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

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

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14

15 **Abstract**

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

34

35

36 Graphical abstract



37

38 Keywords

39 *Haplosporidium pinnae*, *Pinna nobilis*, Mass mortality, Histology, Molecular analyses, 18SrRNA

40 1. Introduction

41 The fan mussel *Pinna nobilis* (Linnaeus 1758) is the largest saltwater bivalve in the Mediterranean
42 Sea, where it is endemic and protected as endangered species (i.e., Annex II of the Barcelona
43 Convention, SPA/BD Protocol 1995, and Annex IV of the EU Habitats Directive 2007) (Darriba,
44 2017, Vázquez-Luis et al., 2017). With a maximum reported age of 27 years, this filter-feeding
45 mollusc usually settles on soft substrates (occasionally on hard ones) from 0.5 to 60 m depth, using
46 byssal threads to anchor. It may reach up to 120 cm in height (Schultz and Huber, 2013, Basso et
47 al., 2015). Populations of *P. nobilis* are distributed in many areas along the Italian coasts and are
48 considered sensitive to anthropogenic and environmental threats, such as high levels of urbanization
49 (Ladisa et al., 2010), urban discharges and freshwater agricultural inputs (Calace et al., 2008,
50 Bellucci et al., 2016). One of the largest populations of *P. nobilis* known so far in Italy was present
51 in the Gulf of Taranto, in the Mar Piccolo basin of the Ionian Sea (Centoducati et al., 2007, Tursi et
52 al., 2018), despite this area being subject to severe anthropogenic impacts (Bracchi et al., 2016). In
53 this area, a high survival rate and a low mortality (i.e., from a minimum of 0.1% up to 8.8%) have
54 been recently observed in optimal conditions (Tursi et al., 2018). Protozoan infection by
55 haplosporidan parasites has been recently implicated in a mass mortality event (MME) of *P. nobilis*
56 occurring in the Spanish coast of the Western Mediterranean Sea (Darriba, 2017, Vázquez-Luis et
57 al., 2017), with *Haplosporidium pinnae* nov. sp. identified as the causative agent of the still on-
58 going MME in this area (Catanese et al., 2018). Haplosporidians are highly pathogenic for marine
59 and freshwater invertebrates with high mortality rates caused, for example in different oyster
60 species, by *Haplosporidium nelsoni*, *Bonamia ostreae* and *Bonamia exitiosa* (Engelsma et al.,
61 2014). In particular, the sporulation of *H. pinnae* occurs in the digestive gland tubules, impairing
62 food absorption and causing severe dysfunction and death of the host (Darriba, 2017, Vázquez-Luis
63 et al., 2017, Catanese et al., 2018). In the early summer of 2018, a MME was recorded in *P. nobilis*
64 populations in the Ionian Sea, with up to 100% mortality in 3 months. We investigated the causes of
65 this sudden MME using bacteriological, parasitological, histopathological and molecular tools.

