken with caution. Furthermore, the low genetic diversity found within the WCC southern areas could be explained by the introduction of the North American crayfish species *P. clarkii* and *P. leniusculus* during the 1970s (Alonso et al., 2000; Martín-Torrijos et al., 2019). These North American crayfish are natural carriers of the crayfish plague pathogen *A. astaci* (Martín-Torrijos et al., 2021) and responsible for the decline of the Iberian WCC until nowadays. In particular, by the end of the 1990s the Southern WCC populations almost had disappeared in the Iberian Peninsula (Alonso et al., 2000; Martín-Torrijos et al., 2019).

The current genetic diversity found in Iberian Peninsula may have been shaped by the LGM, as suggested above and in previous studies. The extensive sampling of this study has allowed us to find the greatest haplotype and nucleotide diversities so far reported for the Iberian WCC populations (Matallanas et al., 2016). We should point out, however, that 27 populations were monomorphic. These monomorphic populations were represented by the four most common haplotypes (H1, H16, H24, and H30). This agrees with the strong phylogeographic structure found. In contrast, the most diverse phylogeographic area was the Central-Eastern. This contains populations highly diverse that are located in the provinces of Burgos, Teruel and Huesca. This high genetic variation found within these populations coincides with the historical records of dense populations of WCC described (Alonso et al., 2000). This abundance evidences the importance that crayfish fisheries used to have in local economies of rural Iberian areas. By 1964, Spanish legislation already had regulated the size, amount of crayfish and the fishing gear allowed for the fishing activities (Torre Cervigón and Rodríguez Marqués, 1964). This regulation might have helped to avoid the overexploitation of the resource, maintaining most of the genetic diversity that remains nowadays. Although recent human translocations might have influenced the current Iberian crayfish distribution, there is, still, a strong phylogeographic structure. During the past years, several authors have suggested that the origin of the Iberian populations might have been the result of an Italian crayfish translocation during the 17th century (Vedia and Miranda, 2013; Clavero et al., 2016). However, the results obtained in the present study show a greater genetic diversity than that described in previous studies, and indicate that is highly structured and difficult to attribute to a 17th century translocation from Italy. Therefore, our results support the native origin of the WCC in the Iberian Peninsula. Moreover, the rapid impact of the crayfish plague during the 1970s' dramatically reduced the number of Iberian WCC populations in less than 2 years (Alonso et al., 2000). These massive declines might have extinguished highly diverse WCC populations, and what we actually come across is a small fraction of its original genetic diversity. In addition, the enormous extinctions due to the crayfish plague may have driven Iberian WCC populations to suffer inbreeding, bottlenecks and genetic drift. Currently, the difficulty of obtaining samples due to the threatened status of the WCC made us to use different number of individuals from each of the analyzed populations. The reduced number of samples obtained from some of the populations revealed the appearance of rare private haplotypes in low frequencies. These rare haplotypes may represent a reflect of the remaining biodiversity within WCC populations.

In addition, the delimited phylogeographic areas seem to explain better the genetic diversity of the Iberian WCC populations. The AMOVA analysis showed more genetic differentiation among the three phylogeographic areas and a slight genetic differentiation among populations within these areas. This suggests substantial gene flow among populations from the same phylogeographic areas. On the other hand, the reduced population sites, the geographical distribution, and the pressure over the remaining Iberian WCC populations by the continuous threat by crayfish plague, for instance, might be favoring their isolation and hindering the gene flow between them. Thus, the unique genetic diversity represented by private haplotypes, which is found in low frequencies, would remain in the same populations instead of being transferred to proximal populations.

