The prevalence of two known pathogens of Atlantic Blue Crab *Callinectes sapidus* (Rathbun, 1896) in coastal Adriatic, Aegean sea, and Atlantic Iberian coast

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Abstract

In the Mediterranean Sea, the abundance of the invasive portunid crab, *Callinectes sapidus*, has dramatically increased in recent years. This raises concerns about damage to ecosystems, but also offers opportunities for exploitation of a new fishery. Newly invasive species may escape from pathogens in their native range, may introduce new pathogens, or can become host to endemic pathogens. Understanding these factors is important for predicting or managing natural resources in the invaded range. This study investigated the prevalence of two pathogens common in *C. sapidus* in its home range of North America: the reovirus CsRV1 and the protozoan parasite *Hematodinium perezi*. In crabs collected from Aegean, Adriatic, and Atlantic waters, the CsRV1 virus was not detected. In contrast, the parasite *H. perezi* was found in crabs from all areas except the Aegean Sea. Sequence analysis of the *H. perezi* ITS1 gene indicated that the strains observed are most related to genotypes already described in Europe and the

Mediterranean, and not to strains from the Americas or Asia. The arrival of new species and new potential pathogens is ongoing through transfer of ballast water to the Atlantic and Mediterranean. Although systems are in place to exchange or inactivate ballast water, it is advisable to continue and expand surveillance for pathogens in introduced species, to inform management of movement of these species between regions.

Introduction

The Atlantic blue crab *Callinectes sapidus* Rathbun,1896 (Brachyura: Portunidae) is a swimming crab native to the western Atlantic in coastal and estuarine waters of New England (USA) to Uruguay, including the Gulf of Mexico and the Caribbean Sea (Mancinelli *et al.,* 2021, 2017). In recent years it has also emerged as one of the most concerning invasive species in the Mediterranean Sea. It was first recorded (as *Neptunus pelagicus*) in Europe in 1900 (Bouvier 1901), and appears to be established on the Atlantic coast of France and England (Pezy *et al.,* 2019). Other records show the species has also moved south to the upper Iberian coast (Ribeiro and Veríssimo 2014). In the Mediterranean Sea it was first recorded in 1949 in the Gulf of Venice, Italy, in the northern Adriatic Sea (Giordani Soika 1951), and possibly as early as 1935 near Turkey (Enzenroß *et al.,* 1997). It has become invasive in the eastern-most and western basins of the Mediterranean and the Black Sea (Vella *et al.,* 2023). Recently, the species was reported in Malta and Sicily (Vella *et al.,* 2023), Sardinia (Culurgioni *et al.,* 2020), Spain (Clavero *et al.,* 2022), the Adriatic and Aegean seas (Glamuzina *et al.,* 2023, 2021; Cilenti *et al.*, 2015; Kevrekidis *et al.,* 2023), and the Mediterranean coast of Africa (Kara and Chaoui, 2021; Taybi and Mabrouki, 2020). Also recently, *C. sapidus* has appeared in abundance on the southern Atlantic Coast of Portugal, and has been reported on the Atlantic coast of Morocco, possibly reflecting an expansion through the straits of Gibraltar (Morais *et al.,* 2019; González-Ortegón *et al.,* 2022; Lamkhalkhal *et al.,* 2024).

The life history of *C. sapidus* includes a 28-35 day oceanic planktonic phase, before metamorphosis to benthic postlarvae (megalopa stage), that contributes to dispersal. Blue crabs are relatively short-lived, with an estimated longevity of 2-3 years in some parts of the US native range (Kahn and Hestler 2005), and a recent estimate of 4.8 years in an Italian lagoon (Mancinelli *et al*., 2024). Therefore, in locations with consistently high crab abundance, there must be recurring sources of larvae, either through local self recruitment or from broodstock sources upstream in oceanic currents. For example, in the south-eastern Adriatic Sea, a strong anti-clockwise gyre connects the coast of Italy and Croatia, while in the Aegean Sea, where blue crabs have been abundant for decades (Holthuis 1961) currents are weak and the many islands ensure promote connectivity and self recruitment (Estournel *et al.,* 2021). The local-recruitment hypothesis is supported by Schubart *et al.,* (2022) who found low genetic connectivity between

