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Abstract

The fan mussel Pinna nobilis (Linnaeus 1758) is an endemic bivalve of the Mediterranean basin, protected by international legislation as an endangered species. In the early summer of 2018, a mass mortality event (MME) of P. nobilis was recorded in the Gulf of Taranto (Southern Italy, Ionian Sea). Moribund specimens of P. nobilis were collected by scuba divers and processed by bacteriological, parasitological, histopathological and molecular analyses to investigate the causes of this MME. Different developmental stages (i.e., plasmodia, spores and sporocysts) of a presumptive haplosporidian parasite were observed during the histological analysis in the epithelium and in the lumen of the digestive tubules, where mature spores occurred either free or in sporocysts. The spores presented an operculum and an ovoid shape measuring 4.4 µm (±0.232) in length and 3.6 µm (± 0.233) in width. BLAST analysis of an 18SrRNA sequence revealed a high nucleotide similarity (99%) with the reference sequence of Haplosporidium pinnae available in GenBank database. Accordingly, at the phylogenetic analysis, 18SrRNA sequence was clustered as a paraphyletic clade with the reference sequence of H. pinnae, excluding other haplosporidians (i.e., Bonamia and Minchinia genera). Based on data reported, H. pinnae was the causative agent of MME in the populations of P. nobilis sampled in the Ionian Sea, where the conservation of this endangered species is heavily threatened by such a protozoan infection. Further investigations should regard the life cycle of H. pinnae, in order to reduce the pathogen spreading and to mitigate the burden of the disease where P. nobilis is facing the risk of extinction.

Keywords	Haplosporidium pinnae; Pinna nobilis; mass mortality; histology; molecular analyses; 18SrRNA.
Taxonomy	Parasitology, Molecular Biology, Biological Sciences

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Dear Dr. Solter,

Please find enclosed the revised version of manuscript JIP_2018_308 entitled "*Haplosporidium pinnae* associated with mass mortality in endangered *Pinna nobilis* (Linnaeus1758) fan mussels" by Rossella Panarese, Perla Tedesco, Giovanni Chimienti, Maria Stefania Latrofa, Francesco Quaglio, Giuseppe Passantino, Canio Buonavoglia, Andrea Gustinelli, Angelo Tursi and myself, which was considered acceptable for publication in Journal of Invertebrate Pathology pending minor revisions.

All the suggestions and comments raised by the Editor-in-Chief have been carefully considered while revising the attached version of the manuscript. You will find the documents named as "TextR3_edited" and "Highlights_edited" containing the revised manuscript and highlights, respectively.

I thank you for your editorial work.

Yours faithfully,
Domenico Otranto

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The revised version of manuscript JIP_2018_308 entitled "*Haplosporidium pinnae* associated with mass mortality in endangered *Pinna nobilis* (Linnaeus1758) fan mussels" by Rossella Panarese, Perla Tedesco, Giovanni Chimienti, Maria Stefania Latrofa, Francesco Quaglio, Giuseppe Passantino, Canio Buonavoglia, Andrea Gustinelli, Angelo Tursi has been downloaded.

All the suggestions and comments raised by the Editor-in-Chief have been carefully considered while revising the attached version of the manuscript. You will find the documents named as "TextR3_edited" and "Highlights_edited" containing the revised manuscript and highlights, respectively.

I thank you for your editorial work.

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Highlights

- A mass mortality event of *Pinna nobilis* was recorded in Southern Italy (Ionian Sea)
- Developmental stages of *Haplosporidium* spp. were present in the digestive glands
- 18SrRNA sequence ~~was~~ identified the pathogen as *Haplosporidium pinnae* ~~by molecular analyses~~
-

Haplosporidium pinnae associated with mass mortality in endangered *Pinna nobilis* (Linnaeus 1758) fan mussels

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Abstract

The fan mussel, *Pinna nobilis* (Linnaeus 1758), is an endemic bivalve of the Mediterranean basin, protected by international legislation as an endangered species. In the early summer of 2018, a mass mortality event (MME) of *P. nobilis* was recorded in the Gulf of Taranto (Southern Italy, Ionian

Sea). Moribund specimens of *P. nobilis* were collected by scuba divers and processed by bacteriological, parasitological, histopathological and molecular analyses to investigate the causes of this MME. Different developmental stages (i.e., plasmodia, spores and sporocysts) of a presumptive haplosporidian parasite were observed during the histological analysis in the epithelium and in the lumen of the digestive tubules, where mature spores occurred either free or in sporocysts. The spores presented an operculum and an ovoid shape measuring 4.4 µm (±0.232) in length and 3.6 µm (± 0.233) in width. BLAST analysis of an 18SrRNA sequence revealed a high nucleotide similarity (99%) with the reference sequence of *Haplosporidium pinnae* available in GenBank database. Phylogenetic analysis using the 18SrRNA sequence clustered the pathogen in a paraphyletic clade with the reference sequence of *H. pinnae,* excluding other haplosporidians (i.e., *Bonamia* and *Minchinia* genera). Based on data reported, *H. pinnae* was the causative agent of MME in the populations of *P. nobilis* sampled in the Ionian Sea, where the conservation of this endangered species is heavily threatened by such a protozoan infection. Further investigations should contribute to knowledge about the life cycle of *H. pinnae* in order to reduce spread of the pathogen and to mitigate the burden of the disease where *P. nobilis* is facing the risk of extinction.

