

Article

New Records of *Callinectes sapidus* (Crustacea, Portunidae) from Malta and the San Leonardo River Estuary in Sicily (Central Mediterranean)

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Abstract: The current study provides new data on the known records of the Atlantic blue crab, *Callinectes sapidus* Rathbun, 1896, from the Maltese Islands through two ovigerous female specimens collected from Salini (Malta), a Natura 2000 site, in August 2020. Additional new records of the species were also presented from the San Leonardo River estuary, Syracuse (Sicily, Italy), in August 2022. The specimens collected from both Malta and Sicily were identified using both morphological and molecular analyses. The latter has shown that all the specimens analysed share their mitochondrial DNA barcode region with the most recorded haplotype of the same species from the Mediterranean Sea. Knowing the new distribution and range expansion records of alien species is important for the continuous monitoring of marine alien species, which is essential for the implementation of the best possible mitigation measures. This is especially relevant for *C. sapidus*, as it is considered one of the worst invaders of the Mediterranean Sea. Therefore, given that this alien species is edible, fishing for it may allow some control over its population size and further expansion. Knowing its distribution is crucial to inform fishers about its exact location, making such a mitigation measure more effective.

Keywords: alien species; Atlantic blue crab; central Mediterranean; distribution; estuary; Natura 2000 sites



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1. Introduction

The Atlantic blue crab, *Callinectes sapidus* Rathbun, 1896, is native to the western Atlantic coasts from northern Argentina to Nova Scotia, including the Gulf of Mexico and the Caribbean Sea [1,2], and in recent years, it has been reported to be expanding its distribution northwards, possibly in response to climate change [3,4]. This species is known to be a euryhaline and eurythermal species that inhabits mainly coastal marine environments, including estuaries and lagoons [4–6], with some records extending to freshwater systems [6]. Apart from its native range along the western Atlantic coasts, it has been reported as an alien in several north-eastern Atlantic regions, including the Mediterranean Sea, North Sea and Baltic Sea, and some areas in the Pacific Ocean [4,5]. The first record for the Mediterranean Sea was collected in 1949 through a female specimen from the Gulf of Venice, Italy, in the Adriatic Sea [7,8]; however, this record may be preceded by earlier arrivals in the Aegean Sea, dating to the 1930s [4,9]. Subsequently, its area of distribution has progressively expanded into the eastern and western basins of the Mediterranean Sea [4,9–15] and into the Black Sea [16].

In Sicilian waters, the Atlantic blue crab was reported in Messina in the 1970s [17,18] and along the eastern coast of Sicily in the late 1980s [19]. Since then, there have been an increasing number of other Sicilian records, and nowadays, *C. sapidus* has a patched distribution along the coasts of Sicily [12,20]. Corresponding to the species' life history [10],

in Sicily, the species seems to be more present in the coastal areas with an input of freshwater, such as estuaries with sandy or muddy bottoms [20,21], a characteristic noted elsewhere in the Mediterranean Sea [22–25] and its native range [26,27]. In Malta, the species was mentioned in 1972 [28], although that record was misidentified and belonged to *Portunus segnis* (Forskål, 1775) [29]. Therefore, the only record for Malta was through a male specimen collected from Marsaxlokk harbour in July 2021 [30].

Currently, there are no scientifically proven data on the vector that led to the initial introduction of this species into the Mediterranean Sea; however, while some argue that this is an intentional introduction for fishery purposes [7], many others associate this alien introduction to ballast waters [10,31,32]. The genetic data indicate that both these two factors played a role in the multiple introductions [33]. Once introduced, ballast waters may be the main contributing factor for further dispersal in the Mediterranean Sea, given the pelagic stages of the species' life history [10,34], while current climatic changes seem to further favour the metabolic response and proliferation of *C. sapidus* in the region [35].