77 different populations of *C. sapidus* in the invaded range, consistent with multiple founder
78 introductions.
79
80 The success of the blue crab in new regions is likely enhanced by its omnivory and ability to
81 adapt to a wide range of salinity, temperature and dissolved oxygen (Glandon *et al.,* 2019).
82 Success of non-native species in a new range is often also attributed to an escape from predators
83 and infectious diseases that suppressed their numbers in the home range (sometimes referred to
84 as the enemy release hypothesis) (Blakeslee *et al.,* 2009; Torchin *et al.,* 2007; Schultz *et al.,*
85 2019). While a lot has been reported on occurrence and the invasiveness of *C. sapidus* in the
86 Mediterranean, there is still little knowledge of the infections that it may acquire in this new
87 range. The enemy release (pathogen escape) concept assumes that newly arrived species have not
88 carried their home-range pathogens with them.
89
90 In its North American range, *C. sapidus* is infected by a number of pathogenic parasites and
91 viruses (Shields and Overstreet 2007). The most studied is the parasitic dinoflagellate,
92 *Hematodinium perezi*, which is known to cause mortality in *C. sapidus*, and is a host generalist
93 that infects a wide range of crustacean species. The type species is described in *Liocarcinus*
94 *depurator* from the Atlantic coast of Europe (Chatton and Poisson, 1931; Small et al. 2102).
95 Three different molecular *H. perezi* clades are recognized, each characteristic of different global
96 regions, and distinguished by genomic changes in the ribosomal RNA gene ITS1 region (Small
97 *et al*., 2012, Pagenkopp-Lohan *et al.,* 2012). The genotype infecting crabs in the Mediterranean
98 and Atlantic coast of Europe is referred at Type I, while those in Asia, often associated with
99 mortality in aquaculture, are type II, whereas *H. perezi* found in *C. sapidus* and other hosts on
100 the Atlantic estuaries of north America are type III (Small *et al*., 2012).
101
102 In the United States, regional population declines of *C. sapidus* have coincided with elevated
103 prevalence *Hematodinium* sp. (Lee and Frischer 2004). A related *Hematodinium* sp. that infects
104 Norway lobster *(Nephrops norvegicus*) is a known driver of mortality in this fishery on the west
105 coast of Scotland (Stentiford and Neil 2011; Molto-Martin *et al*., 2024). In the Mediterranean,
106 there are few reports of *H. perezi* (or other *Hematodinium* sp.) in any host, however it has been
107 reported in *C. sapidus* and other crabs in the south of Italy (Pagliara and Mancinelli 2018;
108 Mancinelli *et al.,* 2013).
109
110 The life cycle of *H. perezi* is complex, with a progression of several morphological cell types
111 within the host, culminating in the release of dinospores that emerge from mouth or gills
112 (Stentiford and Shields, 2005). Transmission of the parasite is thought to be via dinospores,
113 which remain infective for hours to days in the water column (Coffey *et al*., 2012). Prevalence
114 and infectivity of *H. perezi* are responsive to salinity, with higher prevalence in crabs captured in
115 water above 18 psu, and dinospores becoming inactive at salinity below 10 psu (Messick and
116 Shields 2000; Coffey *et al*., 2012).

The blue crab is also infected across its native range by a virus known as CsRV1, which was first observed in electron microscopy as "reo-like virus" (RLV) in the 1970s (Johnson 1977). This virus is associated with the majority of mortality in captive blue crabs in the United States (Bowers *et al.,* 2010; Spitznagel *et al.,* 2019). Surprisingly, the CsRV1 genome has a region of high genetic similarity to a reovirus, named P virus, described in the portunid crab *Liocarcinus depurator*, on the Mediterranean coast of France (Flowers *et al.,* 2016; Montanie *et al.,* 1993; Walton *et al.,* 1999). There has been no publication on evidence of CsRV1 or P virus in the Mediterranean in the intervening 25 years. CsRV1 has been studied thoroughly within the crab's native range (Zhao *et al.,* 2020). CsRV1 is present from Uruguay to New England, but has not been reported in wild capture of any other species than *C. sapidus* (Zhao *et al.,* 2020). The genotype of CsRV1 varies in a manner consistent with geographic origins (Zhao *et al.,* 2023).

As *C. sapidus* continues to expand and increase in abundance across the Mediterranean and Atlantic coast of Europe, several authors point out that there are still considerable gaps in our knowledge of the ecology of this crustacean in the new range, even as the species is being exploited as a fishery resource in some locations (e.g., Mancinelli *et al.,* 2017; Vella *et al.,* 2023; Azzurro *et al.,* 2024; Galil 2024). Given the importance of *H. perezi* and CsRV1 in the ecology of *C. sapidus* in its native range, it is relevant to investigate how these pathogens are distributed in *C. sapidus* in the invaded range. Given the global distribution of *H. perezi* and other *Hematodinium* sp., the bi-hemispheric distribution of CsRV1 and molecular evidence that CsRV1 may have a close relative in Europe, we investigated the prevalence of these two known *C. sapidus* pathogens in the Adriatic and Aegean Seas, where *C. sapidus* has been reported for over a decade, and on the newly-invaded coast of Portugal in the Guadiana estuary.

