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Free Pass Through the Pillars of Hercules? Genetic and Historical Insights Into the Recent Expansion of the Atlantic Blue Crab *Callinectes sapidus* to the West and the East of the Strait of Gibraltar

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The invasive Atlantic blue crab *Callinectes sapidus* has considerably extended its distribution along the Gulf of Cadiz and Moroccan coasts in the northeast Atlantic. This could indicate a new introduction event in the Gulf of Cadiz from the native area, in case of detectable genetic differences from the Mediterranean Sea populations. To test this and reconstruct the invasion, we assessed the genetic structure of crabs in the Gulf of Cadiz and Alboran Sea. We used sequences of the mitochondrial gene cytochrome c oxidase subunit I (COI), in almost its full length (1511 basepairs), of 149 individuals from three sites along the Spanish Mediterranean coast, two in the Alboran Sea, and two in the Gulf of Cadiz. Our data reveal low genetic variability, with only two haplotypes (here referred to as CSWM1 and CSWM2), compared to its known high genetic diversity in the native range, indicating a strong founder effect in the study region. Here we document an inversion of haplotype predominance between regions, haplotype CSWM2 being dominant in most of the Spanish Mediterranean coast, but less frequent in the Gulf of Cadiz and Alboran Sea. This suggests a secondary introduction of propagules into the investigated area, rather than a new introduction from the native area, nor a natural westward expansion with environmental selection of one haplotype over the other. Further studies with additional populations would help to better unveil the history of yet another invasive species in the Gulf of Cadiz.

Keywords: Atlantic blue crab, invasive species, founder effect, gene flow, COI, haplotype

INTRODUCTION

The range expansion of aquatic non-indigenous species (NIS) can be caused by natural phenomena (e.g., migrations, facilitated by other species, oceanographic currents) or be human-mediated (Galil et al., 2019). The most prominent human-mediated dispersal vectors in aquatic ecosystems are intentional introductions, aquaculture, and shipping/boating (e.g., ballast water, hull fouling) (Gollasch, 2006; González-Ortegón et al., 2007; Cuesta et al., 2016). The invasion of exotic species is being accelerated worldwide as a consequence of expanding transport and commerce (Mack et al., 2000); for instance, non-native species have been reported in the Gulf of Cadiz since 1980, but have increased rapidly in the last five years (González-Ortegón et al., 2020). The so-called Atlantic blue crab *Callinectes sapidus* Rathbun, 1896, a species native to the western Atlantic Ocean, is a good example for the introduction and fast growth of new non-native populations in the Euro-African area (Nehring, 2011; Mancinelli et al., 2021). Ballast water has been accepted as the most probable introduction vector of this species in numerous regions (Nehring, 2011).

C. sapidus has a disjunct distribution in the native range, from the coast of Massachusetts (United States) to Venezuela and then in the southwestern Atlantic from Alagoas (Brazil) to northern Argentina (dos Santos and D'Incao, 2004; Windsor et al., 2019). It was introduced in the Eastern Atlantic, including the Mediterranean, Adriatic, North and Baltic seas as well as waters of Hawaii and Japan, *via* human-mediated vectors (Windsor et al., 2019). This species often uses estuaries to complete its life cycle and is a particularly successful invader of marine and estuarine ecosystems, because of its high fecundity and larval survivability, broad environmental tolerance, large body size, aggressive behavior, omnivorous diet, and strong swimming ability (Nehring, 2011). The extended planktonic larval phase of this species maximizes its chances of introduction through ballast water (DiBacco et al., 2012), as well as natural dispersal (Encarnação et al., 2022; Png-Gonzalez et al., 2021).