Some other biological traits of *C. sapidus* include high fecundity, which in its native range has an average of 3.2 million eggs per brood, as well as competitive behaviour, including aggressiveness, versatility in the diet, and high physiological plasticity to a wide range of environmental conditions [5,36–39], which makes it one of the worst 100 invasive alien species in the Mediterranean Sea [40]. This invasive species is becoming a keystone species in estuaries and lagoons [41–43], such as the Ebro Delta in Spain, where its presence led to steep declines in native biodiversity [41]. However, even though in some Mediterranean regions fishermen catch and sell *C. sapidus* [10,23,25,42,44–46], this alien species is reported to damage fishing nets and mutilate commercially caught fish, which will then be discarded, thus lowering the production and value of traditional fisheries, and in some instances, causing injuries to fishermen while removing crabs from nets [12,44,46,47].

The effective conservation of species and habitats in estuaries and coastal areas, protected under the Natura 2000 network (across all EU countries), depends on accurate biodiversity monitoring and the timely interventions associated with the introduction of an alien species, which may disrupt the native communities and habitats. However, while the impact of an alien species is predicted in the majority of the Mediterranean marine Natura 2000 sites, most sites do not feature reports on the threats of biological invasions [48]. This lack of reporting, together with the uncertainties and knowledge gaps in species identification, presents a deficiency in the accurate information required to support conservation policies and marine management in this region [48–50].

The aim of this study is to update the records and distribution of *C. sapidus* from Malta and the eastern coast of Sicily. Furthermore, DNA barcoding, using cytochrome c oxidase subunit I (COI), was used to corroborate the identification of the species and the geographical origin of the specimens analysed, given that specific COI lineages are known to be closely linked to a geographical location [33,51–53].

2. Materials and Methods

2.1. Area of Study

Two areas, one in Malta and one in Sicily (Figure 1), were investigated during this study. Both locations are associated with the Natura 2000 sites and were selected for the study after the authors received reports on the presence of blue crabs, which at the time of the reporting, were not identified down to the species level. The area in Malta is found at Salini Bay, which is in the town of St. Paul's Bay. This site is characterised by one of the few remaining salt marshes in Malta and an estuary with incoming freshwater from a complex valley system that leads to the area. In Sicily, the area under study forms part of the San Leonardo River estuary, a sandy-coastal area on the eastern Sicilian coast between the Catania and Syracuse provinces [54].