Materials and methods

Crab sampling and environmental data

Thirty adult *Callinectes sapidus* were collected from each of 6 estuaries in the Mediterranean and Atlantic Iberian coast between 2021 and 2023 (Figure 1), for a total of 180 crabs.
Wire traps were used in Mediterranean sampling locations of the South Adriatic Sea in Croatia (Parila lagoon in Neretva river delta, Ston saltworks, Mlini in Župa Bay) and Montenegro (Ada Bojana). Blue crabs originating from the Aegean sea (North Euboean Gulf) in Greece were also captured using commercial wire traps and obtained from the fish market. Mediterranean crab samples were transported to laboratory on ice and processed immediately upon arriving.
Temperature and salinity were measured at the time of the sampling in Parila lagoon and Ston saltworks by YSI Pro30 probe, and in Ada Bojana by HANNA HI 98194 multiparameter probe. Environmental data for Mlini were acquired from a year round study of the Župa bay (M. Pećarević, personal communication) while the temperature of the Aegean sea was obtained by daily satellite readings provided by NOAA, some 70 km southeast from the sampling site.

Crabs from Atlantic coast were captured by fishermen using gill and fyke nets in the tidal Guadiana River in Portugal (bordering Spain). Salinity and temperature were collected as a contribution to the International Group for Marine Ecology Time Series (IGMETS) monitoring program, in the Guadiana lower estuary station, southwest Iberian Peninsula. These crabs were transported to shore within 2 hours, and frozen at -20 ˚C until processing in the laboratory.

Table 1. *Callinectes sapidus* sampling sites, dates and environmental conditions

Location	Map ID	Collect date (month-year)	Latitude (°N)	Longitude (°E)	Temp (°C)	Sal (psu)
Parila lagoon, South Adriatic Sea	1	February and March 2021	43.0307	17.4512	15	18
Mlini, South Adriatic Sea	2	February and May 2021	42.8301	17.6975	21.7	38
Ston saltworks, South Adriatic Sea	3	May, June and October 2021	42.6178	18.2031	18.8	38
Ada Bojana, South Adriatic Sea	4	September 2022	41.8538	19.3572	22.5	32
North Euboean Gulf, Aegean Sea	5	April 2023	38.7107	23.2851	15.0	n.a.*
Foz de Odeleite, Guadiana estuary	6	June 2023	37.3534	-7.4409	18.3	13

*n.a. not available

167 **Figure 1.** Sampling locations on the Adriatic, Aegean and Iberian Atlantic coasts. Numbers at
168 each location correspond to numbers in Table 1.
169

170 ***Dissection, nucleic acid extraction and polymerase chain reactions***

171 Crab legs (fresh or partially thawed on ice) were externally cleaned with RNaseZap (Thermo
172 Fisher Scientific) and dissected with new razor blades. Two samples of 50-100 mg muscle tissue
173 were dissected from a walking leg of each crab and stored at 4°C in 96% ethanol (Gram-Mol,
174 Croatia) for DNA analysis, as well as in TriReagent solution (Thermo Fisher Scientific) for RNA
175 analysis. All samples were transported to the Laboratory for Aquaculture of the Institute of
176 Oceanography and Fisheries in Split, Croatia for nucleic acid extraction and histological analysis
177 see Methods 2.3.
178

179 *DNA extraction*

180 Genomic DNA was extracted following a simplified phenol-chloroform-isoamyl DNA isolation
181 procedure (Laird *et al.,* 1991). Resulting DNA pellets were suspended in 30 µl of TE buffer,
182 checked for purity and concentration by NanoPhotometer (Implen) and stored at -20°C until
183 shipment. DNA was then re-precipitated with ethanol and shipped as precipitates to the Institute
184 Marine and Environmental Technology (IMET) in Baltimore, USA for PCR analyses.
185

186 *RNA extraction*

187 For the RNA extraction the tissue was homogenized with 1 mL TriReagent solution (Thermo
188 Fisher Scientific) with ceramic beads using MagNa Lyser (Roche) and then the procedure
189 followed the protocol used by Spitznagel *et al.* (2019). Obtained RNA pellets were suspended in
190 50 µl of nuclease-free water and checked for purity and concentration by NanoPhotometer
191 (Implen). RNA was re-precipitated in ethanol and pelleted nucleic acid samples were shipped to
192 the Institute of Marine and Environmental Technology (IMET) in Baltimore, USA where they
193 were stored at -20°C until resuspension in nuclease-free water for RT-qPCR analysis.
194

195 *RTt-qPCR detection of CsRV1*

196 The presence of CsRV1 RNA was investigated using quantitative reverse transcription PCR (RT-
197 qPCR) methods adapted from Flowers *et al.,* (2016), using a primer pair designed to amplify a
198 158-bp region of the ninth genome segment of CsRV1: 5'-
199 TGCGTTGGATGCGAAGTGACAAAG-3' (RLVset1F) and 5'-
200 GCGCCATACCGAGCAAGTTCAAAT-3' (RLVset1R). Positive controls and standard curves
201 consisted of a 10-fold dilution series of purified double-stranded RNA (dsRNA) purified from
202 crabs infected with CsRV1, and containing 10 to 10^6 CsRV1 genome copies per µl, serially
203 diluted in 25 ng μl^{-1} yeast tRNA carrier. The RT-qPCR cycling conditions and reagents were as
204 described by Spitznagel *et al.* (2019), using qScript XLT One-Step RT-PCR Kit (Quantabio,