Current populations are a remain of what Iberian WCC populations used to be. The massive extinction events that Iberian WCC have been suffering during the past 45 years due to the introduction of North American invasive crayfish carrying the crayfish plague (Martín-Torrijos et al., 2019), and intensive harvesting, might have had irreversible effects on the Iberian WCC genetic diversity. Thus, the majority of the ancient Iberian WCC genetic variation might be already extinct. The reduction of the genetic diversity, and consequently their adaptive potential (Boulding, 2008; Jump et al., 2009), could increase the species extinction risk. To preserve the maximum genetic diversity, we recommend that current conservation and management programs for the WCC in the Iberian Peninsula should consider the patterns of genetic diversity found in this study. Thus, we propose that the three phylogeographic areas revealed in this study should be considered as essential management units to preserve the genetic diversity that characterized them. Furthermore, conservation actions that include breeding and re-stocking programs should consider for each specific area not only the most common but also the private haplotypes. This

will certainly help to preserve the unique genetic pool from the endangered Iberian WCC populations.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

LM-T contributed to the design, with the laboratory work, and wrote the manuscript. DB, ID, AM, and JD-U contributed to the supervision of the manuscript and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

We would like to acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI). JD-U was supported from Project CGL2016-80526-R of the Ministerio de Economía y Competitividad, Spain, and LM-T was supported LIFE MedWetRivers (LIFE11 NAT/ES/000699) and LIFE Potamo Fauna (LIFE12 NAT/ES/001091). This study was also supported by Gobierno de Navarra (20153614 and 20166383), Junta de Comunidades de Castilla-La Mancha (20153074 and 20166103), Gobierno de Aragón (20153608; 20171065 and 20171106), and La Agencia de Medio Ambiente y Agua de Andalucía (20091163).

ACKNOWLEDGMENTS

We would like to thank the assistance in providing us with crayfish samples and information to Jokin Larumbe (Gobierno de Navarra); Fernando Alonso and Javier Valenciano (Junta de Comunidades de Castilla-La Mancha), Sara Lapesa (Gobierno de Aragón), Joan Montserrat (Parque Natural La Garrotxa, Catalunya), Lluis Benejam (CEBCAT-La Balca S. L.), Antonio Pradillo (CCEDCV—EL PALMAR, Generalitat Valenciana), Juan María Herrero (Junta de Castilla y León), Jon Zulaika (Diputación de Gipuzkoa, País Vasco), Borja Nebot (Junta de Andalucía), and Javier Galindo (Agencia Medio Ambiente y Agua de Andalucía). We would also like to thank Marta Higuera and Emilio Cano from the Real Jardín Botánico–Consejo Superior de Investigaciones Científicas (RJB-CSIC) for their technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.669292/full#supplementary-material

REFERENCES

Akhan, S., Bektas, Y., Berber, S., and Kalayci, G. (2014). Population structure and genetic analysis of narrow-clawed crayfish (*Astacus leptodactylus*) populations in Turkey. *Genetica* 142, 381–395. doi: 10.1007/s10709-014-9782-5

Aldabe, J., Esquizabel, J. I., Fernández, M. J., Iruzun, J. C., Mendia, F., Rueda, F., et al. (1991). *Estudio de Distribución y Abundancia de Las Especies de Cangrejo Presentes En Ríos Navarros. Documento Interno Del Servicio de Medio Ambiente Del Gobierno de Navarra*. Pamplona: Gobierno de Navarra.

Alonso, F., Temiño, C., and Diéguez-Uribeondo, J. (2000). Status of the white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858), in Spain: distribution and legislation. *Bull. Fr. Pêche piscic.* 356, 31–53. doi: 10.1051/kmae:2000003

Beroiz, B., Callejas, C., Alonso, F., and Ochando, M. D. (2008). Genetic Structure of Spanish white-clawed crayfish (*Austropotamobius pallipes*) populations as determined by RAPD analysis: reasons for optimism. *Aquat. Conserv. Mar. Freshw. Ecosyst.* 18, 190–201. doi: 10.1002/aqc.829

Bláha, M., Uzhytchak, M., Bondarenko, V., and Policar, T. (2017). The least known European native crayfish *Astacus pachypus* (Rathke, 1837) revealed its phylogenetic position. *Zool. Anz.* 267, 151–154. doi: 10.1016/j.jcz.2017.03.001

Boulding, E. G. (2008). "Genetic diversity, adaptive potential, and population viability in changing environments," in *Fox conservation biology: evolution in action*, ed. S. C. Carroll (New York: Oxford University Press), 199–219.