66 2. Materials and methods

67 2.1. Sampling collection and processing

68 Samples were collected in the Mar Piccolo basin (T: ± 25 °C; salinity: ± 37 ppt), a coastal marine
69 ecosystem with lagoon features (Gulf of Taranto, Southern Italy, Central Mediterranean Sea; Fig.
70 1). The seafloor is dominated by soft sediment, from mud to mixed sand, locally colonised by
71 benthic communities consisting mainly of filter- and suspension-feeders and seaweeds (Matarrese et
72 al., 2004, Mastrototaro et al., 2008). These communities coexist with a suite of anthropogenic
73 impacts, including high level of urbanization, heavy industries, intense maritime traffic, as well as
74 mussel and fish farms (Bracchi et al., 2016). According to recent monitoring programs carried out
75 in this area (Tursi et al., 2018), two sampling sites with the highest density of *P. nobilis* were
76 selected (Fig. 1). Specimens of *P. nobilis* were collected in July 2018, from 3 to 8 m depth, by
77 scuba divers in the two sampling sites. Specimens of *P. nobilis* presented generic symptoms of a
78 disease condition (i.e., slow response to mechanical stimuli, opened valves and high presence of
79 mucous secretions). The sampling of 10 moribund specimens, collected from a subpopulation of
80 7107 *P. nobilis*, was carried out under the permission of the Italian Ministry for Environment, Land
81 and Sea Protection, based on the agreement between the Special Commissioner for Urgent
82 Intervention for Remediation, Environmental Enhancement and Upgrading of Taranto and the
83 University of Bari Aldo Moro (no. 1890, 16/06/2016). Total length and weight of specimens were
84 recorded and a macroscopic examination was conducted to evaluate the external aspect of the
85 specimens, their nutritional state and internal organs, the gross alterations of valves as well as the
86 presence of macroscopic lesions. Samples of hemolymph (2 ml from each specimen) were taken
87 from the anterior adductor muscle and from the heart with a sterile syringe and plated on different
88 culture media (TSA + 2% NaCl, Blood Agar, TCBS e FMM). Fresh smears of hemolymph were
89 stained with May-Grunwald Giemsa and Hemacolor®. The remaining hemolymph (frozen at
90 -20 °C) and the digestive glands (fixed in ethanol 70%) were used for molecular analyses.

91 2.2. Histopathological studies

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

96 2.3. Molecular analyses

97 Genomic DNA was extracted from digestive glands that had been chopped by sterile scissors and
98 washed twice (15 min) with sterile distilled water (800 μ l), and from hemolymph samples (100 μ l),
99 using DNEasy Blood & Tissue kit and QIAampDNA Minikit (Qiagen, Germany), respectively.
100 Pathogen DNA was screened by standard PCR (PCR) using generic primers targeting the 18SrRNA
101 region for *Haplosporidium* spp. (~350 bp) and *Bonamia* spp. (~573 bp) and a specific pair of
102 primers for *H. nelsoni* (~300 bp), as previously described (Cochennec et al., 2000, Renault et al.,
103 2000). PCR products were examined on 2% agarose gels stained with GelRed (VWR International

104 PBI, Milano, Italy) and visualised on a GelLogic 100 gel documentation system (Kodak, New
105 York, USA). The amplicons were purified and sequenced in both directions with the same primers
106 used for PCR, employing the Big Dye Terminator v.3.1 chemistry in a 3130 genetic analyser
107 (Applied Biosystems, California, USA). Sequences were aligned using the ClustalW program
108 (Larkin et al., 2007) and compared with those available in GenBank by Basic Local Alignment
109 Search Tool (BLAST-<http://blast.ncbi.nlm.nih.gov.ezproxy.unibo.it/Blast.cgi>). The evolutionary
110 history was inferred using the Maximum Likelihood method based on the Kimura 2-parameter
111 model (Kimura, 1980). A discrete Gamma distribution was used to model evolutionary rate
112 differences among sites. Evolutionary analyses were tested on 4000 Bootstrap replications, using
113 MEGA6 software (Tamura et al., 2013). The phylogenetic analysis was run by using 18SrRNA
114 sequences of species belonging to Haplosporidia order available in GenBank. *Mikrocytos mackini*
115 (AN:HM563060) and *Marteilia cochillia* (AN: KF278722) were used as outgroups.