205 Beverly MA) in 10 µl reactions containing 0.5 µM of each primer, on a QuantStudio 3 Real Time
206 Thermocycler. Gene target copies were calculated as copies mg^{-1} of crab muscle, and samples
207 with >500 copies mg^{-1} were recorded as CsRV1-positive according to Flowers *et al.* (2016),
208 which is based on empirical measurement of background contamination from field and
209 laboratory procedures. RNA amplification quality was assessed by using endpoint RT-PCR to
210 amplify a fragment the blue crab small subunit ribosomal RNA transcript using primers
211 CrabSSUfor 5'-CATGTCTAAGTACAAGCCGAA-3' and CrabSSURev 5'-
212 GGGTAATTTGCGTGCCT-3', which produce a 405 nucleotide amplicon (GenBank
213 JAHFWG010003967; Bachvaroff *et al.,* 2021). PCR products were assessed by gel
214 electrophoresis. RNA samples that failed to amplify with this rRNA primer set were deemed low
215 quality and were not included in the CsRV1 analyses.
216
217 *PCR detection of Hematodinium sp.*

218 The presence and amount of *Hematodinium* sp. DNA was assessed by the qPCR method of
219 Nagle *et al.,* (2009), which amplifies a 121-nucleotide region of the small subunit ribosomal
220 RNA gene. Analyses were performed in duplicate on a QuantStudio 3 Real Time Thermocycler
221 using PerfeCTa SYBR Green FastMix (Quantabio) with the following thermocycling conditions;
222 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The
223 qPCR reactions included negative (no template) and positive (cloned target gene) controls. The
224 number of *Hematodinium* sp. target copies was quantified by comparison to a standard curve (10
225 to 10^6 copies per µl) of cloned *Hematodinium* sp. DNA (pES103, see Hanif *et al.,* 2013) that was
226 run on each plate. Based on the fraction of total DNA used in the qPCR reaction and the weight
227 of the tissue from which it was extracted, the *Hematodinium* sp. infection intensity was
228 expressed as the number of gene copies mg^{-1} of muscle.
229
230 *Sequencing and analysis of H. perezi ITS1 gene*

231 A 783 bp region of the 18S rRNA gene, from the SSU across the first internal transcribed spacer
232 (ITS1) region to the 5.8S region, was amplified and sequenced for phylogenetic analysis.
233 Amplification of this region was performed using the primers PlaceF (5'-
234 GGGTAATCTTCTGAAAACGCATCGT-3') from Hanif *et al.*, 2013 and a reverse primer (5'-
235 CTGGTCTCAGCGTCTGTTCA -3') from the 5.8S region of the *H. perezi* rRNA gene cluster.
236 The PCR was conducted on a BioRad C1000 thermocycler with AccuStart II PCR ToughMix
237 under the following conditions: initial denaturation at 94°C for 5 minutes, followed by 31 cycles
238 of 94°C for 25 seconds, 58°C for 25 seconds, and 72°C for 1 minute. Amplified products were
239 visualized by 2% (w/v) agarose gel electrophoresis, stained with SYBR Safe DNA Gel Stain, and
240 examined under ultraviolet light. Aliquots of each reaction were sequenced using an ABI 3130
241 genetic analyzer. Quality trimming and removal of primer sequences and low quality sequence
242 was carried out using CLC Genomics Workbench 9.5.2 (Qiagen, Hilden, Germany). Assembled
243 forward plus reverse assemblies were manually inspected, and consensus sequences were
244 exported for further analysis.

245 Comparison of sequences derived from crabs in this study was conducted by alignment tools in
246 CLC Workbench. A BLAST search of the 650 nt region identified candidate *H. perezi* sequence
247 accessions in GenBank for comparison to experimental data. Selected candidates were chosen
248 that represented genotypes I, II, and III (Small *et al*., 2012) and geographic origins of Europe,
249 Asia and North America. The reulsting alignment was trimed to the sequence area in common,
250 visually inspected, and visualized using the tree functions using https://ngphylogeny.fr/. Default
251 parameters were used to create a Neighbor Joining tree with bootstrap values determined using
252 100 iterations.
253
254 *Histological analysis*