Chiesa, S., Scalici, M., Negrini, R., Gibertini, G., and Nonnis Marzano, F. (2011). Fine-scale genetic structure, phylogeny and systematics of threatened crayfish species complex. *Mol. Phylogenet. Evol.* 61, 1–11. doi: 10.1016/j.ympev.201\break1.03.031

Clavero, M., Nores, C., Kubersky-Piredda, S., and Centeno-Cuadros, A. (2016). Interdisciplinarity to reconstruct historical introductions: solving the status of cryptogenic crayfish. *Biol. Rev.* 91, 1036–1049. doi: 10.1111/brv.12205

Clement, M., Posada, D., and Crandall, K. A. (2000). TCS: a computer program to estimate gene genealogies. *Mol. Ecol.* 9, 1657–1659. doi: 10.1046/j.1365-294x.2000.01020.x

Crandall, K. A., and Fitzpatrick, J. F. (1996). Crayfish molecular systematics: using a combination of procedures to estimate phylogeny. *Syst. Biol.* 45, 1–26. doi: 10.1093/sysbio/45.1.1

Diéguez-Uribeondo, J., Royo, F., Souty-Grosset, C., Ropiquet, A., and Grandjean, F. (2008). Low genetic variability of the white-clawed crayfish in the Iberian Peninsula: its origin and management implications. *Aquat. Conserv. Mar. Freshw. Ecosyst.* 18, 19–31. doi: 10.1002/aqc.811

Dupanloup, I., Schneider, S., and Excoffier, L. (2002). A simulated annealing approach to define the genetic structure of populations. *Mol. Ecol.* 11, 2571–2581. doi: 10.1046/j.1365-294X.2002.01650.x

Excoffier, L., Laval, G., and Schneider, S. (2005). Arlequin (Version 3.0): An integrated software package for population genetics data analysis. *Evol. Bioinform. Online* 1:47.

Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* 3, 294–299.

Fratini, S., Zaccara, S., Barbaresi, S., Grandjean, F., Souty-Grosset, C., Crosa, G., et al. (2005). Phylogeography of the threatened crayfish (Genus *Austropotamobius*) in Italy: implications for its taxonomy and conservation. *Heredity* 94, 108–118. doi: 10.1038/sj.hdy.6800581

Füreder, L., Gherardi, F., Holdich, D., Reynolds, J., Sibley, P., and Souty-Grosset, C. (2010). Austropotamobius pallipes. *The IUCN Red List of Threatened Species 2010: E.T2430A9438817*. Available Online at: http://www.iucnredlist.org/details/2430/0 (accessed December 28, 2020).

García-París, M., Alcobendas, M., Buckley, D., and Wake, D. B. (2003). Dispersal of viviparity across contact zones in Iberian populations of fire salamanders (*Salamandra*) inferred from discordance of genetic and morphological traits. *Evolution* 57, 129–143. doi: 10.1111/j.0014-3820.2003.tb00221.x

Gómez, A., and Lunt, D. H. (2007). "Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula," in *Phylogeography of Southern European refugia: evolutionary perspectives on the origins and conservation of European biodiversity*, eds S. Weiss and N. Ferrand (Dordrecht: Springer Netherlands), 155–188. doi: 10.1007/1-4020-4904-8_5

Gopurenko, D., Hughes, J. M., and Keenan, C. P. (1999). Mitochondrial DNA evidence for rapid colonisation of the Indo-West Pacific by the mudcrab *Scylla serrata*. *Mar. Biol.* 134, 227–233. doi: 10.1007/s002270050541