116 3. Results

117 The average length of *P. nobilis* processed was 30 cm (SD: ± 1.58) and the average weight was
118 212 g (± 5.48). Several epibionts were attached to the valves, such as the polychaete *Eulalia ornata*
119 (Saint-Joseph, 1888), unidentified bryozoans, the common limpet *Patella vulgata* (Linnaeus, 1758),
120 the crab *Tumidotheres maculatus* (Say, 1818) and the starfish *Asterina pancerii* (Gasco, 1876). On
121 macroscopic observation the specimens of *P. nobilis* appeared emaciated, but no other gross
122 alterations were observed. A large watery vesicle was found in the visceral mass of one specimen
123 from Site 2. In all the specimens, the gills were collapsed and appeared pale brownish in color,
124 while the digestive glands were darker and softer than expected for healthy specimens. Fresh
125 preparations of digestive gland showed the presence of mature spores occurring either free or in
126 sporocysts (Fig. 2). Spores were ovoid with visible operculum and measured $4.4 \mu\text{m}$ (± 0.232) in
127 length \times $3.6 \mu\text{m}$ (± 0.233) (mean values calculated on $n = 50$ individuals). Culturing on TCBS,
128 MacConkey agar and *Aeromonas* agar base to detect *Vibrio* spp., *Escherichia coli* and *Aeromonas*
129 spp., respectively, was negative. Histopathological analyses showed diffused degenerative lesions in
130 the presence of different developmental stages of a haplosporidan parasite in the epithelium and in
131 the lumen of digestive tubules (Fig. 3A). Some larger sporocysts were protruding in the lumen of
132 the tubules causing atrophy in the surrounding cells as a consequence of compression. In some
133 tubules the epithelium was completely detached leaving only the basal lamina (Fig. 3B). Detached
134 cells appeared in coagulative necrosis. Spherical binucleate stages were also observed within the
135 epithelium of digestive tubules (Fig. 3C). In the interstitial space between tubules, an intense
136 inflammatory response characterized by the presence of hemocyte infiltration was observed.
137 Parasitic stages were present in the lumen of the intestine (Fig. 3D, arrow) associated with necrotic
138 sloughing of digestive cells with loss of the cilia, although they were absent in the intestinal
139 mucosa. The same response was observed in the mantle tissue, along with the presence of
140 uninucleate developmental stages inside host hemocytes (Fig. 3E). Numerous brown cells were
141 observed in the connective tissue (Fig. 3F) around the digestive gland and in the lumen of digestive
142 tubules, as well as in the mantle, gills and gonads. No other parasites were observed. All digestive
143 glands and hemolymph samples scored positive in PCR using degenerate primers for
144 *Haplosporidium* spp., resulting in amplicons of the expected size (~ 350 bp). No amplification was
145 obtained using primers for *H. nelsoni* and *Bonamia* spp. The BLAST analysis of the 18SrRNA

146 sequences of all the specimens tested revealed highest nucleotide identity, 99%, with the reference
147 sequence of *H. pinnae* in the GenBank database (AN: LC338065). The molecular identity was
148 confirmed by clustering of the 18SrRNA sequence obtained with that of *H. pinnae* reference strain,
149 supported by high bootstrap value (99%, Fig. 4). The *H. pinnae* clade clustered in a paraphyletic
150 group, appearing distinct from the *Bonamia/Minchinia* clade and from the clade containing most of
151 the other *Haplosporidium* species (Fig. 4). The sequence was deposited in GenBank under
152 accession number MK163629.

153 **4. Discussion**

154 We used molecular analysis to identify *H. pinnae* as the agent of the MME in *P. nobilis* populations
155 in the investigated area of Ionian Sea, and observed haplosporidia in pathological lesions in the
156 host. The presence of *H. pinnae* in examined *P. nobilis* with no other pathogens present, the
157 observed lesions in the digestive gland and the absence of inflammatory nodular lesions typical of
158 micobacteria indicate that this MME is due to this protozoan infection as observed in the Western
159 Coast of Mediterranean Sea in Spain (Darriba, 2017, Vázquez-Luis et al., 2017, Catanese et al.,
160 2018, Carella et al., 2019). Histological analysis showed that the presence of *H. pinnae* in all
161 specimens was associated with heavy lesions of the digestive gland structure and severe tubular
162 necrosis. The spores developed in the epithelium of the digestive gland and appeared to be released
163 in the lumen of the gland's tubules, reaching the intestine of the host for elimination into the
164 environment. In addition, the presence of different stages of sporulation of the protozoa (Hine and
165 Thorne, 2002) in the digestive gland confirmed *Haplosporidium* sp. as the agent of the lesions in
166 the examined specimens of *P. nobilis* (Catanese et al., 2018). Similar pathological conditions of the
167 digestive gland have also been associated with the sporulation of *H. nelsoni* and *Haplosporidium*
168 *tuxtlensis* in eastern oyster *Crassostrea virginica* (Gmelin, 1791) and the striped false limpet
169 *Siphonaria pectinata* (Linnaeus, 1758) (Couch et al., 1966, Vea and Siddall, 2011). Before the
170 MME in Spain, haplosporidan parasites were detected infecting species of bivalves, gastropods,
171 crustacean, worms, ascidians and even hyperparasite trematode larvae (Burreson and Ford, 2004,
172 Arzul and Carnegie, 2015), but never in a member of the Pinnoidea Superfamily. The spreading of
173 this parasite into non-endemic areas is still unknown, but it may be argued that the outbreak spread
174 from Spain, being transported in the summer marine currents (Fernández et al., 2005). Nonetheless,
175 it cannot be ruled out that anthropic activity, such as maritime transport, ballast waters and trade of
176 living bivalves may have enhanced dispersal of the protozoa. Dynamics of haplosporidians in their
177 hosts suggest that these parasites could be seasonal, depending on environmental parameters such as
178 temperature and salinity (Darriba, 2017).