255 At the time of dissection for DNA and RNA extractions, pieces of hepatopancreas were excised
256 from the crab samples and fixed for 24 hours in modified Davidson's fixative, prepared with
257 filtered seawater to maintain isotonicity. After fixation, the samples were transferred to 70%
258 ethanol and stored at 4°C. Selected samples of fixed tissue were dehydrated in graded ethanol
259 series, cleared in xylene and paraffin-embedded. Tissue sections were cut at 3-5 µm thickness,
260 stained with Mayer's hematoxylin and eosin (MHE) and coverslipped in NeoMount. Stained
261 sections were inspected under a Leica DM3000 LED microscope. Images were captured with a
262 Leica DMC 4500 camera and assembled and annotated in PhotoShop CS5 (Adobe Systems).
263
264
265 **Results**
266
267 There was no evidence found for CsRV1 virus RNA in the samples of *C. sapidus* collected in
268 this study, from the Aegean Sea, Adriatic Sea or Guadiana estuary. This represented a total of
269 166 individual samples from 6 locations. CsRV1 RT-qPCR assays were conducted on all RNA
270 samples, but of these, several RNA samples (10 from Portugal and 4 from Greece), did not
271 produce RT-PCR amplicons with the positive control SSU primers, and so were excluded from
272 analysis.

273 DNA of the parasite *H. perezi* was detected in crabs from the Adriatic Sea and Guadiana estuary,
274 but not from the Aegean Sea. The proportion of infected crabs and the mean intensity (copies of
275 *H. perezi* target DNA mg^{-1} muscle tissue) are listed in Table 2. Individual crabs had *H. perezi*
276 DNA intensity ranging from 30 copies to over 100,000 copies of target DNA mg^{-1} of tissue
277 (supplemental material available).

278

279 **Table 2.** Prevalence and mean copy number of *H. perezi* and CsRV1 as determined by qPCR.
280 Copy number of cloned target genes per mg tissue.

	Hematodinium perezi			**CsRV1**	
Location	**Sample**	**Prevalence**	**Mean**	**Sample**	**Prevalence**

	size	(%)	intensity (copies mg^{-1})	size*	(%)
Parila lagoon, South Adriatic Sea	30	23	1.28 x 10^3	30	0
Ston saltworks, South Adriatic Sea	30	40	1.54 x 10^5	30	0
Mlini, South Adriatic Sea	30	67	3.00 x 10^5	30	0
Ada Bojana, South Adriatic Sea	30	10	3.72 x 10^3	30	0
North Euboean Gulf, Aegean Sea	30	0	n.a.	26	0
Foz de Odeleite, Guadiana estuary	30	87	3.25 x 10^4	20	0

*some samples were excluded because they failed the rRNA amplification quality check.

Histological analysis of the hepatopancreas of a crab from Ada Bojana Montenegro that had 1.8 x10e3 gene copies mg^{-1} of fresh weight is shown in **Figure 2**. The interstitial spaces between tubules is distended and heavily invaded by parasite cells. Occasionally, cellular aggregates, possibly fixed phagocytes, are seen. Some of the tubules (T) are depleted of lipid inclusions (arrows).

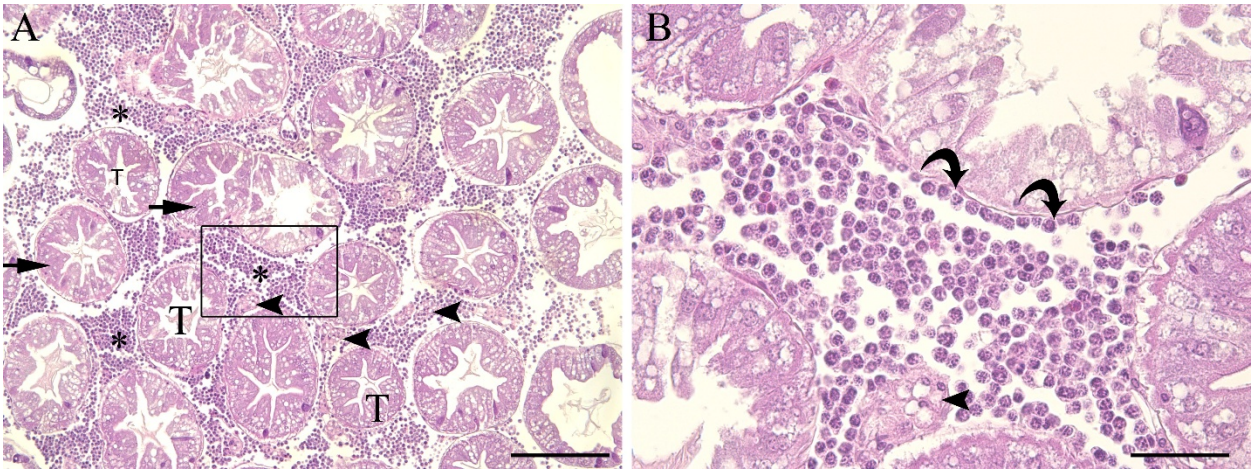

Figure 2. Representative micrographs of a hepatopancreas of the *C. sapidus* from Ada Bojana Montenegro infected with *H. perezi* at high infection intensity. **(A)** The intertubular space is distended and the interstitial tissue is almost completely replaced by vegetative stages of the parasite (asterisks). Occasionally, cellular aggregates, presumably fixed phagocytes, can be seen (arrowheads). Some of the tubules (T) show a depletion of lipid inclusions (arrows). **(B)** High

magnification (400x) showing details of the developmental stages of the parasite with fixed phagocytes (arrowhead) and parasites adhering to the tubule epithelium (curved arrows). H&E staining. Scale bar: (A) 200 µm, (B) 50 µm