In European Atlantic waters, *C. sapidus* was detected for the first time along the Atlantic coast of France in 1901 (Bouvier, 1901). In the Mediterranean Sea, it was reported in the Aegean Sea as early as the 1930s, and in the adjacent Turkish coast in 1949 (Enzenroß et al., 1997). Since then, this species has greatly expanded its distribution along the entire Mediterranean Sea and adjacent seas, but also in the North Sea and the Atlantic coast of the Iberian Peninsula and northern Africa (Encarnação et al., 2021; Mancinelli et al., 2021; Chaouti et al., 2022; González-Ortegón and Chairi [in press)]. On the western coast of the Iberian Peninsula, the first record dates back to 1967 in the Tagus Estuary (Portugal), but only a few sporadic records were made since then in this region (Encarnação et al., 2022). Along the Mediterranean coast of the Iberian Peninsula, the first record was made in the Ebro Delta in 2012-2013 (Castejón & Guerao, 2013), and since then its distribution expanded rapidly towards the Strait of Gibraltar where it has been registered since 2017 (Cabal et al., 2006; Nehring, 2011; Mancinelli et al., 2017a; Mancinelli et al., 2017b; González-Ortegón et al., 2020; Mancinelli et al., 2021). In the Gulf of Cadiz, the first published record of Atlantic blue crab dates back to 2016 (Morais et al., 2019), however, a male adult specimen was already collected in the Guadalquivir Estuary in 2002 (Rodríguez & Cuesta, unpublished data). Since 2016, the species expanded rapidly westwards along the southern coast of Portugal and has now reached the southwestern coast of Portugal (Encarnação et al., 2022; Vasconcelos et al., 2019; Encarnação et al., 2021). Meanwhile, in the Alboran Sea, the first record was made in 2017 in Nador Lagoon (Morocco, Mediterranean Sea) (Chartosia et al., 2018), and the species later expanded to the east and west (Taybi and Mabrouki, 2020). Today, the species is also present in the Moroccan Atlantic coast (Chaouti et al., 2022; González-Ortegón and Chairi [in press)].

Complex oceanographic patterns exist in the Gulf of Cadiz and Strait of Gibraltar, which are influenced by surface currents and the Mediterranean outflow, all of which vary seasonally (García-Lafuente et al., 2006; Peliz et al., 2007; Sánchez-Leal et al., 2017). A recent study demonstrated that the westward expansion of the Atlantic blue crab along the southern Portuguese coast towards the western coast (north of Cape St. Vincent) could be due to a seasonal coastal counter-current that coincides with the species reproductive period (Encarnação et al., 2022), similar to the passive transport of particles in summer under a strong and persistent counter-current event in the Gulf of Cadiz (see **Figure 1** in Casaucao et al., 2021). This observation prompted two hypotheses about the origin of *C. sapidus* in the study regions: 1) new and independent introduction into the Gulf of Cadiz from the native area in the Western Atlantic, or 2) a metapopulation expansion across the Gulf of Cadiz, Alboran Sea, and western Mediterranean Sea. To test these hypotheses, we used genetic tools to compare DNA-sequences of the gene cytochrome c oxidase subunit I (COI) of Atlantic blue crab specimens collected from the Mediterranean coast of eastern of Spain, Alboran Sea, and Gulf of Cadiz and compared them with sequences of individuals from native populations.

METHODS

Sampling Sites

Atlantic blue crab specimens were collected from two sites in the Gulf of Cadiz (Guadiana and Larache estuaries), two in the Sea of Alboran (Algeciras and Nador Lagoon), and three in the eastern Mediterranean Spanish coast (Mar Menor, Gandía, Ebro Delta) (**Figure 1**). Details about the samples and sampling sites are described in **Table 1**.

Genetic and Data Analyses

In total, 149 DNA sequences were analysed from individuals collected in these seven locations. In the frame of the present study, the pereiopod muscle tissue from 110 individuals were excised for DNA isolation using a modified Chelex 10% protocol (Estoup et al., 1996). The mitochondrial DNA (mtDNA) of up to 1511 basepairs encoding the enzyme COI were amplified to compare