Keywords: *Haplosporidium pinnae*, *Pinna nobilis*, mass mortality, histology, molecular analyses, 18SrRNA.

1. Introduction

The fan mussel *Pinna nobilis* (Linnaeus 1758) is the largest saltwater bivalve in the Mediterranean Sea, where it is endemic and protected as endangered species (i.e., Annex II of the Barcelona Convention, SPA/BD Protocol 1995, and Annex IV of the EU Habitats Directive 2007) (Darriba, 2017; Vázquez et al., 2017). With a maximum reported age of 27 years, this filter-feeding mollusc usually settles on soft substrates (occasionally on hard ones) from 0.5 to 60 m depth, using byssal threads to anchor. It may reach up to 120 cm in height (Schultz and Huber, 2013; Basso et al.,

2015). Populations of *P. nobilis* are distributed in many areas along the Italian coasts and are considered sensitive to anthropogenic and environmental threats, such as high levels of urbanization (Ladisa et al., 2010), urban discharges and freshwater agricultural inputs (Calace et al., 2008; Bellucci et al., 2016). One of the largest populations of *P. nobilis* known so far in Italy was present in the Gulf of Taranto, in the Mar Piccolo basin of the Ionian Sea (Centoducati et al., 2007; Tursi et al., 2018), despite this area being subject to severe anthropogenic impacts (Bracchi et al., 2016). In this area, a high survival rate and a low mortality (i.e., from a minimum of 0.1% up to 8.8%) have been recently observed in optimal conditions (Tursi et al., 2018). Protozoan infection by haplosporidan parasites has been recently implicated in a mass mortality event (MME) of *P. nobilis* occurring in the Spanish coast of the Western Mediterranean Sea (Darriba, 2017; Vázquez et al., 2017), with *Haplosporidium pinnae* nov. sp. identified as the causative agent of the still on-going MME in this area (Catanese et al., 2018). Haplosporidians are highly pathogenic for marine and freshwater invertebrates with high mortality rates caused, for example in different oyster species, by *Haplosporidium nelsoni*, *Bonamia ostreae* and *Bonamia exitiosa* (Engelsma et al., 2014). In particular, the sporulation of *H. pinnae* occurs in the digestive gland tubules, impairing food absorption and causing severe dysfunction and death of the host (Darriba, 2017; Vázquez et al., 2017; Catanese et al., 2018). In the early summer of 2018, a MME was recorded in *P. nobilis* populations in the Ionian Sea, with up to 100% mortality in 3 months. We investigated the causes of this sudden MME using bacteriological, parasitological, histopathological and molecular tools.

2. Materials and methods

2.1. Sampling collection and processing

Samples were collected in the Mar Piccolo basin (T: ±25°C; salinity: ±37ppt), a coastal marine ecosystem with lagoon features (Gulf of Taranto, Southern Italy, Central Mediterranean Sea; Fig. 1). The seafloor is dominated by soft sediment, from mud to mixed sand, locally colonised by benthic communities consisting mainly of filter- and suspension-feeders and seaweeds (Matarrese et

al., 2004; Mastrototaro et al., 2008). These communities coexist with a suite of anthropogenic impacts, including high level of urbanization, heavy industries, intense maritime traffic, as well as mussel and fish farms (Bracchi et al., 2016). According to recent monitoring programs carried out in this area (Tursi et al., 2018), two sampling sites with the highest density of *P. nobilis* were selected (Fig. 1). Specimens of *P. nobilis* were collected in July 2018, from 3 to 8 m depth, by scuba divers in the two sampling sites. Specimens of *P. nobilis* presented generic symptoms of a disease condition (i.e., slow response to mechanical stimuli, opened valves and high presence of mucous secretions). The sampling of 10 moribund specimens, collected from a subpopulation of 7,107 *P. nobilis*, was carried out under the permission of the Italian Ministry for Environment, Land and Sea Protection, based on the agreement between the Special Commissioner for Urgent Intervention for Remediation, Environmental Enhancement and Upgrading of Taranto and the University of Bari "Aldo Moro" (no. 1890, 16/06/2016). Total length and weight of specimens were recorded and a macroscopic examination was conducted to evaluate the external aspect of the specimens, their nutritional state and internal organs, the gross alterations of valves as well as the presence of macroscopic lesions. Samples of hemolymph (2 ml from each specimen) were taken from the anterior adductor muscle and from the heart with a sterile syringe and plated on different culture media (TSA+2%NaCl, Blood Agar, TCBS e FMM). Fresh smears of hemolymph were stained with May-Grunwald Giemsa and Hemacolor®. The remaining hemolymph (frozen at -20°C) and the digestive glands (fixed in ethanol 70%) were used for molecular analyses.

