



# First Detection of the Crayfish Plague Pathogen *Aphanomyces astaci* in Costa Rica: European Mistakes Should Not Be Repeated

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## OPEN ACCESS

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### 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, Wehrmann IS and  
Diéguez-Urbeondo 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

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

## 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*, *Austropotamobius*, *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.

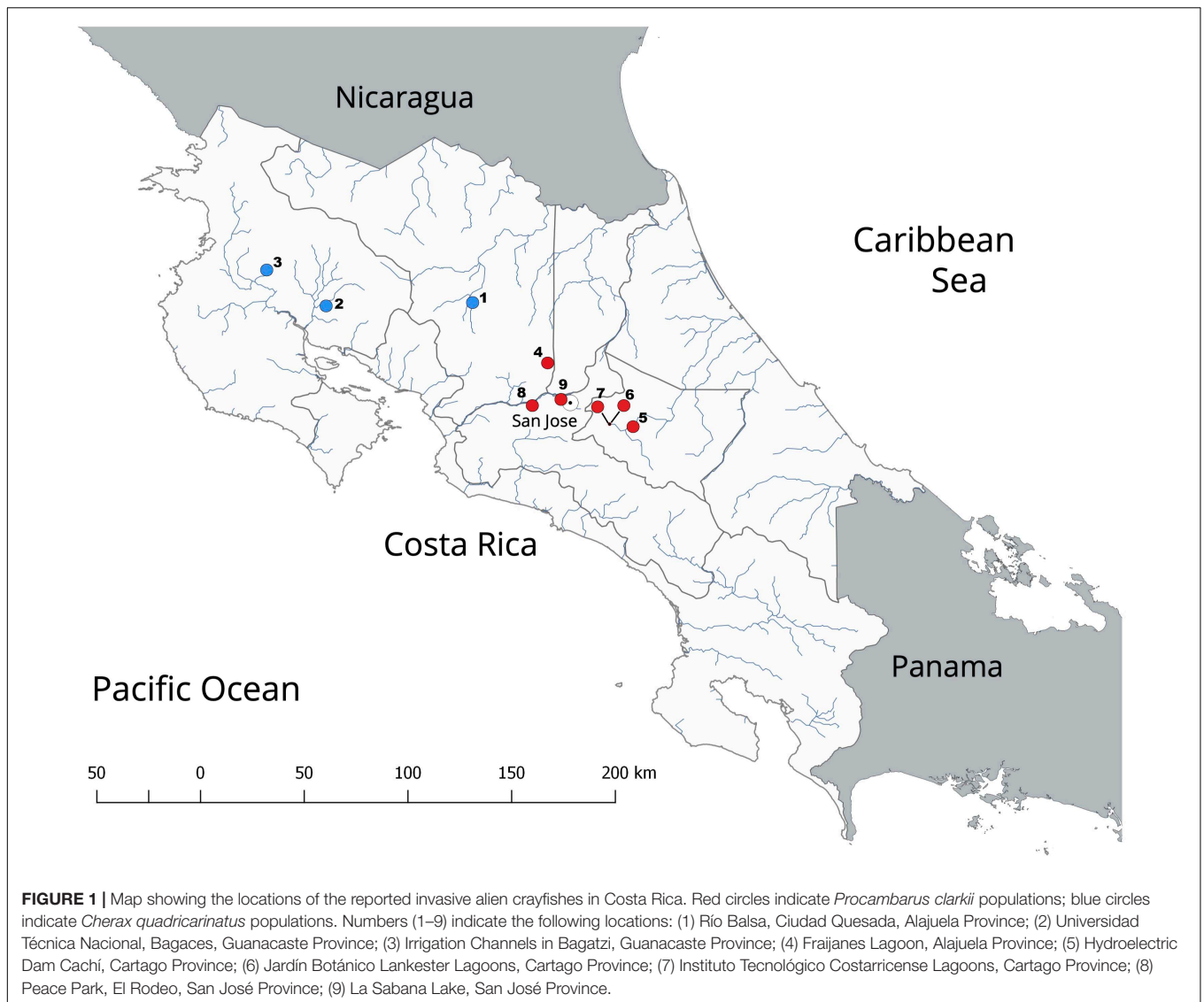
Several cases of the introduction of alien animal species have been reported for Costa Rica (e.g., Barrientos-Llosa and Monge-Nájera, 2010; Barquero and Araya, 2016), a known biodiversity hotspot (Myers et al., 2000). However, to date, only two invasive crayfish species, *P. clarkii* and *Cherax quadricarinatus*, 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