Grandjean, F., Bouchon, D., and Souty-Grosset, C. (2002). Systematic of the European endangered crayfish species *Austropotamobius pallipes* (Decapoda: Astacidae) with a re-examination of the status of *Austropotamobius berndhauseri*. *J. Crust. Biol.* 22, 677–681. doi: 10.1651/0278-0372(2002)022[0677:soteec]2.0.co;2

Grandjean, F., Gouin, N., Souty-Grosset, C., and Diéguez-Uribeondo, J. (2001). Drastic bottlenecks in the endangered crayfish species *Austropotamobius pallipes* in Spain and implications for its colonization history. *Heredity* 86, 431–438. doi: 10.1046/j.1365-2540.2001.00849.x

Grandjean, F., Harris, D. J., Souty-Grosset, C., and Crandall, K. A. (2000). Systematics of the European endangered crayfish species *Austropotamobius pallipes* (Decapoda: Astacidae). *J. Crust. Biol.* 20, 522–529. doi: 10.1651/0278-0372(2000)020[0522:soteec]2.0.co;2

Hasegawa, M., Kishino, H., and Yano, T. (1985). Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* 22, 160–174. doi: 10.1007/bf02101694

Hewitt, G. M. (2000). The genetic legacy of the Quaternary ice ages. *Nature* 405, 907–913. doi: 10.1038/35016000

Hewitt, G. M. (2004). Genetic consequences of climatic oscillations in the Quaternary. *Philos. Trans. R. Soc. Lond.* 359, 183–195. doi: 10.1098/rstb.2003.1388

IUCN. (2021). *The IUCN Red List of Threatened Species. Version 2021-1*. Available Online at: https://www.iucnredlist.org (accessed July 28, 2021).

Jelić, M., Klobučar, G. I. V., Grandjean, F., Puillandre, N., Franjević, D., Futo, M., et al. (2016). Insights into the molecular phylogeny and historical biogeography of the white-clawed crayfish (Decapoda, Astacidae). *Mol. Phylogenet. Evol.* 103, 26–40. doi: 10.1016/j.ympev.2016.07.009

Jukes, T. H., and Cantor, C. R. (1969). "Evolution of Protein Molecules," in *Mammalian Protein Metabolism*, ed. H. N. Munro ((New York: Academic Press), 21–132. doi: 10.1016/b978-1-4832-3211-9.50009-7

Jump, A. S., Marchant, R., and Penuelas, J. (2009). Environmental change and the option value of genetic diversity. *Trends Plant Sci.* 14, 51–58. doi: 10.1016/j.tplants.2008.10.002

Jussila, J., Edsman, L., Maguire, I., Diéguez-Uribeondo, J., and Theissinger, K. (2021). Money kills native ecosystems: European crayfish as an example. *Front. Ecol. Evol.* 9:648495. doi: 10.3389/fevo.2021.648495

Katoh, K., Misawa, K., Kuma, K., and Miyata, T. (2002). MAFFT: A novel method for rapid multiple sequence alignment based on fast fourier transform. *Nucleic Acids Res.* 30, 3059–3066. doi: 10.1093/nar/gkf436

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., et al. (2012). Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28, 1647–1649. doi: 10.1093/bioinformatics/bts199

Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T., and Calcott, B. (2016). PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Mol. Biol. Evol.* 34, 772–773. doi: 10.1093/molbev/msw260

Largiadèr, C. R., Herger, F., Lörtscher, M., and Scholl, A. (2000). Assessment of natural and artificial propagation of the white-clawed crayfish (*Austropotamobius pallipes* species complex) in the Alpine region with nuclear and mitochondrial markers. *Mol. Ecol.* 9, 25–37. doi: 10.1046/j.1365-294x.2000.00830.x

Leigh, J. W., and Bryant, D. (2015). POPART: full-feature software for haplotype network construction. *Methods Ecol. Evol.* 6, 1110–1116. doi: 10.1111/2041-210X.12410 doi: 10.1111/2041-210x.12410

Librado, P., and Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25, 1451–1452. doi: 10.1093/bioinformatics/btp187

Lovrenčić, L., Bonassin, L., Boštjančić, L. L., Podnar, M., Jelić, M., Klobučar, G., et al. (2020). New insights into the genetic diversity of the stone crayfish: taxonomic and conservation implications. *BMC Evol. Biol.* 20:146. doi: 10.1186/s12862-020-01709-1

Magurran, A. E., and McGill, B. J. (2011). *Biological diversity: frontiers in measurement and assessment*. Oxford: Oxford University Press.