2.2. Histopathological studies

Portions of digestive gland, mantle, gills, gonads and muscle of fan mussels were preserved in buffered formalin 10% for histological analyses. Samples were dehydrated in an increasing ethanol gradient, embedded in paraffin wax, sectioned at 3-4 μm with a rotary microtome, and stained with Hematoxylin and Eosin, following standard methods (Culling et al., 1985).

105 **2.3. Molecular analyses**

106 Genomic DNA was extracted from digestive glands that had been chopped by sterile scissors and
107 washed twice (15 min) with sterile distilled water (800µl), and from hemolymph samples (100 µl),
108 using DNEasy Blood & Tissue kit and QIAampDNA Minikit (Qiagen, Germany), respectively.
109 Pathogen DNA was screened by standard PCR (PCR) using generic primers targeting the 18SrRNA
110 region for *Haplosporidium* spp. (~350 bp) and *Bonamia* spp. (~573 bp) and a specific pair of
111 primers for *H. nelsoni* (~300 bp), as previously described (Cochennec et al., 2000; Renault et al.,
112 2000). PCR products were examined on 2% agarose gels stained with GelRed (VWR International
113 PBI, Milano, Italy) and visualised on a GelLogic 100 gel documentation system (Kodak, New
114 York, USA). The amplicons were purified and sequenced in both directions with the same primers
115 used for PCR, employing the Big Dye Terminator v.3.1 chemistry in a 3130 genetic analyser
116 (Applied Biosystems, California, USA). Sequences were aligned using the ClustalW program
117 (Larkin et al., 2007) and compared with those available in GenBank by Basic Local Alignment
118 Search Tool (BLAST-http://blast.ncbi.nlm.nih.gov/Blast.cgi). The evolutionary history was inferred
119 using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). A
120 discrete Gamma distribution was used to model evolutionary rate differences among sites.
121 Evolutionary analyses were tested on 4,000 Bootstrap replications, using MEGA6 software
122 (Tamura et al., 2013). The phylogenetic analysis was run by using 18SrRNA sequences of species
123 belonging to Haplosporidia order available in GenBank. *Mikrocytos mackini* (AN:HM563060) and
124 *Marteilia cochillia* (AN: KF278722) were used as outgroups.

125

126 **3. Results**

127 The average length of *P. nobilis* processed was 30 cm (SD: ±1.58) and the average weight was 212
128 g (±5.48). Several epibionts were attached to the valves, such as the polychaete *Eulalia ornata*
129 (Saint-Joseph, 1888), unidentified bryozoans, the common limpet *Patella vulgata* (Linnaeus, 1758),
130 the crab *Tumidotheres maculatus* (Say, 1818) and the starfish *Asterina pancerii* (Gasco, 1876). On

131 macroscopic observation the specimens of *P. nobilis* appeared emaciated, but no other gross
132 alterations were observed. A large watery vesicle was found in the visceral mass of one specimen
133 from Site 2. In all the specimens, the gills were collapsed and appeared pale brownish in color,
134 while the digestive glands were darker and softer than expected for healthy specimens. Fresh
135 preparations of digestive gland showed the presence of mature spores occurring either free or in
136 sporocysts (Fig. 2). Spores were ovoid with visible operculum and measured 4.4 µm (±0.232) in
137 length x 3.6 µm (± 0.233) (mean values calculated on n = 50 individuals). Culturing on TCBS,
138 MacConkey agar and *Aeromonas* agar base to detect *Vibrio* spp., *Escherichia coli* and *Aeromonas*
139 spp., respectively, was negative. Histopathological analyses showed diffused degenerative lesions in
140 the presence of different developmental stages of a haplosporidan parasite in the epithelium and in
141 the lumen of digestive tubules (Fig. 3A). Some larger sporocysts were protruding in the lumen of
142 the tubules causing atrophy in the surrounding cells as a consequence of compression. In some
143 tubules the epithelium was completely detached leaving only the basal lamina (Fig. 3B). Detached
144 cells appeared in coagulative necrosis. Spherical binucleate stages were also observed within the
145 epithelium of digestive tubules (Fig. 3C). In the interstitial space between tubules, an intense
146 inflammatory response characterized by the presence of hemocyte infiltration was observed.
147 Parasitic stages were present in the lumen of the intestine (Fig. 3D, arrow) associated with necrotic
148 sloughing of digestive cells with loss of the cilia, although they were absent in the intestinal
149 mucosa. The same response was observed in the mantle tissue, along with the presence of
150 uninucleate developmental stages inside host hemocytes (Fig. 3E). Numerous brown cells were
151 observed in the connective tissue (Fig. 3F) around the digestive gland and in the lumen of digestive
152 tubules, as well as in the mantle, gills and gonads. No other parasites were observed. All digestive
153 glands and hemolymph samples scored positive in PCR using degenerate primers for
154 *Haplosporidium* spp., resulting in amplicons of the expected size (~350bp). No amplification was
155 obtained using primers for *H. nelsoni* and *Bonamia* spp. The BLAST analysis of the 18SrRNA
156 sequences of all the specimens tested revealed highest nucleotide identity, 99%, with the reference