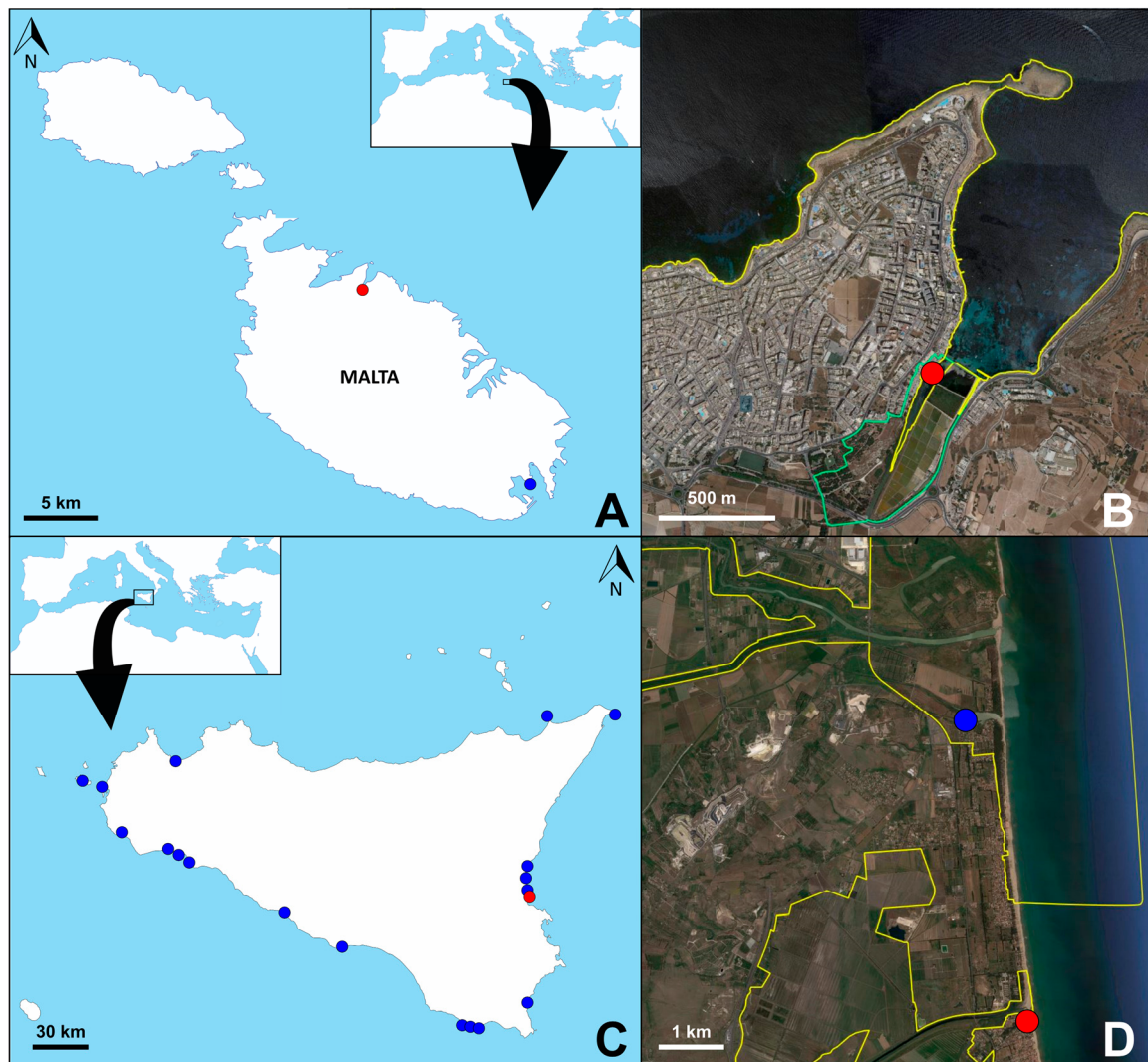


Figure 1. (A) Map of Malta showing the 2020 record (red) and the 2021 record (blue [30]); (B) map of the Is-Salini area, Malta showing the location of the current new record (red), including the outlines of the Natura 2000 sites MT0000007 (green) and MT0000112 (yellow); (C) map of Sicily showing the current record (red) and previous records (blue [20]); (D) map of the San Leonardo River estuary, Sicily showing the location of a previous record (blue) and current record (red), including the outline of the Natura 2000 site ITA070029 (yellow). (Source: <http://www.google.com/earth/index.html> and <https://natura2000.eea.europa.eu> (accessed on 7 April 2023)).

2.2. Specimens Collection

MALTA: On the 1st and 2nd of August 2020, two specimens of *Callinectes sapidus* (Figure 2) were collected from St. Paul's Bay, Malta (GPS: 35°56'53.8" N, 14°25'23.3" E; Figure 1A,B) by a Maltese recreational fisherman using a crab trap. These two specimens were collected from the last 130 m of one of the canals at *Is-Salini* (Natura 2000 site code: MT0000007, [54]), which leads to the open sea. This canal forms part of the Marine Protected Area, *Żona fil-Baħar madwar Għawdex* (Natura 2000 site code: MT0000112, [54]). The specimens, forwarded for research, were photographed, measured, and deposited at the specimen collection held by the Conservation Biology Research Group of the University of Malta (CBRG-UM) as voucher specimens SMM015 and SMM016, as part of the long-term ongoing research on marine biodiversity and non-native species.