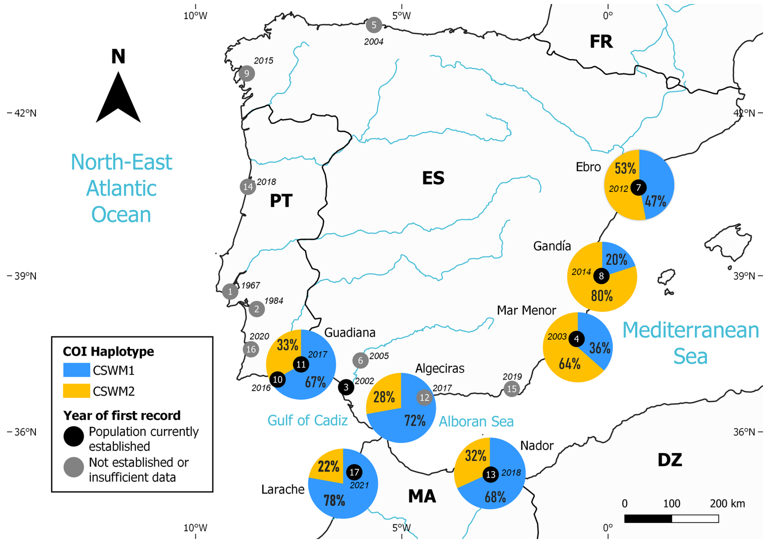

FIGURE 1 | Location of the sampling sites and proportion of COI haplotypes – CSWM1 and CSWM2 – identified in the invasive populations of Atlantic blue crab *Callinectes sapidus* Rathbun, 1896 analysed for this study. Locations and year of first record are presented for the Atlantic and Mediterranean coasts of the Iberian Peninsula and Morocco. Populations were considered established if evidence of completion of life cycle exists (juveniles, males, females, ovigerous females) or recurrent reports for at least 2 years. The year represents the first known records. Colour grey or black represent the current-day status of populations based on available literature. 1, 2, 16: Encarnação et al. (2022); 3: Rodríguez & Cuesta, (unpublished data); 4: Chic and Garrabou (2020); 5: Cabal et al. (2006); 6: ICES (2007); 7: Castejón and Guerao (2013); 8: Izquierdo-Gómez and Izquierdo-Muñoz in Karachle et al. (2016); 9: Bañón et al. (2016); 10, 11: Morais et al. (2019); 12: González-Ortegón et al. (2020); 13: Oussellam and Bazairi in Chartosia et al. (2018); 14: Izquierdo-Gómez (2022); 15: de Vries and Lemmens (2022); 17: Chaouti et al. (2022); González-Ortegón and Chairi (in press).

haplotype frequencies between populations. The amplification temperature profile was: i) initial denaturation for 4 min. at 94°C; ii) 40 cycles of 45 s at 95°C for denaturation; iii) 1 min. at 50°C for primer annealing; iv) 1 min. at 72°C for elongation; v) final extension for 4 min. at 72°C. The primers used were COL6a (5'-CAAATCATAAAGAYATTGG-3') as forward primer and COH16 (5'-CATYWTTCTGCCATTTTAGA-3') as reverse primers (Schubart, 2009), in addition to the newly designed internal primers COL4Cs (5'-TAGCAGGTGCTATTACTATACT-3') and COH900Cs (5'-CTAAAAATTTTAATACCAGTGG-3'). PCR products were sent to Stab-Vida Laboratories to be purified and then bidirectionally sequenced. Sequence files were proofread using the trace viewer software Chromas Lite 2.6.4 (Technelysium Pty Ltd 2017), aligned with Clustal W, and implemented in BioEdit 7.2.6.1 (Hall, 1999).

The similarity of haplotypes of the populations of *Callinectes sapidus* from the Gulf of Cadiz and Alboran Sea and their possible geographic origin were investigated by 'blasting' the haplotype sequences in GenBank and searched in BOLD for the same sequences (100% fitting) deposited in these databases.

RESULTS

Only two haplotypes of the mtDNA COI gene were identified in the seven Atlantic blue crab populations analysed in this study, herein referred to as CSWM1 and CSWM2. Sequences of these haplotypes were deposited in GenBank under the accession numbers ON248058 (CSWM1) and ON248059 (CSWM2) and corresponding voucher specimens were deposited in the Decapoda and Stomatopoda Crustacean Collection (Spanish Institute of Oceanography in Cadiz, IEO-CSIC, Spain) under catalogue numbers IEO-CD-ICMAN/2936 (CSWM1) and IEO-CD-ICMAN/2937 (CSWM2).The Gulf of Cadiz and Alboran Sea were predominantly CSWM1 (67-78%), while the Mediterranean Spanish coast was dominated by CSWM2 (53-80%) (**Figure 1**).