157 sequence of *H. pinnae* in the GenBank database (AN: LC338065). The molecular identity was
158 confirmed by clustering of the 18SrRNA sequence obtained with that of *H. pinnae* reference strain,
159 supported by high bootstrap value (99%, Fig. 4). The *H. pinnae* clade clustered in a paraphyletic
160 group, appearing distinct from the *Bonamia/Minchinia* clade and from the clade containing most of
161 the other *Haplosporidium* species (Fig. 4). The sequence was deposited in GenBank under
162 accession number MK163629.
163
164 **4. Discussion**
165 We used molecular analysis to identify *Haplosporidium pinnae* as the agent of the MME in *P.*
166 *nobilis* populations in the investigated area of Ionian Sea, and observed haplosporidia in
167 pathological lesions in the host. The presence of *H. pinnae* in examined *P. nobilis* with no other
168 pathogens present, the observed lesions in the digestive gland and the absence of inflammatory
169 nodular lesions typical of micobacteria indicate that this MME is due to this protozoan infection as
170 observed in the Western Coast of Mediterranean Sea in Spain (Darriba, 2017; Vázquez et al., 2017;
171 Catanese et al., 2018; Carella et al., 2019). Histological analysis showed that the presence of *H.*
172 *pinnae* in all specimens was associated with heavy lesions of the digestive gland structure and
173 severe tubular necrosis. The spores developed in the epithelium of the digestive gland and appeared
174 to be released in the lumen of the gland's tubules, reaching the intestine of the host for elimination
175 into the environment. In addition, the presence of different stages of sporulation of the protozoa
176 (Hine and Thorne, 2002) in the digestive gland confirmed *Haplosporidium* sp. as the agent of the
177 lesions in the examined specimens of *P. nobilis* (Catanese et al., 2018). Similar pathological
178 conditions of the digestive gland have also been associated with the sporulation of *H. nelsoni* and *H.*
179 *tuxtlensis* in eastern oyster *Crassostrea virginica* (Gmelin, 1791) and the striped false limpet
180 *Siphonaria pectinata* (Linnaeus, 1758) (Couch et al., 1966; Vea and Siddall, 2011). Before the
181 MME in Spain, haplosporidan parasites were detected infecting species of bivalves, gastropods,
182 crustacean, worms, ascidians and even hyperparasite trematode larvae (Burreson and Ford, 2004;

183 Arzul and Carnegie, 2015), but never in a member of the Pinnoidea Superfamily. The spreading of
184 this parasite into non-endemic areas is still unknown, but it may be argued that the outbreak spread
185 from Spain, being transported in the summer marine currents (Fernández et al., 2005). Nonetheless,
186 it cannot be ruled out that anthropic activity, such as maritime transport, ballast waters and trade of
187 living bivalves may have enhanced dispersal of the protozoa. Dynamics of haplosporidians in their
188 hosts suggest that these parasites could be seasonal, depending on environmental parameters such as
189 temperature and salinity (Darriba, 2017).
190 First evidence of unexpected mortality of *P. nobilis* (40% of the individuals) in the study area was
191 observed during the summer 2017, followed by a low mortality period during the winter
192 (unpublished data) and by the drastic decline of the population in the following summer of 2018.
193 Based on our observations, environmental conditions such as warm temperatures may be an
194 important driver for the development of *H. pinnae*, suggesting that the impact of global warming
195 could enhance the spreading of this parasite all over the Mediterranean Sea. Control of spread is
196 difficult due to the lack of an adaptive immune system of the host and the rapid death of infected
197 individuals, resulting in up to 100% mortality in a few months. Furthermore, the administration of
198 treatment is impossible to carry out because of the potential impact on the marine ecosystem, as
199 well as the restrictions by European legislation (Guardiola et al., 2012). Therefore, resettlement of
200 *P. nobilis* populations at the end of the MME seems to be the only option available to mitigate the
201 on-going local extinction of this protected species.
202 Some aspects of the life cycle of *H. pinnae* remain unknown, including the potential of an
203 intermediate host, the role of other definitive hosts, such as *Pinna rudis* (Linnaeus, 1758) or *Atrina*
204 spp., and the persistence of infective spores in the environment. Further studies are needed to
205 improve knowledge about the life cycle of *H. pinnae* in order to mitigate the ongoing disease and
206 plan proper repopulation strategies for *P. nobilis* in areas where the MME caused the extinction of
207 the species.
208

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References

Arzul, I., Carnegie, R.B., 2015. New perspective on the haplosporidian parasites of molluscs. J. Invertebr. Pathol. 131, 32-42. https://doi.org/10.1016/j.jip.2015.07.014.

Basso, L., Vázquez-Luis, M., Gracía-March, J.R., Deudero, S., Álvarez, E., Vicente, N., Duarte, C:M., Hendriks, I.E., 2015. Chapter three – the Pen Shell, *Pinna nobilis*: a review of population status and recommended research priorities in the Mediterranean Sea. Adv. Mar. Biol. 71, 109–160. https://dx.doi.org/10.1016/bs.amb.2015.06.002.