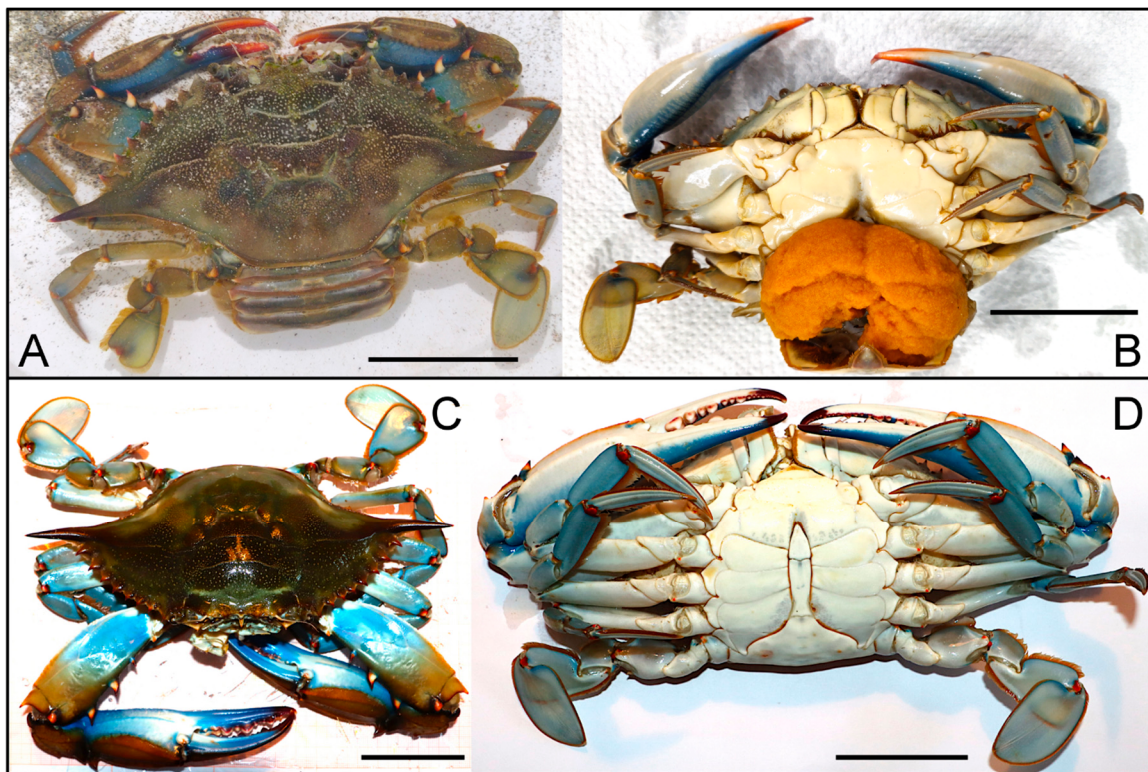


Figure 2. *Callinectes sapidus*. Ovigerous female from Malta, caught on 1 August 2020 (SMM015): (A) dorsal view, (B) ventral view; male from Sicily, caught on 30 August 2022 (SM11): (C) dorsal view, (D) ventral view. Scale bars = 5 cm. Photographs are by A.V., S.A.D. and E.G.

SICILY: On the 30th August 2022, three specimens of *C. sapidus* (Figure 2) were collected from the San Leonardo River estuary (GPS: 37°20′38.0″ N, 15°05′31.1″ E; Figure 1C,D) in the province of Syracuse. This area is located 4 km south of the Marine Protected Area, Simeto River Oasis (Natura 2000 site code: ITA070029, [54]), where this species has been previously reported ([20]; Figure 1). These specimens were caught by Sicilian recreational fishers using fishing rods, with shrimps and chicken skin used as bait. The catch area was 50 m upstream from the river mouth at a depth ranging between 0.5 m and 1 m. These specimens were photographed and measured, and the tissue samples were deposited at the marine biodiversity and non-native species collection of the CBRG-UM as tissues from the voucher specimens SM09, SM10 and SM11 for further research.

2.3. Morphological and Genetic Analyses

The specimens were identified morphologically following published keys [10,36,55]. The biometric measures were taken in accordance with Millikin and Williams [36] to the nearest 1 mm using an electronic calliper. Additionally, a tissue sample was taken from each specimen and preserved in 100% ethanol.