H. perezi infections (qPCR signal) in three locations were intense enough to enable amplification of a 888 nt region of the ITS and gene. Each of these sequences is deposited in GenBank, under the accession numbers PP854984 (Portugal), PP854699 (Montenegro), PP854700 (Zupa). As can be seen in **Figure 3**, a Maximum Likelihood phylogenetic analysis of aligned ITS1 regions of these sequences with corresponding regions of 256 nucleotides of the *H. perezi* ITS1 from other regions of the eastern Atlantic, North America, and Asia shows that the two *H. perezi* sequences from the Adriatic are identical, and slightly less similar to the sequence from Portugal (98% identity). All of them are more similar to *H. perezi* sequences from European waters than from Asia or North America. Bootstrap values are below 75 for all nodes except those at the branchpoints between continental regions.

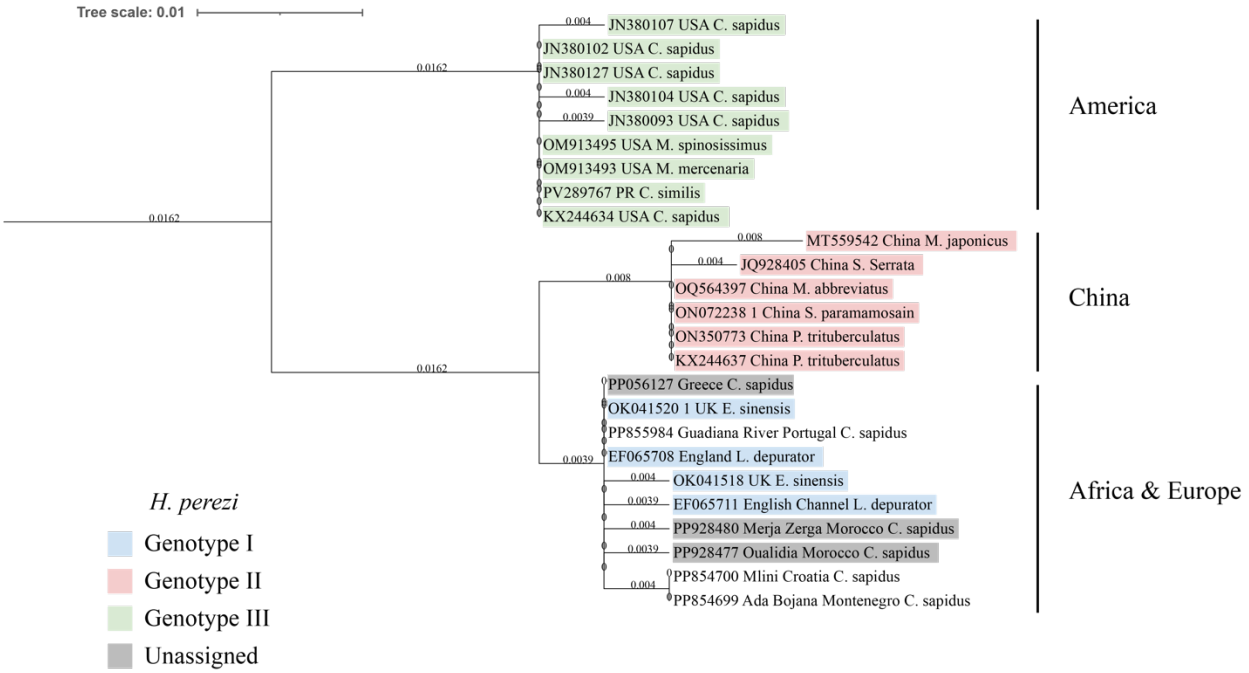

Figure 3. Neighbor joining tree from alignment (261 nt) of *H. perezi* ITS1 sequences from this study (no shading) as compared to *H. perezi* sequences in hosts from the Atlantic coast of Europe and North Africa, Mediterranean, Asia and North America. Numbers at branches are bootstrap values for 100 replicates. Genbank accession of *H. perezi* representing genotypes I, II and III are shaded with different colors as indicated in the legend.