Bellucci, L.G., Cassin, D., Giuliani, S., Botter, M., Zonta, R., 2016. Sediment pollution and dynamic in the Mar Piccolo of Taranto (Southern Italy): insights from bottom sediment traps and surficial sediments. Mar Pollut Bull 23:12554–12565. https://doi.org/10.1007/s11356-016-6738-6.

Bracchi, V.A., Marchese, F., Savini, A., Chimienti, G., Mastrototaro, F., Tessarolo, C., Cardone, F., Tursi, A., Corselli, C., 2016. Seafloor integrity of the Mar Piccolo Basin (Southern Italy): quantifying anthropogenic impact. J Maps. https ://doi.org/10.1080/17445 647.2016.11529 20.

Burreson, E.M., Ford, S.E., 2004. A review of recent information on the Haplosporidia, with special reference to *Haplosporidium nelsoni* (MSX disease). Aquat. Living Resour.17, 499–517. https://doi.org/10.1051/alr:2004056

Calace, N., Cardellicchio, N., Ciardullo, S., Petronio, B.M., Pietrantonio, M., Pietroletti, M., 2008. Metal distribution in marine sediments of the Mar Piccolo in Taranto (Ionic Sea, Southern Italy). Toxicol Environ Chem 90: 549–564. https://doi.org/10.1080/02772240701570420.

Carella, F., Aceto, S., Pollaro, F., Miccio, A., Iaria, C., Carrasco, N., Prado, P., De Vico, G., 2019. A mycobacterial disease is associated with the silent mass mortality of the pen shell *Pinna nobilis* along the Tyrrhenian coastline of Italy. Scientific reports 9: 2725. https://doi.org/10.1038/s41598-018-37217-y.

Catanese, G., Grau, A., Valencia, J.M., Garcia-March, J.R., Alvarez, E., Vázquez-Luis, M., Deudero, S., Darriba, S., Carballal, M.J., Villalba, A., 2018.*Haplosporidium pinnae* sp. nov., a haplosporidan parasite associated with mass mortalities of the fan mussel, *Pinna nobilis*, in the Western Mediterranean Sea. J. Inv. Pathol. 157: 9–24. https://doi.org/10.1016/j.jip.2018.07.006.

Centoducati, G., Tarsitano, E., Bottalico, A., Marvulli, M., Lai, O.R., Crescenzo, G., 2007. Monitoring of the endangered *Pinna nobilis* Linné, 1758 in the Mar Grande of Taranto (Ionian Sea, Italy). Environ. Monit. Assess. 131, 339. https://doi.org/10.1007/s10661-006-9479-z.

Cochennec, N., Le Roux, F., Berthe, F., Gerard, A., 2000. Detection of *Bonamia ostreae* based on small subunit ribosomal probe. J. Invertebr. Pathol. 76, 26–32. http://dx.doi.org/10.1006/jipa.2000.4939.

Couch, J.A., Farley, C.A., Rosenfield, A., 1966. Sporulation of *Minchinia nelsoni* (Haplosporida, Haplospordiidae) in *Crassostrea virginica* (Gmelin). Science 153, 1529-1531. https://doi.org/10.1126/science.153.3743.1529.

261 Culling, C.F.A., Allison, R.T., Barr, W.T., 1985. Cellular pathology techniques, fourth ed.
262 Butterworths, London.
263
264 Darriba, S., 2017. First haplosporidan parasite reported infecting a member of the Superfamily
265 Pinnoidea (*Pinna noblis*) during a mortality event in Alicante (Spain, Western Mediterranean). J.
266 Inv. Pathol. 148, 14-19. https://doi.org/10.1016/j.jip.2017.05.006.
267
268 Engelsma, M.Y., Culloty, S.C., Lynch, S.A., Arzul, I., Carnegie, R.B., 2014. *Bonamia* parasites: a
269 rapidly changing perspective on a genus of important mollusc pathogens. Dis. Aquat. Org. 110, 5–
270 23. https://doi.org/10.3354/dao02741.
271
272 Fernández, V., Dietrich, D.E., Haney, R.L., Tintoré, J., 2005. Mesoscale, seasonal and interannual
273 variability in the Mediterranean Sea using a numerical ocean model. *Progress in Oceanography*, 66,
274 321-340. https://doi.org/10.1016/j.pocean.2004.07.010.
275
276 Guardiola, F.A., Cuesta, A., Meseguer, J., Esteban, A.M., 2012. Risks of Using Antifouling
277 Biocides in Aquaculture. Int J Mol Sci. 13, 1541-1560. https://doi: 10.3390/ijms13021541.
278
279 Hine, P.M., Thorne, T., 2002. *Haplosporidium* sp. (Alveolata: Haplosporidia) associated with
280 mortalities among rock oysters *Saccostrea cucculata* in north Western Australia. Dis. Aquat. Org.
281 Vol. 51, 123-133. https://doi.org/10.3354/dao051123.
282
283 Kimura, M., 1980. A Simple Method for Estimating Evolutionary Rates of Base Substitutions
284 Through Comparative Studies of Nucleotide Sequence. J. Mol. Evol. 16, 111-20.
285 https://doi.org/10.1007/BF01731581.
286