The total genomic DNA was extracted from 10 mg of each tissue sample using the GF-1 Tissue DNA Extraction Kit (Vivantis, Shah Alam, Malaysia). The PCR amplifications were carried out for COI, utilising HCO1490 and LCO2198 [56] with an amplification protocol of 95 °C for 15 min, followed by 7 cycles at 95 °C for 1 min, 45 °C for 1 min 15 s and 72 °C for 1 min 15 s, followed by 37 cycles at 95 °C for 1 min, 50 °C for 1 min 15 s and 72 °C for 1 min 15 s, and with a final extension at 72 °C for 10 min. The PCR products were visually verified on 1.5% agarose gels stained with ethidium bromide, and the purified PCR products were sequenced using both the forward and reverse primers via ABI3730XL. The quality check, editing and assembly of the complementary sequences were carried out using Geneious

R10 [57]. The genetic data generated during this study were deposited at GenBank under the accession numbers OQ735408–OQ735412.

The sequences generated during this study were compared against each other and the already available data on both the NCBI GenBank database via Blastn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (accessed on 7 April 2023) and the BOLD database using the Species Level Barcode Records Identification Engine (<http://www.boldsystems.org>) (accessed on 7 April 2023); [58]). The genetic distances from the closest matches were noted. The integration of the current genetic data with other publicly available data allowed for comparative analyses of haplotypes. Sequences from GenBank were chosen and aligned using MUSCLE [59] as embedded in Geneious R10 [57]. The model of best fit, as identified by jModel [60], was then used to construct a phylogenetic tree using Bayesian inference via MrBayes, v3.2 [61]. This analysis used 5×10^5 generations with a sampling frequency of 1000 generations and a burn-in of 25% to stabilise the log-likelihood scores, allowing for better visualisation of the genetic relationship between the currently analysed specimens and publicly available data.

3. Results

Two specimens of *Callinectes sapidus* were collected from *Baħar iċ-Ċagħaq*, Malta, on the 1st and 2nd of August 2020. These two specimens were both ovigerous females and had carapace widths of 149 mm and 134 mm and weighed 164.7 g and 157.9 g, respectively (Table 1; Figure 1). The weight of their egg masses was 24.7 g and 37.9 g, accounting for 15.0% and 24.0% of the total body weight, respectively. The first specimen's egg mass was orange in colour, while that of the second specimen was brownish and comprised smaller eggs compared to those found in the first specimen. The collection location, *Is-Salini*, is a Natura 2000 site characterised by a salt marsh with incoming freshwater through canals on either side of the marsh. The specimens were collected around 130 m upstream from the end of the canal, which leads into the sea in an area having a sandy and muddy bottom. Additionally, the canal forms part of the southern-east part of the Marine Protected Area and Natura 2000 site that surrounds the island of Gozo, which encompasses the northern coast of Malta [54].

Table 1. Morphometric data of the collected *Callinectes sapidus* analysed in this study.

	SMM015	SMM016	SM9	SM10	SM11
Sampling location ¹	Malta	Malta	Sicily	Sicily	Sicily
Sampling date	1 August 2020	2 August 2020	30 August 2022	30 August 2022	30 August 2022
Weight ²	164.7	157.9	364.0	466.0	408.0
Maximum carapace width ³	149	134	200	205	210
Carapace length	61	57	90	90	90
Abdomen width	49	45	40	44	37
Left pollex length	69	59	90	100	100
Left pollex width	15	12	20	25	25
Left pollex depth	18	16	27	32	30
Left dactyl length	37	31	50	50	55
Right pollex length	56	62	97	110	100
Right pollex width	10	15	27	30	28
Right pollex depth	15	19	30	35	28
Right dactyl length	37	31	55	55	50
Sex	Female (ovigerous)	Female (ovigerous)	Male	Male	Male

¹ Malta (GPS: 35°56′53.8″ N, 14°25′23.3″ E); Sicily (37°20′38.0″ N, 15°05′31.1″ E); ² weight in grams; ³ lengths in mm.

The three crab specimens, collected from the San Leonardo River estuary, Sicily, near a Natura 2000 site, had carapace widths of 200 mm, 205 mm and 210 mm and weighed 364.0 g, 466.0 g and 408.0 g, respectively. All three specimens collected from Sicily were males (Table 1; Figure 2).