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  $-80^{\circ}\text{C}$  and then lyophilized in a VirTis BenchTop K freeze dryer for 24 h ( $\leq -50^{\circ}\text{C}$ ;  $\leq 20$  mTorr). Subsequently, samples were mechanically ruptured using a TissueLyser (QIAGEN, Venlo, Netherlands). Genomic DNA was isolated using the E.Z.N.A.<sup>®</sup> 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 (rns) and large (rnl) 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	<i>Cherax quadricarinatus</i>
2 Universidad Técnica Nacional, Bagaces, Guanacaste Province	10.33497	−85.13975	<i>Cherax quadricarinatus</i>
3 Irrigation Channels in Bagatzi, Guanacaste Province	10.37946	−85.27321	<i>Cherax quadricarinatus</i>
4 Fraijanes Lagoon, Alajuela Province	10.12471	−84.19105	<i>Procambarus clarkii</i>
5 Hydroelectric Dam Cachí, Cartago Province	9.8316	−83.80968	<i>Procambarus clarkii</i>
6 Jardín Botánico Lankester Lagoons, Cartago Province	9.839553	−83.888691	<i>Procambarus clarkii</i>
7 Instituto Tecnológico Costarricense Lagoons, Cartago Province	9.854629	−83.91052	<i>Procambarus clarkii</i>
8 Peace Park, El Rodeo, San José Province	9.91911	−84.2768	<i>Procambarus clarkii</i>
9 La Sabana Lake, San José Province	9.934766	−84.10359	<i>Procambarus clarkii</i>

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  $\mu$ M 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 pereopods characteristic of infection by *A. astaci*. Moreover, under microscopic observation, we found melanized hyphae growing within the pereopods 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

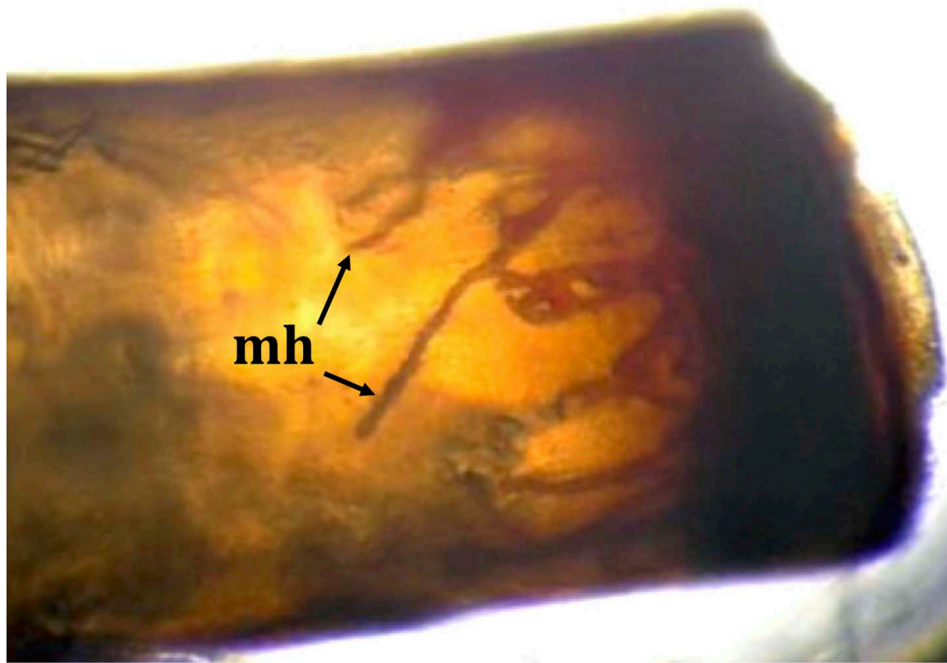
*A. astaci* sequences from *P. clarkii* in Costa Rica with the D-haplogroup.

## 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-Urbeondo 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 Pseudoscorpionidae) (Lara and Wehrtmann, 2011; Lara et al., 2013; Magalhães et al., 2015). Despite the seemingly narrow host range of *A. astaci* (Unestam, 1972; Diéguez-Urbeondo 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 *Procamburus 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-Urbeondo, 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 (INCOPECA, *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-Madriral, 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 Martínez-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>

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**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.

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