287 Ladisa, G., Todorovic, M., Trisorio Liuzzi, G., 2010. Assessment of desertification in semi-arid
288 Mediterranean environments: the case study of Apulia region (Southern Italy), in: Zdruli, P.,
289 Pagliai, M., Kapur, S., Faz Cano, A. (Eds.), Land degradation and desertification: assessment,
290 mitigation and remediation. Springer, New York, pp. 493–516.
291
292 Lande, R., 1993. Risks of population extinction from demographic and environmental stochasticity
293 and random catastrophes. Am. Nat. 142, 911–927. https://doi.org/10.1086/285580.
294
295 Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H.,
296 Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G.,
297 2007. Clustal W and Clustal X version 2.0. Bioinformatics, 23, 2947-2948.
298 https://doi.org/10.1093/bioinformatics/btm404.
299
300 Mastrototaro, F., Giove, A., D'Onghia, G., Tursi, A., Matarrese, A., Gadaleta, M. V., 2008. Benthic
301 diversity of the soft bottoms in a semi-enclosed basin of the Mediterranean Sea. Journal of the
302 Marine Biological Association, UK, 88, 247–252. https://doi.org/10.1017/S0025315408000726.
303
304 Matarrese, A., Mastrototaro, F., D'Onghia, G., Maiorano, P., Tursi, A., 2004. Mapping of the
305 benthic communities in the Taranto seas using side-scan Sonar and an underwater video camera.
306 Chemistry and Ecology, 20, 377–386. https://doi.org/10.1080/02757540410001727981.
307
308 Renault, T., Stokes, N.A., Chollet, B., Cochennec, N., Berthe, F., Burreson, E.M., 2000.
309 Haplosporidiosis in the Pacific oyster, *Crassostrea gigas*, from the French Atlantic coast. Dis.
310 Aquat. Org. 42, 207–214. https://doi.org/10.3354/dao042207.
311