179 First evidence of unexpected mortality of *P. nobilis* (40% of the individuals) in the study area was
180 observed during the summer 2017, followed by a low mortality period during the winter
181 (unpublished data) and by the drastic decline of the population in the following summer of 2018.
182 Based on our observations, environmental conditions such as warm temperatures may be an
183 important driver for the development of *H. pinnae*, suggesting that the impact of global warming
184 could enhance the spreading of this parasite all over the Mediterranean Sea. Control of spread is
185 difficult due to the lack of an adaptive immune system of the host and the rapid death of infected
186 individuals, resulting in up to 100% mortality in a few months. Furthermore, the administration of
187 treatment is impossible to carry out because of the potential impact on the marine ecosystem, as

188 well as the restrictions by European legislation (Guardiola et al., 2012). Therefore, resettlement of
189 *P. nobilis* populations at the end of the MME seems to be the only option available to mitigate the
190 on-going local extinction of this protected species.

191 Some aspects of the life cycle of *H. pinnae* remain unknown, including the potential of an
192 intermediate host, the role of other definitive hosts, such as *Pinna rudis* (Linnaeus, 1758) or *Atrina*
193 spp., and the persistence of infective spores in the environment. Further studies are needed to
194 improve knowledge about the life cycle of *H. pinnae* in order to mitigate the ongoing disease and
195 plan proper repopulation strategies for *P. nobilis* in areas where the MME caused the extinction of
196 the species.

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200 Vera Corbelli) and the University of Bari Aldo Moro.