The colour of the carapaces of all five sampled crabs was a bluish-green with orange-red spines. Their carapaces had a granulated surface, and their shape was broad, with the anterolateral margin being characterised by nine teeth. The first eight teeth were short, but the ninth one was very prominent, as it was the longest and pointed directly outwards. The chelipeds were longer than walking legs, with the merus having three spines on the inner margin and another subdistal spine. The walking legs were compressed, with the fifth walking leg having paddle-like distal segments, typical of the portunid species. Furthermore, segments 3 to 5 of the male abdomen were fused, and the abdomen had the shape of a narrow inverted T. The morphological features matched the descriptions by Rathbun [55], Millikin and Williams [36] and Galil et al. [10]. The female specimens analysed in this study had red claw dactyls, which is considered a visual cue towards mate choice [62,63], while the claw of the analysed male specimens had an exterior white and interior blue colouration, matching the colour description for the mature males in Baldwin and Johnsen [63].

The species identification was also confirmed through genetic analyses, where the five specimens produced the same 658 bp haplotype. This matched 100% with the data from GenBank (OQ108525—Spain; ON248058—Spain; MG462571—USA; MG462529—Turkey), fitting in BIN BOLD:AAB6460, which is the only BOLD BIN for the species. This BOLD BIN has an average p -distance of 1.57%, with the maximum p -distance within the BIN being 5.52%. The haplotype identified in this study matched 100% with the specimens from lineage 1, as identified in Rodrigues et al. [51] and Windsor et al. [52] in its native range, while matching to CSWM1, which is the most common haplotype for the specimens in Spain, along its Atlantic and Mediterranean coast, as identified by González-Ortegón et al. [53], as well as other regions of the Mediterranean Sea, including Albania, Italy and Turkey, as identified in Schubart et al. [33] (Figure 3).

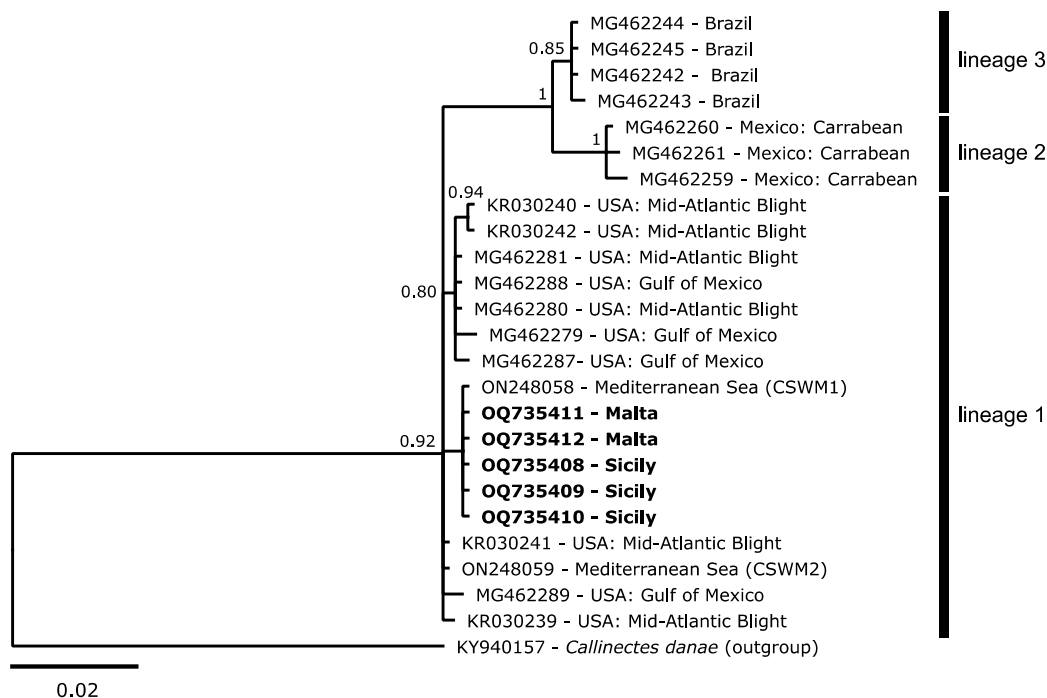


Figure 3. Bayesian inference analyses show the genetic relationship between the currently analysed specimens (in bold) and the three lineages identified for *Callinectes sapidus* in Windsor et al. [52]. CSWM1 and CSWM2 are the two main haplotypes identified in the Mediterranean and represent various specimens from the region [33,46]. *Callinectes danae* was used as an outgroup [64]. The numbers at the nodes indicate the posterior probability values.