Martín-Torrijos, L., Kokko, H., Makkonen, J., Jussila, J., and Diéguez-Uribeondo, J. (2019). Mapping 15 years of crayfish plague in the Iberian Peninsula: The impact of two invasive species on the endangered native crayfish. *PloS One* 14:e0219223. doi: 10.1371/journal.pone.0219223

Martín-Torrijos, L., Martínez-Ríos, M., Casabella-Herrero, G., Adams, S. B., Jackson, C. R., and Diéguez-Uribeondo, J. (2021). Tracing the origin of the crayfish plague pathogen, *Aphanomyces astaci*, to the Southeastern United States. *Sci. Rep.* 11:9332. doi: 10.1038/s41598-021-88704-8

Matallanas, B., Ochando, M. D., Alonso, F., and Callejas, C. (2016). Update of genetic information for the white-clawed crayfish in Spain, with new insights into its population genetics and origin. *Org. Divers. Evol.* 16, 533–547. doi: 10.1007/s13127-016-0268-4

Matallanas, B., Ochando, M. D., Vivero, A., Beroiz, B., Alonso, F., and Callejas, C. (2011). Mitochondrial DNA variability in Spanish populations of *A. italicus* inferred from the analysis of a COI region. *Knowl. Manag. Aquat. Ecosyst.* 401:30. doi: 10.1051/kmae/2011052

Oliva, M., Palacios, D., Fernández-Fernández, J. M., Rodríguez-Rodríguez, L., García-Ruiz, J. M., Andrés, N., et al. (2019). Late quaternary glacial phases in the Iberian Peninsula. *Earth Sci. Rev.* 192, 564–600. doi: 10.1016/j.earscirev.2019.03.015

Pârvulescu, L. (2019). Introducing a new *Austropotamobius* crayfish species (Crustacea, Decapoda, Astacidae): A miocene endemism of the Apuseni Mountains, Romania. *Zool. Anz.* 279, 94–102. doi: 10.1016/j.jcz.2019.01.006

Pedraza-Lara, C., Alda, F., Carranza, S., and Doadrio, I. (2010). Mitochondrial DNA structure of the Iberian populations of the white-clawed crayfish, *Austropotamobius italicus italicus* (Faxon, 1914). *Mol. Phylogenet. Evol.* 57, 327–342. doi: 10.1016/j.ympev.2010.06.007

Perea, S., Böhme, M., Zupančič, P., Freyhof, J., Šanda, R., Özuluğ, M., et al. (2010). Phylogenetic relationships and biogeographical patterns in circum-Mediterranean subfamily leuciscinae (Teleostei, Cyprinidae) inferred from both mitochondrial and nuclear data. *BMC Evol. Biol.* 10:265. doi: 10.1186/1471-2148-10-265

Rambaut, A. (2012). *FigTree_v1.4.0*. Available Online at: http://tree.bio.ed.ac.uk/software/figtree/

Rambaut, A., Suchard, M. A., Xie, D., and Drummond, A. J. (2014). *Tracer v1.6*. Available Online at: http://tree.bio.ed.ac.uk/software/tracer/

Recuero, E., Buckley, D., García-París, M., Arntzen, J. W., Cogalniceanu, D., and Martínez-Solano, I. (2014). Evolutionary history of *Ichthyosaura alpestris* (Caudata, Salamandridae) inferred from the combined analysis of nuclear and mitochondrial markers. *Mol. Phylogenet. Evol.* 81, 207–220. doi: 10.1016/j.ympev.2014.09.014