The Blast and BOLD search for the CSWM1 haplotype showed a 100% similarity with GenBank records Turkey-1 (MG462529)

TABLE 1 | Location of the sampling sites where Atlantic blue crab *Callinectes sapidus* Rathbun, 1896 specimens were collected for mtDNA analyses, date of capture, and number of sequenced specimens (N).

Sampling sites	Latitude	Longitude	Date	N
Mar Menor (Mediterranean Sea, Spain)	37°38'09.6"N	0°44'58.1"W	07-10/2019	22
Ebro Delta (Mediterranean Sea, Spain)	40°38'18.6"N	0°39'20.7"E	2019	19
Gandía (Mediterranean Sea, Spain)	38°59'27.2"N	0°09'08.9"W	2019-2021	20
Nador (Sea of Alboran, Morocco)	35°10'04.3"N	2°51'52.5"W	02/2021	22
Algeciras (Strait of Gibraltar, Spain)	36°10'28.4"N	5°26'12.8"W	07-08/2021	18
Guadiana estuary (Atlantic Ocean, Portugal/Spain)	37°11'13.4"N	7°24'20.5"W	07/2018	21
Larache (Atlantic, Morocco)	35°12'15.3"N	6° 8' 24" W	02/2021	27

(origin: Turkey) from whole frozen uncooked crabs, and USA-2-5 (MG462571) (origin: United States of America) from commercial crabmeat products (Windsor et al., 2019). Two other sequences showed a 100% identity [not considering the primers area] with GenBank sequences KT282079 (Segura River, Mediterranean Sea coast, Spain) (Gonzalez-Wangüemert and Pujol, 2016) and MN759041 (Ordu, Black Sea coast, Turkey) (Öztürk et al., 2020). On the other hand, CSWM2 only fits 100% with the Genbank COI sequence KR030241, corresponding to voucher specimen USNM : IZ:1286742 from Rhode River (Maryland, USA) (Aguilar et al., unpublished).

DISCUSSION

The Gulf of Cadiz is now the most southwestern point in the distribution of *Callinectes sapidus* along the European coast. The genetic data obtained in this study showed that the populations observed in recent years along the Gulf of Cadiz and Alboran Sea have a low genetic diversity and strong population connectivity. All sampled populations include two haplotypes (CSWM1, CSWM2) that differ in their frequencies between the Spanish Mediterranean coast metapopulation and the Gulf of Cadiz-Alboran Sea metapopulation.

New Introduction Event Versus Expansion or Secondary Introduction?

Invasive species often exhibit low genetic diversity due to a strong founder effect, while the absence of genetic variability between invasive populations is most likely the indication of a single introduction event, with a subsequent expansion along the non-native range or secondary introduction within the non-native range (Harrison et al., 2013). Although cryptic intraspecific invasions are more common than acknowledged, since most biological invasion studies rely on morphological identifications rather than on molecular analyses, a new introduction event into a distinct area would more likely imply that genetic diversity would be distinct between non-native populations (Morais and Reichard, 2018). For example, the northward expansion of the invasive European green crab *Carcinus maenas* (Linnaeus, 1758) along the northwest Atlantic coast was apparently due to the introduction of a new lineage and not to its expansion northward by climate change or its acclimatization to cold water (Roman, 2006). Thus, although changes in the distribution of an exotic species may become obvious, molecular analyses could warn us when an adaptation of the present genotypes or the introduction of new lineages occur, being able to further increase its invasive potential in the respective area.

The genetic diversity of *Callinectes sapidus* in its native range is substantially higher than in our study area. On the U.S. East Coast there are at least 67 haplotypes and three genetic lineages along the entire native area (Windsor et al., 2019). Lineage 1 is predominant along the Atlantic coast of North America and Gulf of Mexico, lineage 2 is predominant in the Caribbean Sea, while lineage 3 is predominant and exclusive to the southwestern Atlantic (Windsor et al., 2019). CSWM1 and CSWM2 are the only haplotypes observed in our study area and are rare in lineage 1 (Windsor et al., 2019). In the invaded range, haplotype CSWM1 has been found in the Eastern Mediterranean Sea, Black Sea, and Turkey (Windsor et al., 2019; Öztürk et al., 2020, Berger et al., unpublished), as well as in the Segura River (Gonzalez-Wangüemert and Pujol, 2016).

Considering these genetic data, and the fact that adult blue crabs have a considerable ability to swim and travel up to hundred kilometers (Kara and Chaoui, 2021) or the high fecundity of females, which would allow the passive transport of a huge amount of larvae by oceanic currents between the Mediterranean Sea and the Gulf of Cadiz, it is more likely that the populations of *C. sapidus* in the Gulf of Cadiz and Alboran Sea result from the expansion of the species along the Mediterranean Sea – single introduction – or to a secondary introduction event within the invaded range. For example, the timeline of the first reports along the southern coast of the Iberian Peninsula were made sequentially from the east to the west, between the Ebro Delta and the Gulf of Cadiz, in just seven years (Castejón & Guerao, 2013; Morais et al., 2019; González-Ortegón et al., 2020; Encarnação et al., 2021). Additionally, the haplotype diversity is extremely low between populations – only two haplotypes are present. The inversion in haplotypic frequency could be explained by two processes, a secondary introduction of few individuals from the Spanish Mediterranean coast to the Gulf of Cadiz or Alboran Sea, or to changes in abiotic conditions since estuarine ecosystems are absent between Mar Menor and the Strait of Gibraltar. In case of a new introduction into the Gulf of Cadiz and Alboran Sea, a different diversity of haplotypes would have been expected and, probably, one of the most common haplotypes in the Atlantic blue crab native area (e.g. H34, see Windsor et al., 2019) would have been present in these two adjacent non-native areas, but this was not the case.

Population Connectivity

The presence of only two haplotypes of Atlantic blue crabs collected at the west and the east of the Strait of Gibraltar suggests a lack of genetic structuring and high connectivity between the Gulf of Cadiz and Alboran Sea populations. This may be explained by two significant autecological traits, the species protracted pelagic larval duration and adult migration, which are known to favor the gene flow of marine species over long distances (Cordero et al., 2014; Lacerda et al., 2016). Besides these two traits, ship transport may contribute significantly to the gene flow in the southwestern coast of the Iberian Peninsula (Lacerda et al., 2016).

The haplotype frequency discontinuity between CSWM1 and CSWM2 coincides with the Almeria-Oran Front that is known to act as a gene flow barrier between the Alboran Sea and the Mediterranean Basin to the east, as observed for other marine crustaceans (Patarnello et al., 2007), such as the rockpool shrimp *Palaemon elegans* (Reuschel et al., 2010) and harbour crab *Liocarcinus depurator* (Pascual et al., 2016). If this marked haplotype cline between these two metapopulations subsists, then it will show that the Almeria-Oran Front can also act as a barrier to the widespread admixture between metapopulations of a migratory crustacean species (Reuschel et al., 2010).

CONCLUSIONS

The Atlantic blue crab populations present east and west of the Strait of Gibraltar currently consist of two COI haplotypes – CSWM1 and CSWM2. In the Gulf of Cadiz and Alboran Sea, haplotype CSWM1 is predominant, while haplotype CSWM2 is predominant in the Spanish Mediterranean coast populations. These data suggest that these populations are the product of a single invasion event into the Mediterranean Sea, with subsequent westward expansion or secondary introduction to other areas. Additional studies are needed to continue unveiling the details about the Atlantic blue crab invasion in the southwestern Iberian Peninsula coast.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the https://www.ncbi.nlm.nih.gov/genbank/ repository, accession numbers ON248058 and ON248059.

AUTHOR CONTRIBUTIONS

EGO, JE, HC, PM, MAT, CDS and JAC contributed to conception and design of the study. EGO, JE, HC, FOP and CS contributed to the collection of the crabs. JAC and SB analysed the samples. EGO wrote the first draft of the manuscript. EGO, PM, JE, CDS and JAC contributed to manuscript revision. All authors read and approved the submitted version.

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