Discussion

The spread of *C. sapidus* throughout the Mediterranean and eastern Atlantic is highly consequential for the ecology and economy of the region. Measuring, predicting and adapting to the specific impacts of this invasive species is important for practical reasons, and presents a rare opportunity to evaluate ecological and evolutionary theories about invasive species escaping predators and disease. There has been some indication that blue crabs can dramatically decrease the population of aquatic prey (Céspedes *et al.,* 2024; Nardelli *et al.,* 2024). In Italy and Spain, bivalve farmers have abandoned the practice because of damage caused by *C. sapidus* predation (Chiesa *et al*., 2025). Often, invasive species exhibit a surge in abundance, followed by a decline and leveling of abundance in a new region after some years (Simberloff and Gibbons, 2004). In some cases this could be a consequence of the introduced species disrupting its own prey or habitat resources. Additionally, within the "enemy escape" framework/concept, this could be a result of both external predators and internal pathogens adapting to and exploiting the presence of the new species.

The current study gives insight to the presence of two known infectious agents of the Atlantic blue crab *C. sapidus* in selected locations of western Mediterranean and Atlantic Iberian estuaries. The opportunity to analyze crabs from the Aegean Sea, Adriatic, and Guadiana estuary represents an east-to-west range that corresponds to the possible expansion trajectory of the species over time (Mancinelli *et al.,* 2021). The two infectious agents studied represent very different life history strategies for pathogens. The CsRV1 virus is described only in the *C. sapidus* native range, and has not been yet been reported in any other species of host (Zhao *et al.,* 2022). In contrast, the protozoan parasite (parasitic dinoflagellate) *H. perezi* is a globally-distributed crustacean host generalist (Small and Li 2024).

There are relatively few studies related to the health status of *C. sapidus* in the Mediterranean and adjacent waters. At the time of sampling, ours was the first study to investigate the virus CsRV1 in the Mediterranean or eastern Atlantic. Although we did not detect CsRV1 in any of the *C. sapidus* tested, our limited study cannot be taken as evidence that the virus is entirely absent from the Mediterranean or eastern Atlantic. Perhaps the most intriguing suggestion that CsRV1 could be present in the Mediterranean is the report by Walton *et al.,* (1999) on the sequence of a segment of the P virus genome in the portunid crab *Liocarcinus depurator*. The segment of P virus has over 95% identity to segment 4 of CsRV1 (Flowers *et al.,* 2016). This level of identity is in the range of genome difference between CsRV1 ecotypes in north America versus south America (Zhao *et al.,* 2020). Future investigations of CsRV1 in the Mediterranean should include assessments of portunid species such as *Liocarcinus* sp. and *Carcinus* sp.

On the North American Atlantic coast, studies have shown that CsRV1 prevalence is highly variable across time and space, often as low as 0% or as high as 75% prevalence (Flowers *et al.,* 2018). The only consistent trend seen for CsRV1 in the native range is the near absence in the

361 tropics (Zhao *et al.,* 2020). Flowers *et al.,* (2018) noted that high prevalence of CsRV1was
362 coincident with higher *C. sapidus* populations in Chesapeake Bay, which is consistent with the
363 principles of host-dependent prevalence (Cortez and Duffy 2021). All of the locations surveyed
364 in the current study are temperate, but the overall blue crab population numbers are also
365 unknown in each of the areas. In the coming 5 – 10 years, it will be informative to re-survey for
366 the presence of CsRV1 virus in areas with particularly high *C. sapidus* populations or where the
367 species has been recorded for a long time.
368
369 The presence of *H. perezi* in Mediterranean *C. sapidus* was first reported by Mancinelli *et al.,*
370 (2013) in ecosystems of the Apulia coastline. In that study, over 70% of the crabs examined (*C.*
371 *sapidus, Eriphia verrucosa, Pachygrapsus marmoratus* and *Carcinus mediterraneus*) in the
372 Torre Colimena (Ionean Sea) basin and Acquatina lagoon (West Adriatic Sea) were infected by
373 *Hematodinium* sp. (Mancinelli *et al.,* 2013). Our study showed that just across the southern
374 Adriatic, prevalence of *H. perezi* was moderate to high at all locations. These two coasts are
375 highly connected by an anticlockwise gyre in the southern (Estournel *et al.,* 2021), which would
376 have the potential to rapidly convey both crab larvae and parasite propagules between them.
377
378 In the Aegean Sea, Lattos *et al.,* (2024) recently reported 85% prevalence of *H. perezi* in *C.*
379 *sapidus* that were collected in November following 2020 mortality events. They suggest this high
380 prevalence is responsible for recent declines in *C. sapidus* abundance in that region of Greece.
381 These authors pointed out that there were no external signs of disease observed during the
382 sampling of *C. sapidus* but that infected crabs had a characteristic "milky" coloration of
383 hemolymph. In our study of Aegean Sea crabs harvested in April of 2021, we detected no *H.*
384 *perezi.* Our collection was from a local fish market, so lacked fine scale specificity of geographic
385 origin. We are aware that the use of commercially harvested crabs may bias against heavily
386 infected or moribund crabs, the work of Lattos *et al.* (2024) reported that both "moribund and
387 healthy individuals" were found with *H. perezi*. The association of crab declines with *H. perezi* is
388 still relevant to our current study; there are anecdotal reports from fishermen in Mlini, which had
389 the 2nd highest prevalence (76%), that they didn't encounter blue crabs after the summer of 2021
390 for almost a year (M. Pećarević, personal communication). While there are many possible
391 reasons for this, one is that infections by *H. perezi* reduced the abundance of blue crabs, similar
392 to what has been reported in the *C. sapidus* native range (Lee & Frischer 2004).
393
394 On the Atlantic coast of north Morroco, Lamkhalkhal *et al.,* (2024) also recently reported *H.*
395 *perezi* in *C. sapidus* at 25-60% prevalence. Moreover, they show that the sequence of the ITS1
396 gene of *H. perezi* from Morocco is similar to that in strains from Europe and the Mediterranean.
397 Our study is the first in our knowledge to document *H. perezi* on the Atlantic coast of Portugal.
398 We are aware that a 2021 metagenomic investigation of *C. sapidus* stomach contents from the
399 Rio Formosa estuary near Faro, Portugal also revealed evidence of *H. perezi* DNA. In this case,
400 the sequence was a match to the mitochondrial COX1 gene (J. Encarnacao and P. Morais,