Recuero, E., and García-París, M. (2011). Evolutionary history of *Lissotriton helveticus*: multilocus assessment of ancestral vs. recent colonization of the Iberian Peninsula. *Mol. Phylogenet. Evol.* 60, 170–182. doi: 10.1016/j.ympev.2011.04.006

Robinson, C. A., Thom, T. J., and Lucas, M. C. (2000). Ranging behaviour of a large freshwater invertebrate, the white-clawed crayfish *Austropotamobius pallipes*. *Freshw. Biol.* 44, 509–521. doi: 10.1046/j.1365-2427.2000.00603.x

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Hohna, S., et al. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* 61, 539–542. doi: 10.1093/sysbio/sys029

Santucci, F., Iaconelli, M., Andreani, P., Cianchi, R., Nascetti, G., and Bullini, L. (1997). Allozyme diversity of european freshwater crayfish of the genus *Austropotamobius*. *Bull. Fr. Pêche piscic.* 347, 663–676. doi: 10.1051/kmae/1997045

Scalici, M., and Bravi, R. (2012). Solving alpha-diversity by morphological markers contributes to arranging the systematic status of a crayfish species complex (Crustacea, Decapoda). *J. Zoolog. Syst. Evol. Res.* 50, 89–98. doi: 10.1111/j.1439-0469.2011.00643.x

Schrimpf, A., Piscione, M., Cammaerts, R., Collas, M., Herman, D., Jung, A., et al. (2017). Genetic characterization of western European noble

crayfish populations (*Astacus astacus*) for advanced conservation management strategies. *Conserv. Genet.* 18, 1299–1315. doi: 10.1007/s10592-017-0981-3

Schubart, C. D., and Huber, M. G. J. (2006). Genetic comparisons of German populations of the stone crayfish, *Austropotamobius torrentium* (Crustacea: Astacidae). *Bull. Fr. Pêche Piscic.* 380–381, 1019–1028. doi: 10.1051/kmae:2006008

Silvestro, D., and Michalak, I. (2012). RaxmlGUI: a graphical front-end for RAxML. *Org. Divers. Evol.* 12, 335–337. doi: 10.1007/s13127-011-0056-0

Stamatakis, A. (2014). Raxml version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–1313. doi: 10.1093/bioinformatics/btu033

Temiño, C. and Sáez-Royuela, M. (1998). *Resumen de los Inventarios de Cangrejo Autóctono Austropotamobius Pallipes Realizados en la Provincia de Burgos Entre 1984 y 1997. Documento Interno del Servicio Territorial de Medio Ambiente de Burgos*. Burgos: Junta de Castilla y León.

Toon, A., Finley, M., Staples, J., and Crandall, K. A. (2009). "Decapod phylogenetics and molecular evolution," in *Decapod Crustacean Phylogenetics (Crustacean Issues)*, eds J. W. Martin, K. A. Crandall, and D. L. Felder (Boca Raton: CRC Press), 15–29. doi: 10.1201/9781420092592-c2

Torre Cervigón, M., and Rodríguez Marqués, P. (1964). *El cangrejo de río en España. Documento Interno del Servicio Nacional de Pesca Fluvial y Caza*. Madrid: Ministerio de Agricultura.

Trontelj, P., Machino, Y., and Sket, B. (2005). Phylogenetic and phylogeographic relationships in the crayfish genus *Austropotamobius* inferred from mitochondrial COI gene sequences. *Mol. Phylogenet. Evol.* 34, 212–226. doi: 10.1016/j.ympev.2004.09.010

Vedia, I., and Miranda, R. (2013). Review of the state of knowledge of crayfish species in the Iberian Peninsula. *Limnetica* 32, 269–286. doi: 10.23818/limn.32.22

Zardoya, R., and Doadrio, I. (1999). Molecular evidence on the evolutionary and biogeographical patterns of European cyprinids. *J. Mol. Evol.* 49, 227–237. doi: 10.1007/PL00006545

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership