Infections in the 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 endemic 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* sp. In crabs collected from Aegean, Adriatic, and Atlantic waters, the CsRV1 virus was not detected. In contrast, the parasite *Hematodinium* sp. was found in all areas except the Aegean Sea. Sequence analysis of the *Hematodinium* sp. ITS1 gene indicated that the strains observed are most related to *Hematodinium* sp. strains already described in Europe and the Mediterranean, and not to strains from the Americas. 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 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 (Mancinelli *et al.,* 2017), and possibly as early as 1935 (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, 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) and enter estuaries. Blue crabs are relatively short-lived, with an estimated longevity of 2-3 years (Kahn and Hestler 2005). 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 different populations of *C. sapidus* in the invaded range, consistent with multiple founder introductions.

The success of the blue crab in new regions is likely enhanced by its omnivory and ability to adapt to a wide range of salinity, temperature and dissolved oxygen (Glandon *et al.,* 2019). Success of non-native species in a new range is often also attributed to an escape from predators and infectious diseases that suppressed their numbers in the home range (sometimes referred to

as the enemy release hypothesis) (Blakeslee *et al.,* 2009; Torchin *et al.,* 2007; Schultz *et al.,* 2019). While a lot has been reported on occurrence and the invasiveness of *C. sapidus* in the Mediterranean, there is still little knowledge of the infections that it may acquire in this new range. The enemy release (pathogen escape) concept assumes that newly arrived species have not carried their home-range pathogens with them.

In its home range, *C. sapidus* is infected by a number of parasites and viruses (Shields and Overstreet 2007). The most well characterized is the parasitic dinoflagellate, *Hematodinium perezi*, which is a host generalist that also infect other crustacean species. Different *Hematodinium* sp. genotypes and sub-species are characteristic of different hosts and global region, and identifiable by genomic changes in the ribosomal RNA genes (Small 2012, Messick and Shields 2000, Pagenkopp-Lohan *et al.,* 2012). In the United States, regional population declines of *C. sapidus* have coincided with elevated prevalence *Hematodinium* sp. and in northern Europe (Irish Clyde Sea) the parasite is associated with declines in Norway lobster (Lee and Frischer 2004, Stentifod and Neil, 2012). In the Mediterranean, there are few reports of this parasite in any crustacean, however it is reported in *C. sapidus* in the south of Italy (Pagliara and Mancinelli 2018; Mancinelli *et al.,* 2013). Intriguingly, Lamkhalkhal *et al.,* (2024) recently described *Hematodinium perezi* in *C. sapidus* from the Atlantic coast of Morocco.

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 valuable fishery resource in some locations (e.g., Mancinelli *et al.,* 2017; Vella *et al.,* 2023; Azzurro *et al.,* 2024). One interesting question is: What pathogens can be found in *C. sapidus* in the new range? Given the cosmopolitan nature of *Hematodinium* sp., and the 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, 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 Portuguese Atlantic coast between 2021 and 2023 (Figure 1), for a total of 180 crabs. Wire crab traps were used in Eastern Adriatic Sea (Parila lagoon in Neretva river delta, Ston saltworks, and Mlini in Župa Bay), and traps in the Aegean sea in Greece and Ada Bojana in Montenegro, while gill nets were used in the Guadiana River estuary in Portugal. Temperature and salinity were measured by YSI Pro30 probe in Parila lagoon and Ston saltworks and by HANNA HI 98194 multiparameter probe in Ada Bojana at the time of the crab sampling, while environmental data for Mlini were obtained from a year round study of the Župa bay (M. Pećarević, personal communication). Temperature for Greece was obtained by daily satellite readings provided by a NOAA, some 70 km southeast from the sampling site. Crabs from Portugal were captured by fishermen in the tidal Guadiana River (bordering Spain), Atlantic Iberian coast using gill nets and fyke nets. 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. 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	April 2023	37.3534	-7.4409	18.3	13

*n.a. not available

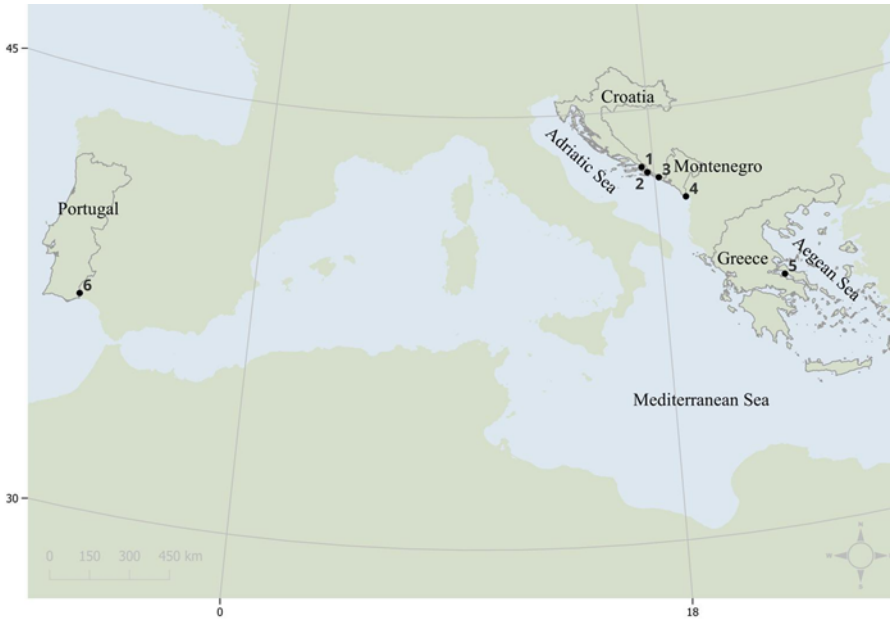


Figure 1. Sampling locations on the Adriatic, Aegean and Iberian Atlantic coasts. Numbers at each location correspond to numbers in Table 1.

Dissection, nucleic acid extraction and polymerase chain reactions

Freshly caught or crabs were cleaned with RNaseZap (Thermo Fisher Scientific) and dissected with razor blades. Two samples of 50-100 mg muscle tissue was dissected from a walking leg of each crab and stored at 4°C in 96% ethanol (Gram-Mol, Croatia) for DNA analysis, as well as in TriReagent solution (Thermo Fisher Scientific) for RNA analysis. All samples were transported to the Laboratory for Aquaculture of the Institute of Oceanography and Fisheries in Split, Croatia for nucleic acid extraction and histological analysis see Methods 2.3.

DNA extraction

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

RNA extraction

For the RNA extraction the tissue was homogenized with 1 mL TriReagent solution (Thermo Fisher Scientific) with ceramic beads using MagNa Lyser (Roche) and then the procedure followed the protocol used by Spitznagel *et al.,* (2019). Obtained RNA pellets were suspended in 50 µl of nuclease-free water and checked for purity and concentration by NanoPhotometer (Implen). RNA was re-precipitated in ethanol and pelleted nucleic acid samples were shipped to

167 the Institute of Marine and Environmental Technology (IMET) in Baltimore, USA where they
168 were stored at -20°C until resuspension in nuclease-free water for Rt-qPCR analysis.
169
170 *Rt-qPCR detection of CsRV1*

171 The presence of CsRV1 RNA was investigated using quantitative reverse transcription PCR (Rt-
172 qPCR) methods adapted from Flowers *et al.,* (2016), using a primer pair designed to amplify a
173 158-bp region of the ninth genome segment of CsRV1: 5'-TGC GTT GGA TGC GAA GTG
174 ACA AAG-3' (RLVset1F) and 5'-GCG CCA TAC CGA GCA AGT TCA AAT-3' (RLVset1R).
175 Positive controls and standard curves consisted of a 10-fold dilution series of purified double-
176 stranded RNA (dsRNA) purified from crabs infected with CsRV1, and containing 10 to 10^6
177 CsRV1 genome copies per µl, serially diluted in 25 ng μl^{-1} yeast tRNA carrier. The qPCR
178 cycling conditions and reagents were as described by Spitznagel *et al.,* (2019), using qScript
179 XLT One-Step RT-PCR Kit (Quantabio, Beverly MA) in 10 µl reactions containing 0.5 µM of
180 each primer, on a QuantStudio 3 Real Time Thermocycler. Gene target copies were calculated as
181 copies per mg of crab muscle, and samples with >500 copies mg−1 were recorded as CsRV1-
182 positive according to Flowers *et al.* (2016), which is based on empirical measurement of
183 background contamination from field and laboratory procedures. RNA amplification quality was
184 assessed by using Rt-PCR to amplify a fragment the blue crab small subunit ribosomal RNA
185 transcript using primers CrabSSUfor 5'-CATGTCTAAGTACAAGCCGAA-3' and
186 CrabSSURev 5'-GGGTAATTTGCGTGCCT-3', which produce a 405 nucleotide amplicon
187 (GenBank JAHFWG010003967; Bachvaroff *et al.,* 2021). RNA samples that failed to amplify
188 with this rRNA primer set were deemed low quality and were not included in the CsRV1
189 analyses.
190
191 *PCR detection of* Hematodinium *sp.*

192 The presence and amount of *Hematodinium* sp. DNA was assessed by the method of Nagle *et al.,*
193 (2009), which amplifies a 121-nucleotide region of the small subunit ribosomal RNA gene.
194 Analyses were performed in duplicate on a QuantStudio 3 Real Time Thermocycler using
195 PerfeCTa SYBR Green FastMix (Quantabio) with the following thermocycling conditions; 50ºC
196 for 2 min, 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min. PCR
197 reactions included negative (no template) and positive (cloned target gene) controls. The number
198 of *Hematodinium* sp. target copies was quantified by comparison to a standard curve (10 to 10^6
199 copies) of cloned *Hematodinium* sp. DNA (pES103, see Hanif *et al.,* 2013) that was run on each
200 plate. Based on the fraction of total DNA used in the qPCR reaction and the weight of the tissue
201 from which it was extracted, the *Hematodinium* sp. infection intensity was expressed as the
202 number of gene copies per mg of muscle.
203
204 *Sequencing and analysis of* Hematodinium *ITS1 locus*

205 A 750 bp region of the 18S rRNA gene, from the SSU to the first internal transcribed spacer
206 (ITS1) region, was amplified and sequenced for phylogenetic analysis. Amplification of this
207 region was performed using the forward primer PlaceF and reverse ITS1 primers (5'-
208 GACCCAGCCTTCACGATAAA-3'). The PCR was conducted on a BioRad C1000
209 thermocycler with AccuStart II PCR ToughMix under the following conditions: initial
210 denaturation at 94°C for 5 minutes, followed by 31 cycles of 94°C for 25 seconds, 58°C for 25
211 seconds, and 72°C for 1 minute. Amplified products were visualized by 2% (w/v) agarose gel
212 electrophoresis, stained with SYBR Safe DNA Gel Stain, and examined under ultraviolet light.
213 Aliquots of each reaction were sequenced using an ABI 3130 genetic analyzer. Quality trimming
214 and removal of barcode sequences from the raw reads were carried out using CLC Genomics
215 Workbench 9.5.2 (Qiagen, Hilden, Germany). The assemblies were manually inspected, and
216 consensus sequences were exported for further analysis. Sequences were aligned using CLC
217 Workbench, using default parameters, and used to create a neighbor Joining tree with boostrap
218 values determined using 100 iterations.
219
220 *Histological analysis*

221 At the time of dissection for DNA extractions, pieces of Croatian crab hepatopancreas were
222 excised from freshly caught crabs and fixed for 24 hours in modified Davidson's fixative,
223 prepared with filtered seawater to maintain isotonicity. Crabs from Greece and Portugal were not
224 assessed by histology. After fixation, the samples were transferred to 70% ethanol and stored at
225 4°C. Selected samples of fixed tissue were dehydrated in graded ethanol series, cleared in xylene
226 and paraffin-embedded. Tissue sections were cut at 3-5 µm thickness, stained with Mayer's
227 hematoxylin and eosin (MHE) and coverslipped in NeoMount. Stained sections were inspected
228 under a Leica DM3000 LED microscope. Images were captured with a Leica DMC 4500 camera
229 and assembled and annotated in PhotoShop CS5 (Adobe Systems).
230
231 **Results**
232
233 There was no evidence found for CsRV1 virus RNA in the samples of *C. sapidus* collected in
234 this study, from the Aegean Sea, Adriatic Sea or Guadiana estuary. This represented a total of
235 166 individual samples from 6 locations. CsRV1 PCR assays were conducted on all 30 RNA
236 samples, but of these, several RNA samples, especially from Portugal, did not produce RT-PCR
237 amplicons with the positive control SSU primers, and so were excluded from analysis.

238 DNA of the parasite *Hematodinium* sp. was detected in crabs from the Adriatic Sea and
239 Guadiana estuary, but not from the Aegean Sea. The proportion of infected crabs and the mean
240 intensity (copies of *Hematodinium* sp. target DNA per mg muscle tissue) are listed in Table 2.
241 Individual crabs had *Hematodinium* sp. DNA intensity ranging from 30 copies to over 1 million
242 copies of target DNA per mg of tissue (supplemental material available).

243

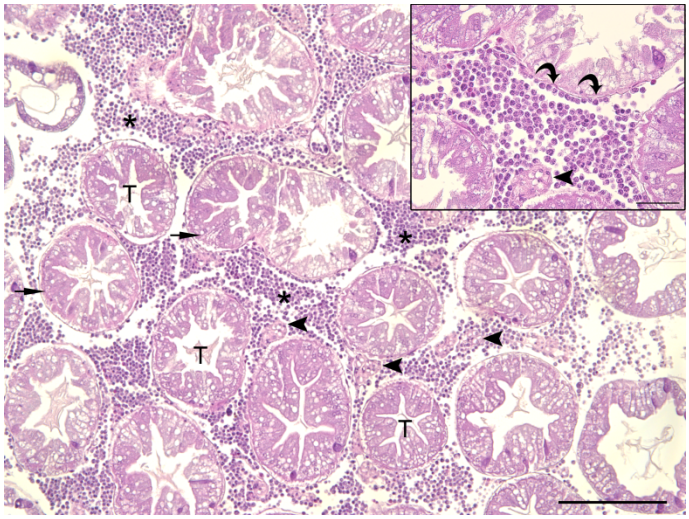
244 **Table 2.** Prevalence and mean copy number of *Hematodinium perezi* and CsRV1 as determined
245 by qPCR. Copy number of *Hematodinium* target genes per mg tissue.

Location	*Hematodinium perezi*			CsRV1	
	Sample size	Prevalence (%)	Mean intensity (copies mg^{-1})	Sample size*	Prevalence (%)
Parila lagoon, South Adriatic Sea	30	23	1.28E+03	30	0
Ston saltworks, South Adriatic Sea	30	40	1.54E+05	30	0
Mlini, South Adriatic Sea	30	67	3.03E+05	30	0
Ada Bojana, South Adriatic Sea	30	10	3.72E+03	30	0
North Euboean Gulf, Aegean Sea	30	0	n.a.	26	0
Foz de Odeleite, Guadiana estuary	30	87	3.25E+04	20	0

246

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

251



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253

Figure 2. Micrograph (100X magnification), with H&E staining, of *Hematodinium* sp. PCR-positive *C. sapidus* hepatopancreas. The intertubular space contains numerous parasite cells (asterisk). Cellular aggregates, possibly fixed phagocytes, are seen (arrowheads). Some tubules (T) lack lipid inclusions (arrows). **Inset**: 400X magnification showing trophonts and fixed phagocytes (arrowhead) adjacent to tubular epithelium (curved arrows). Scale bar: 200 µm, inset: 50 µm.

Hematodinium sp. infections (PCR signal) in three locations were intense enough to enable amplification of a 650 nt region of the rRNA gene. Each of these sequences is deposited in GenBank, under the accession numbers PP854984 (Portugal), PP854699 (Montenegro), PP854700 (Zupa). A Maximum Likelihood phylogenetic analysis of aligned ITS1 regions of these sequences with corresponding regions of 256 nucleotides of the *Hematodinium* ITS1 from other regions of the eastern Atlantic, North America, and Asia shows that the two *Hematodinium* sp. sequences from the Adriatic are identical, and slightly less similar to the sequence from Portugal (98% identity). All of them are more similar to *Hematodinium* sp. 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.

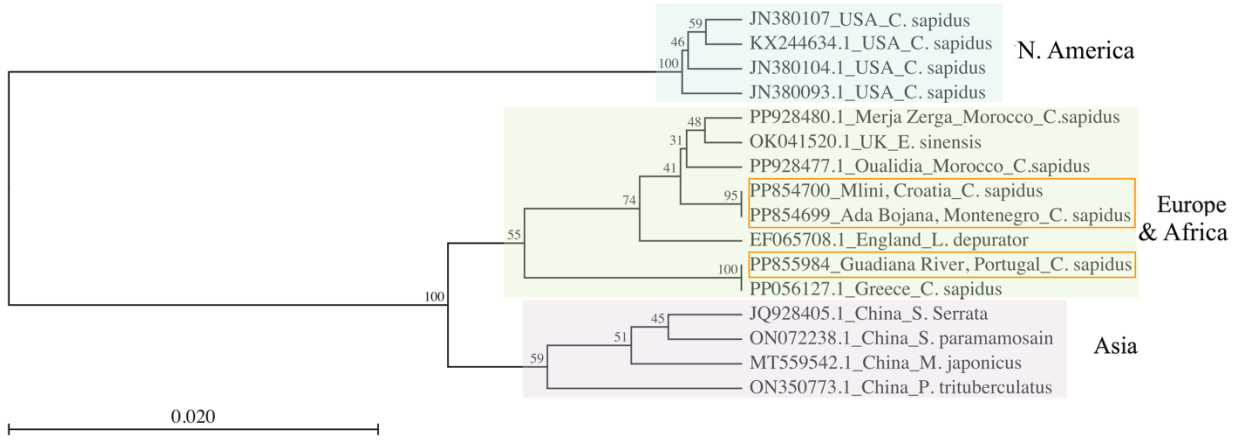

Figure 3. Neighbor joining tree from an alignment (256 nt) of *Hematodinium* sp. ITS1 sequences from this study (within boxes) as compared to *Hematodinium* sp. sequences in hosts from the Atlantic coast of Europe and North Africa, Mediterranean, Asia and North America. Numbers at branches are bootstrap values for 100 replicates.

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. Often, invasive species exhibit a surge in abundance, followed by a