4. Discussion

The two currently analysed specimens of *Callinectes sapidus*, collected in August 2020, represent the earliest record of the species in Maltese waters. Previous references to alien blue crab species in Malta show the presence of *Portunus segnis*, which was first recorded in Malta in 1972 [28,29]. The first published record of *C. sapidus* in Malta was through a specimen collected in July 2021 [30], which was caught around 25 km away and almost a year after the current reported record. Moreover, the currently analysed specimens were two ovigerous females, indicating that this species is reproducing in Maltese waters. Although *C. sapidus* is a marine species, it is known to spend periods in low saline environments, especially juvenile males that use such areas for moulting, while the oligohaline and mesohaline regions of estuaries are associated with mating [36,42,65,66]. After copulation, sperm is stored in females, and the eggs undergo internal fertilisation [38]. Subsequently, females migrate to the lower estuary and adjoining sea to externally brood the eggs before hatching as planktonic larvae [26,38,67]. Zoea larvae then develop in seawater due to their intolerance to low salinity, while post-larvae, they are transported to the entrances of estuaries for further development [68,69]. Therefore, environmental conditions, such as those found in the canal at *Is-Salini*, Malta, and other estuaries, such as that of San Leonardo River, Sicily, are crucial for copulation, while being in contact with the marine habitat allows for the right environmental conditions for the completion of the full life cycle for this species. These areas would therefore need to be monitored more closely, especially considering their contribution to the Natura 2000 sites designated for native species protection.

The currently available COI data shows that, in its native range, *C. sapidus* exhibits three distinct lineages mostly linked to geographic origins. The first lineage encompasses mainly specimens of North Atlantic origin and the Gulf of Mexico, with some presence in the Caribbean Sea and Brazil. Another lineage is mainly found in the Caribbean Sea, with some overlapping at the southern regions of the first lineage, while the third lineage is restricted to Brazil [51,52]. These two studies show that in its native range, *C. sapidus* is characterised by a high level of genetic diversity; however, in the Mediterranean and the Black Sea, it is characterised by low genetic diversity, with all recorded COI haplotypes being related to the first lineage [33,53,70]. These observations indicate that introductions into the Mediterranean and the Black Sea are of North Atlantic origin or from the Gulf of Mexico. In the Mediterranean Sea, almost all specimens of *C. sapidus* genetically group within two haplotypes, CSWM1 and CSWM2 [33,53], except for the specimens from Turkey, which tend to exhibit different haplotypes and have a higher level of genetic diversity, possibly linked to stocking events [33]. In our study, we only found specimens belonging to CSWM1, which is the most common haplotype for the Mediterranean Sea [33,53], thus showing that the specimens analysed in this study are unlikely to be new reintroductions into the Mediterranean Sea but rather range from the expansion of already present populations in the region.

In the Mediterranean, most reported records of *C. sapidus* link its range expansion to marine traffic, including both ballast water and hull fouling [10,12]; yet, one cannot exclude that the first Maltese record might be linked to the possible natural expansion of the species from neighbouring established populations, such as those found in Sicily. *Callinectes sapidus* is characterised by a long planktonic larval development, which may last a couple of weeks [26,34,67], while its megalopa is able to actively swim against the current flow in search of estuarine water [71,72]. Moreover, *C. sapidus* has been associated with jellyfish species [73–75]. A recent study in the north-western Gulf of Mexico, which forms part of its native range, *C. sapidus* megalopae, and in some instances small juveniles, have been recorded on Medusa, of *Chrysaora chesapeakei* (Papenfuss, 1936) (Cnidaria: Pelagiidae), with an occurrence ranging from 1 to 301 individuals of *C. sapidus* megalopae per jellyfish [75]. This transitory association with jellyfish may facilitate transport towards shallower waters and act as a temporary nursery habitat, providing food and protection to megalopae [75]. While this specific jellyfish species has not been recorded in the Mediterranean Sea, con-