312 Schultz, P.W., Huber, M., 2013. Revision of the worldwide Recent Pinnidae and some remarks on
313 fossil European Pinnidae. *Acta Conch.* 13, 1–164.
314
315 Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular
316 Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution30: 2725-2729.
317 https://doi.org/10.1093/molbev/mst197.
318
319 Tursi, A., Corbelli, V., Cipriano, G., Capasso, G., Velardo, R., Chimienti, G., 2018. Mega-litter and
320 remediation: the case of Mar Piccolo of Taranto (Ionian Sea). Rend. Lincei Sci. Fis. Nat. pp 1-8.
321 https://doi.org/10.1007/s12210-018-0738-z.
322
323 Vázquez-Luis, M., Álvarez, E., Barrajón, A., García-March, J.R., Grau, A., Hendriks, I.E., Jimenez,
324 S., Kersting, D., Moreno, D., Perez, M., Ruiz, J.M., Sánchez, J., Villalba, A., Deudero, S., 2017.
325 SOS *Pinna nobilis*: A Mass Mortality Event in Western Mediterranean Sea. Front. Mar. Sci,4, 220.
326 https://doi.org/ 10.3389/fmars.2017.00220.
327
328 Vea, I.M., Siddall, M.E., 2011. Scanning electron microscopy and molecular characterization of a
329 new *Haplosporidium* species (Haplosporidia), a parasite of the marine gastropod *Siphonaria*
330 *pectinata* (Mollusca: Gastropoda: Siphonariidae) in the Gulf of Mexico. J. Parasitol. 97, 1062–
331 1066. https://doi.org/ 10.1645/GE-2850.1.
332
333 **Figure legend**
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335 **Figure 1.** Map of the Mar Piccolo of Taranto (Ionian Sea, Southern Italy) indicating the two
336 sampling sites (dots).
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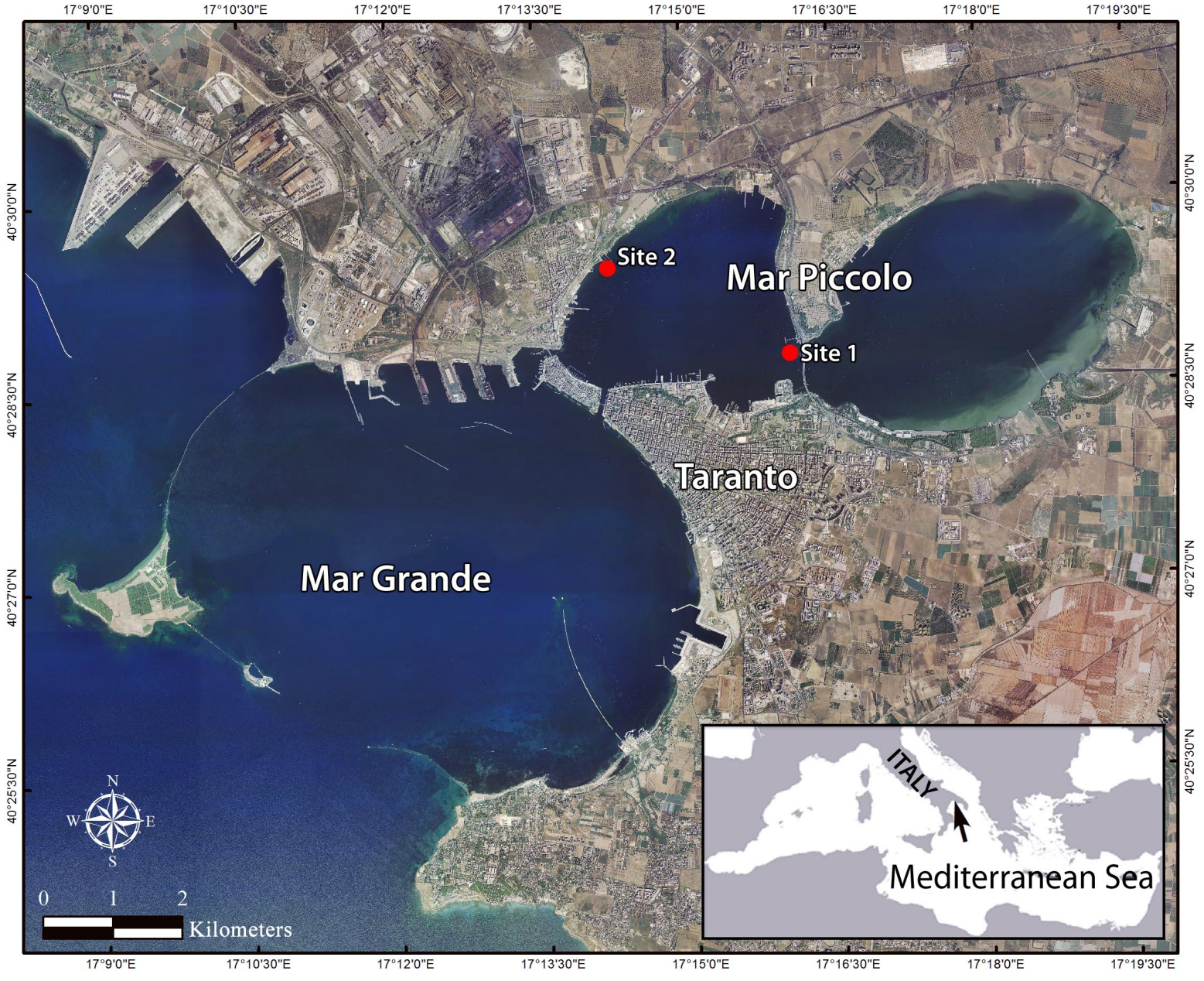
Figure 2. Spores and sporocysts (arrow) of *Haplosporidium pinnae* in fresh preparation of a digestive gland.

Figure 3. (A) Histological section of a digestive gland showing detachment of epithelial cells and *Haplosporidium pinnae* spores in the epithelium and lumen of digestive tubules (arrows); (B) histological section of digestive gland showing detachment from the basal lamina; (C) spherical binucleate stages of *H. pinnae* in the epithelial cells of digestive tubules (arrows); (D) histological section of intestine showing the presence of parasitic stages in the intestinal lumen (arrow) and necrotic cells; (E) developmental stages of *H. pinnae* (arrows) in the cytoplasm of haemocytes in the mantle; (F) numerous brown cells (arrows) in the connective tissue around the digestive gland and in the lumen of digestive tubules.