286 decline and leveling of abundance in a new region after some years (Simberloff and Gibbons,
287 2004). In some cases this could be a consequence of the introduced species disrupting its own
288 prey or habitat resources. Additionally, within the "enemy escape" framework/concept, this
289 could be a result of both external predators and internal pathogens adapting to and exploiting the
290 presence of the new species. There has been some indication that blue crabs can dramatically
291 decrease the population of aquatic prey (Céspedes *et al.,* 2024; Nardelli *et al.,* 2024).
292
293 The current study gives insight to the presence of two known infectious agents of the Atlantic
294 blue crab *C. sapidus* in selected locations of western Mediterranean and Atlantic Iberian
295 estuaries. The opportunity to analyze crabs from the Aegean Sea, Adriatic, and Guadiana estuary
296 represents an east-to-west range that corresponds to the possible expansion trajectory of the
297 species over time (Mancinelli *et al.,* 2021). The two infectious agents studied represent very
298 different life history strategies for pathogens. The CsRV1 virus is described only in the *C.*
299 *sapidus* native range, and has not been yet been reported in any other species of host (Zhao *et al.,*
300 2022). In contrast, the protozoan parasite (parasitic dinoflagellate) *Hematodinium* sp., is a
301 globally-distributed crustacean host generalist (Small 2012).
302
303 There are relatively few studies related to the health status of *C. sapidus* in the Mediterranean
304 and adjacent waters. To our knowledge, ours is the first study to investigate the virus CsRV1 in
305 the Mediterranean or eastern Atlantic. Although we did not detect CsRV1 in any of the *C.*
306 *sapidus* tested, our limited study cannot be taken as evidence that the virus is entirely absent
307 from the Mediterranean or eastern Atlantic. Perhaps the most intriguing suggestion of CsRV1
308 being present in the Mediterranean is the report by Walton *et al.,* (1999) on the sequence of a
309 segment of the P virus genome in the portunid crab *Liocarcinus depurator*. The segment of P
310 virus has over 95% identity to segment 4 of CsRV1 (Flowers *et al.,* 2016). This level of identity
311 is in the range of genome difference between CsRV1 ecotypes in north America versus south
312 America (Zhao *et al.,* 2020). Future investigations of CsRV1 in the Mediterranean should
313 include assessments of portunid species such as *Liocarcinus* sp. and *Carcinus* sp.
314
315 On the North American Atlantic coast, studies have shown that CsRV1 prevalence is highly
316 variable across time and space, often as low as 0% as well as 50 to 75% prevalence (Flowers *et*
317 *al.,* 2018). The only consistent trend seen for CsRV1 in the native range is the near absence in
318 the tropics (Zhao *et al.,* 2020). Flowers *et al.,* (2018) noted that high prevalence of CsRV1was
319 coincident with higher *C. sapidus* populations in Chesapeake Bay, which is consistent with the
320 principles of host-dependent prevalence (Cortez and Duffy 2021). All of the locations surveyed
321 in the current study are temperate, but the overall blue crab population numbers are also
322 unknown in all of the areas. It will be important to continue to survey for the CsRV1 virus in
323 areas with high *C. sapidus* populations and where the species has been recorded for a long time.
324

The presence of *Hematodinium* sp. in Mediterranean *C. sapidus* was first reported by Mancinelli *et al.,* (2013) in ecosystems of the Apulia coastline. In that study, over 70% of the crabs examined (*C. sapidus, Eriphia verrucosa, Pachygrapsus marmoratus* and *Carcinus mediterraneus*) in the Torre Colimena (West Aegean sea) basin and Acquatina lagoon (West Adriatic Sea) were infected by *Hematodinium* sp. (Mancinelli *et al.,* 2013). Our study showed that just across the southern Adriatic, prevalence of *Hematodinium* sp. was moderate to high at all locations. These two coasts are highly connected by an anticlockwise gyre in the southern (Estournel *et al.,* 2021), which would have the potential to rapidly convey both crab larvae and *Hematodinium* sp. propagules between them.

In the Aegean Sea, Lattos *et al.,* (2024) recently reported 85% prevalence of *Hematodinium* sp. in *C. sapidus* that were collected during 2020 mortality events. They suggest this high prevalence is responsible for recent declines in *C. sapidus* abundance in that region of Greece. In our study of Aegean Sea crabs harvested in 2021, we detected no *Hematodinium* sp. Our collection was from a local fish market, so lacked specificity of geographic origin. The association of crab declines with *Hematodinium* sp. is still relevant to our current study; there are anecdotal reports from fishermen in Mlini, which had the 2nd highest prevalence (76%), that they didn't encounter blue crabs after the summer of 2021 for almost a year (M. Pećarević, personal communication). While there are many possible reasons for this, one is that infections by *Hematodinium* sp. reduced the abundance of blue crabs, similar to what has been reported in the *C. sapidus* native range (Lee & Frischer 2004).