generic species [76–79] and other species from the family Pelagiidae, commonly represented by increasingly frequent blooms of *Pelagia noctiluca* (Forsskal, 1775) [80,81], are known in the region. In its native range, the presence of juveniles has also been recorded on floating leaves in estuaries [82]. In view of this, further work is needed to evaluate the link between *C. sapidus* and floating biota, especially for the Pelagiidae species, where the latter may act as vectors of spread during the post-larval stages and possibly delay the megalopae settlement of *C. sapidus* and extend its expansion in the Mediterranean Sea.

Given that *C. sapidus* is edible, this species already represents an exploitable fisheries resource in a number of Mediterranean countries [4,23,25,42,44–46]. The increasing catches of the Atlantic blue crab for human consumption may represent a resource for coastal communities while offering an effective mitigating measure that may control the population size and invasiveness of this crab, thus reducing its ecological impact [83]. However, it must be noted that the presence of *C. sapidus* is widespread, even in areas where fishing is unrestricted, such as certain parts of the Marine Protected Areas and terrestrial Natural Reserves, which include estuaries. Thus, the level of protection in these areas, including the restrictions on harvesting to protect local biodiversity, may inadvertently encourage invasive alien species to spread, allowing for increasing population sizes of *C. sapidus* in these protected Natura 2000 sites. This may have been the case for the San Leonardo River estuary, where the already known presence of *C. sapidus* from the Simeto River Oasis area, an Oriented Nature Reserve that is included in the protected site ITA070029 [20], may have allowed for the expansion of the species' range, as further highlighted in this study. In Malta, the species has also been recorded within a Natura 2000 site that forms part of MT0000007 and MT0000112 and has unique characteristics for the island; thus, immediate mitigation measures are required to control this invasive species.

These additional records from two central Mediterranean islands provide new insights into this species' distribution and provide the first genetic data for *C. sapidus* from the southern central Mediterranean Sea. This work highlights the need for more research to better address and reduce knowledge gaps related to the biology, impacts of this invasive species on native Mediterranean biodiversity, and mitigation measures required, especially in sensitive Natura 2000 sites. Using these management requirements, together with the use of effective tools to closely monitor this species for timely interventions, are in accordance with Descriptor 2 of the European Marine Strategy Framework Directive and other regional regulations on invasive alien species.

5. Conclusions

The Mediterranean Sea is a biodiversity hot spot that is suffering from multiple anthropogenic pressures exacerbated by climate change effects. Invasive alien species are a strong threat to native biodiversity; however, given the current maritime transportation demands, such incidences continue to pose an ever-increasing threat. In view of this, biodiversity and conservation research play a crucial role in efficiently identifying new records of invasive alien species. These results reduce the knowledge gaps and update the status and distribution of such species so that mitigation measures, such as fishing for edible invasive alien species, can be made more effective. Through these results, fishers can be informed directly of the exact distribution of these species, reducing the need for these fishermen to waste fuel in search of this resource, thereby reducing the carbon footprint of this mitigation measure itself. The new distributional information, combined with genetic data of these alien species records, provides an even more effective alien monitoring and mitigation tool. The results of the genetic analyses provide additional insights into the possible geographical origin and vector of introduction and help to elucidate the mechanisms by which these species expand their geographical range, supplying further support for precaution and mitigation measures to reduce the impact of invasive alien species.

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Institutional Review Board Statement: Ethical review and approval were waived for this study, as the specimens were caught by local fishermen during their fishing activities and were deceased when collected by the researchers.

Data Availability Statement: The genetic data are available on GenBank under the accession numbers OQ735408–OQ735412.

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Conflicts of Interest: The authors declare no conflict of interest.

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