Figure 4. Maximum likelihood tree based on 18SrRNA sequences of *Haplosporidium pinnae* generated with those of other haplosporidans parasite available from GenBank. Bootstrap values are based on 4000 replicates and only bootstraps > 50% are indicated. Accession number, host and country of haplosporidians, *Mikrocytos mackini* and *Marteilia cochillia* 18SrRNA sequences used as outgroups are reported.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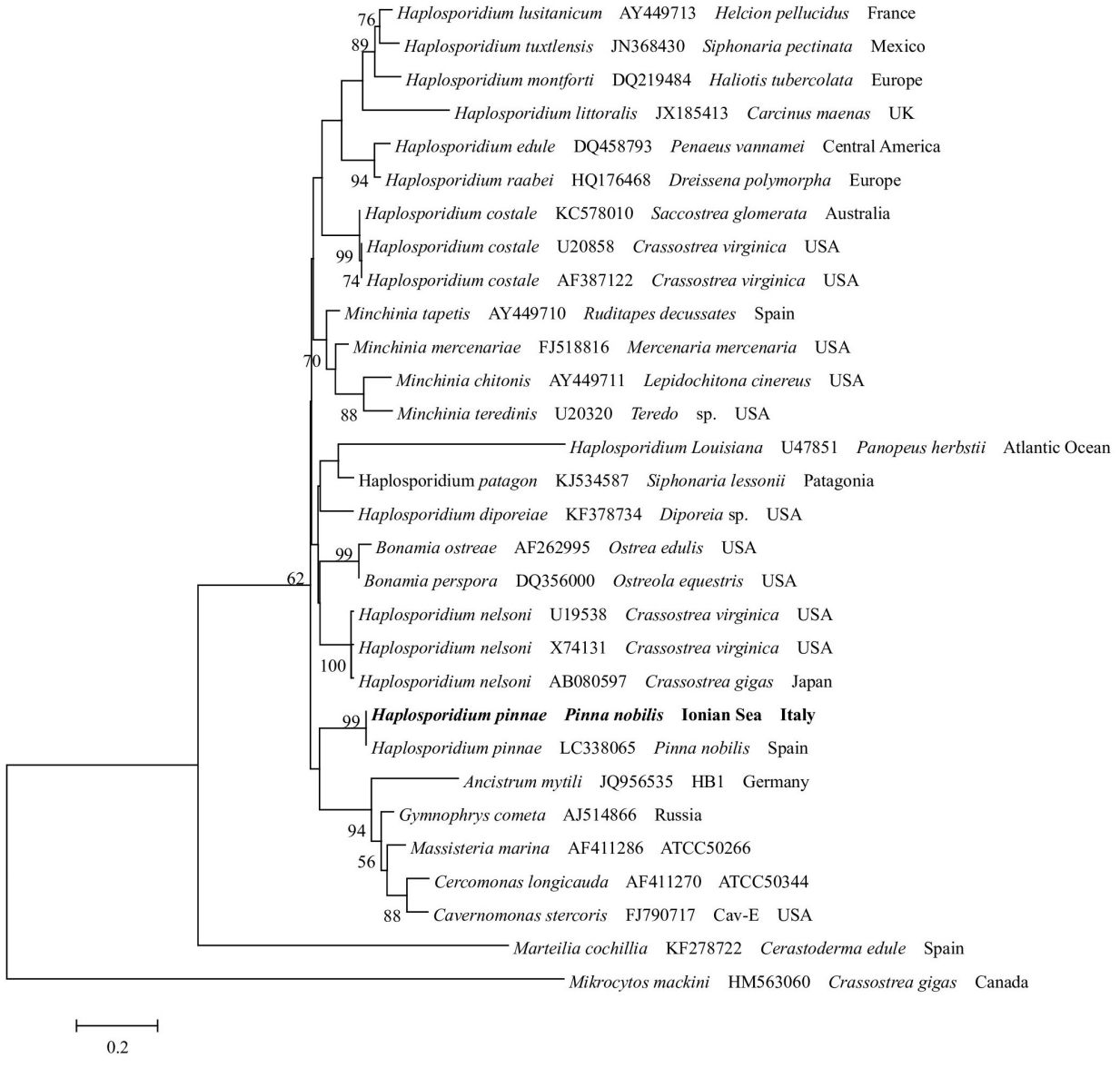
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Haplosporidium lusitanicum AY449713 Helcion pellucidus France
Haplosporidium tuxtlensis JN368430 Siphonaria pectinata Mexico
Haplosporidium montforti DQ219484 Haliotis tubercolata Europe
Haplosporidium littoralis JX185413 Carcinus maenas UK
Haplosporidium edule DQ458793 Penaeus vannamei Central America
Haplosporidium raabei HQ176468 Dreissena polymorpha Europe
Haplosporidium costale KC578010 Saccostrea glomerata Australia
Haplosporidium costale U20858 Crassostrea virginica USA
Haplosporidium costale AF387122 Crassostrea virginica USA
Minchinia tapetis AY449710 Ruditapes decussates Spain
Minchinia mercenariae FJ518816 Mercenaria mercenaria USA
Minchinia chitonis AY449711 Lepidochitona cinereus USA
Minchinia teredinis U20320 Teredo sp. USA
Haplosporidium Louisiana U47851 Panopeus herbstii Atlantic Ocean
Haplosporidium patagon KJ534587 Siphonaria lessonii Patagonia
Haplosporidium diporeiae KF378734 Diporeia sp. USA
Bonamia ostreae AF262995 Ostrea edulis USA
Bonamia perspora DQ356000 Ostreola equestris USA
Haplosporidium nelsoni U19538 Crassostrea virginica USA
Haplosporidium nelsoni X74131 Crassostrea virginica USA
Haplosporidium nelsoni AB080597 Crassostrea gigas Japan
Haplosporidium pinnae Pinna nobilis Ionian Sea Italy
Haplosporidium pinnae LC338065 Pinna nobilis Spain
Ancistrum mytili JQ956535 HB1 Germany
Gymnophrys cometa AJ514866 Russia
Massisteria marina AF411286 ATCC50266
Cercomonas longicauda AF411270 ATCC50344
Cavernomonas stercoris FJ790717 Cav-E USA
Marteilia cochillia KF278722 Cerastoderma edule Spain
Mikrocytos mackini HM563060 Crassostrea gigas Canada
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