401 personal communication), so no direct comparison can be made to the ITS1 sequences.
402 Nevertheless, this suggests that the parasite has been in Atlantic coastal estuaries of Portugal
403 since at least 2021.
404
405 In the *C. sapidus* native range, the prevalence of *H. perezi* is typically associated with high
406 salinity (Messick and Shields 2000; Gandy *et al.,* 2015). In our study, there is no strict
407 correspondence between salinity at the sites sampled and the prevalence of *Hematodinium* sp. In
408 fact, the highest prevalence was found in the Guadiana River, which had locally low salinity but
409 is less than 20 km away from the ocean. In the Eastern Adriatic Sea, salinity was above 30 psu
410 except in Parila lagoon (18 psu), which also had the lowest *Hematodinium* sp. prevalence in that
411 region. The only sample without detection of *H. perezi* was in the Aegean Sea, where crabs were
412 obtained from a fish market. Therefore precise location and salinity information was not
413 available. This is in contrast to the recent report by Lattos *et al.,* (2024) of *H. perezi* prevalence
414 over 80% across the Aegean Sea, which they note was coincident with a decline in the
415 abundance of *C. sapidus* as reported by fishermen. The contrasting prevalence found in our study
416 and that of Lattos *et al.,* (2024) is not unusual for marine disease studies, where diverse
417 environmental factors interact with shifting population size and connectivity to produce highly
418 variable prevalence.
419
420 The relationship of *H. perezi* ITS1 sequences in our study with corresponding regions of *H.*
421 *perezi* strains across the globe is consistent with prior observations that *H. perezi* within different
422 continents are similar to one another, and distinct from other regions, regardless of host. The
423 high identity of ITS1 sequences between Greece and Portugal strains stands out, however, and
424 may suggest a rapid movement from one location to another. One possible mechanism for such
425 transport of a parasite strain is ballast water (Nehring, 2011; Cantrell *et al*., 2020). To comply
426 with the International Convention for the Control and Management of Ships' Ballast Water and
427 Sediments (IMO, 2004), prior to entering the Mediterranean, cargo ships exchange ballast water
428 in the Atlantic. Therefore, it is possible that a strain of *H. perezi* or a host infected with it was
429 transferred in ballast from the Atlantic to the Aegean Sea. Additional comparisons of *H. perezi*
430 genetics will be needed to lend support or refute the idea that that the parasite has recently
431 moved.
432
433 It is important to understand how endemic infectious agents find or adapt to newly arrived
434 invasive species for at least two reasons. One is the effect that these agents may have on the
435 health of native species; will they proliferate in a new reservoir host, or perhaps the new host will
436 constitute a dead end that lowers transmission of the infectious agent. Evidence is that the
437 genotype I of *H. perezi* is well- adapted to *C. sapidus*. A second reason to understand infectious
438 agents is the impact on the invasive species over time. In many regions of Europe, there is still a
439 policy to reduce the number of *C. sapidus* in the invaded range. As income from *C. sapidus*
440 fisheries grow, however, there may be concern about disease-related mortality if it affects

harvests. In North America, the CsRV1 virus is associated with stress and high populations of *C. sapidus* (Spitznagel *et al.,* 2019; Flowers *et al.,* 2018). Elsewhere in the Americas, other viruses have been recently identified in *C. sapidus* (Tavares *et al*., 2022; Zhao *et al*., 2022), illustrating that there is a potential for various pathogens to be transferred to or within European waters by ballast water transfer or movement of live crustaceans. Surveillance of *C. sapidus* health is therefore advisable in all regions of Europe.

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