On the Atlantic coast of north Morroco, Lamkhalkhal *et al.,* (2024) also recently reported *Hematodinium* sp. in *C. sapidus* at 25-60% prevalence. Moreover, they show that the sequence of the ITS1 gene of *Hematodinium* sp. from Morocco is nearly identical to that in strains from Europe and the Mediterranean. Our study is the first in our knowledge to document *Hematodinium* sp. on the Atlantic coast of Portugal. We are aware that a 2021 metagenomic investigation of *C. sapidus* stomach contents from the Rio Formosa estuary near Faro, Portugal also revealed evidence of *Hematodinium* sp. DNA. In this case, the sequence was a match to the mitochondrial COX1 gene (J. Encarnacao and P. Morais, personal communication), so no direct comparison can be made to the ITS1 sequences. Nevertheless, this suggests that the parasite has been in Atlantic coastal estuaries of Portugal since at least 2021.

In the *C. sapidus* native range, the prevalence of *Hematodinium* sp. is typically associated with high salinity (Messick and Shields 2000; Gandy *et al.,* 2015). In our study, there is no strict correspondence between salinity at the sites sampled and the prevalence of *Hematodinium* sp. In fact, the highest prevalence was found in the Guadiana River, which had locally low salinity but is less than 20 km away from the ocean. In the Eastern Adriatic Sea, salinity was above 30 psu except in Parila lagoon (18 psu), which also had the lowest *Hematodinium* sp. prevalence in that region. The only sample without detection of *Hematodinium* sp. was in the Aegean Sea, where

crabs were obtained from a fish market. Therefore precise location and salinity information was not available. This is in contrast to the recent report by Lattos *et al.,* (2024) of *Hematodinium* sp. prevalence over 80% across the Aegean Sea, which they note was coincident with a decline in the abundance of *C. sapidus* as reported by fishermen. The contrasting prevalence found in our study and that of Lattos *et al.,* is not unusual for marine disease studies, where diverse environmental factors interact with shifting population size and connectivity to produce highly variable prevalence.

The relationship of *Hematodinium* sp. ITS1 sequences in our study with corresponding regions of *Hematodinium* sp. strains across the globe is consistent with prior observations that *Hematodinium* sp. within different continents are similar to one another, and distinct from other regions, regardless of host. The high identity of ITS1 sequences between Greece and Portugal *Hematodinium* sp. strains stands out, however, and may suggest a rapid movement from one location to another. One possible mechanism for such transport of a parasite strain is ballast water. To comply with the International Convention for the Control and Management of Ships' Ballast Water and Sediments (IMO, 2004), prior to entering the Mediterranean, cargo ships exchange ballast water in the Atlantic. Therefore, it is possible that a strain of *Hematodinium* sp. or a host infected with it was transferred in ballast from the Atlantic to the Aegean Sea. Additional comparisons of *Hematodinium* sp. genetics will be needed to lend support or refute the idea that that the parasite has recently moved.

It is important to understand how endemic infectious agents find or adapt to newly arrived invasive species for at least two reasons. One is the effect that these agents may have on the health of native species; will they proliferate in a new reservoir host, or perhaps the new host will constitute a dead end that lowers transmission of the infectious agent. Evidence is that the host generalist *Hematodinium* sp. has already adapted to *C. sapidus*. A second reason to understand infectious agents is the impact on the invasive species over time. In many regions of Europe, there is still a policy to reduce the number of *C. sapidus* in the invaded range. As income from *C. sapidus* 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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