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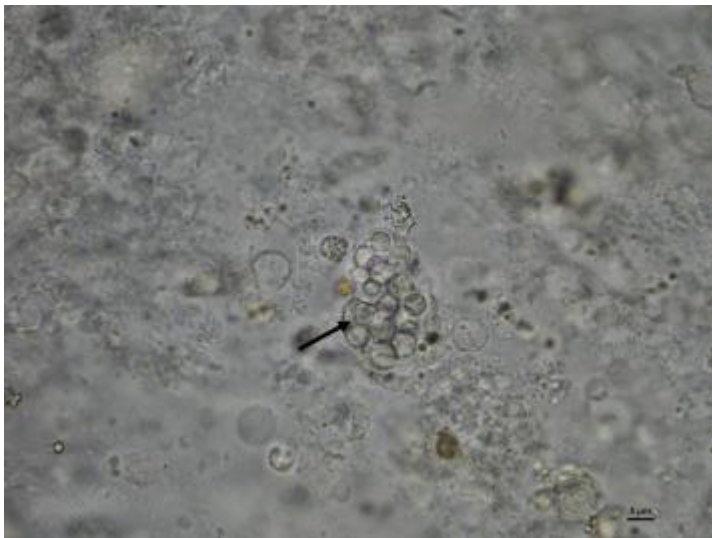
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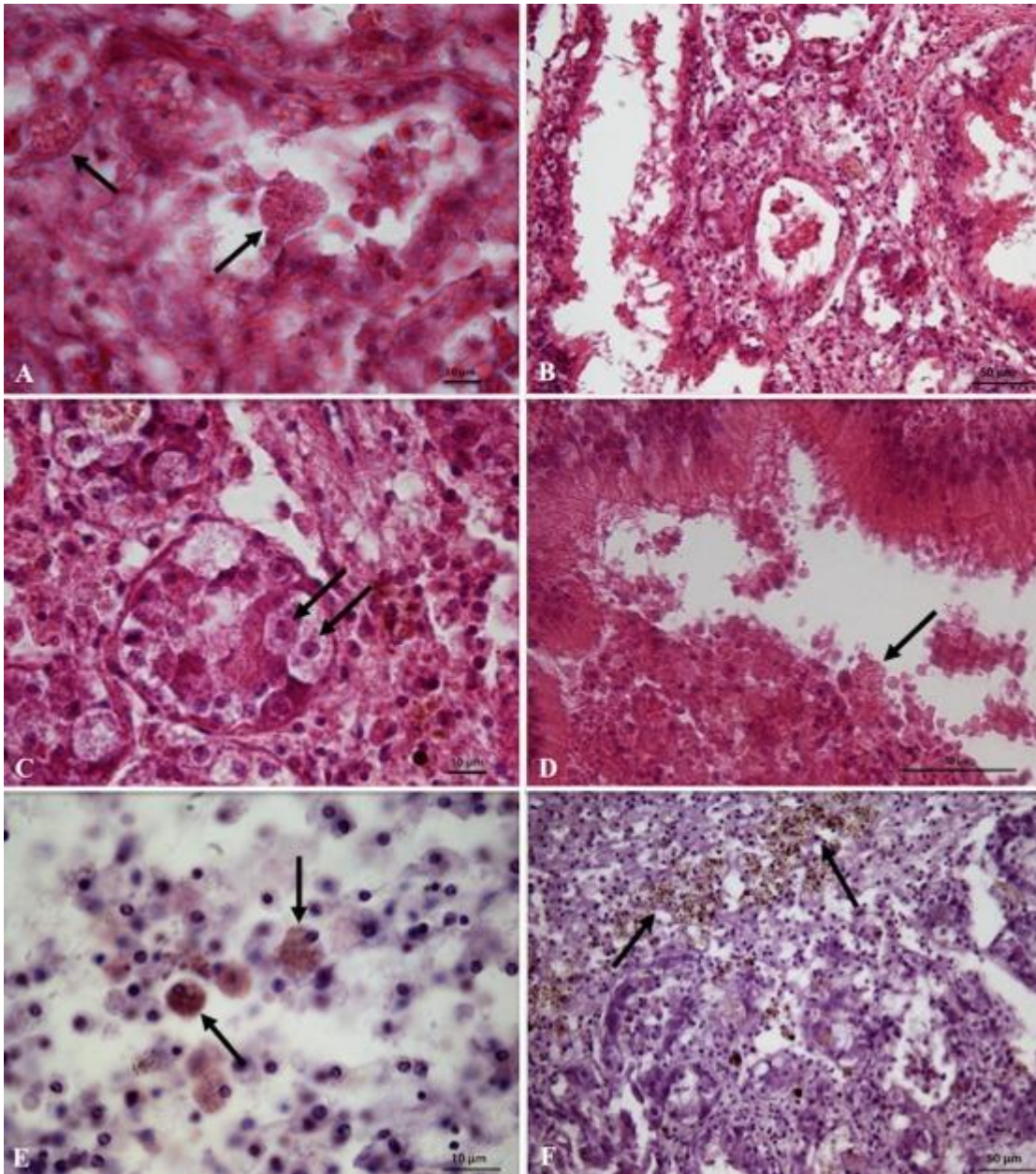
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 347 Fig. 1. Map of the Mar Piccolo of Taranto (Ionian Sea, Southern Italy) indicating the two sampling sites
 348 (dots).

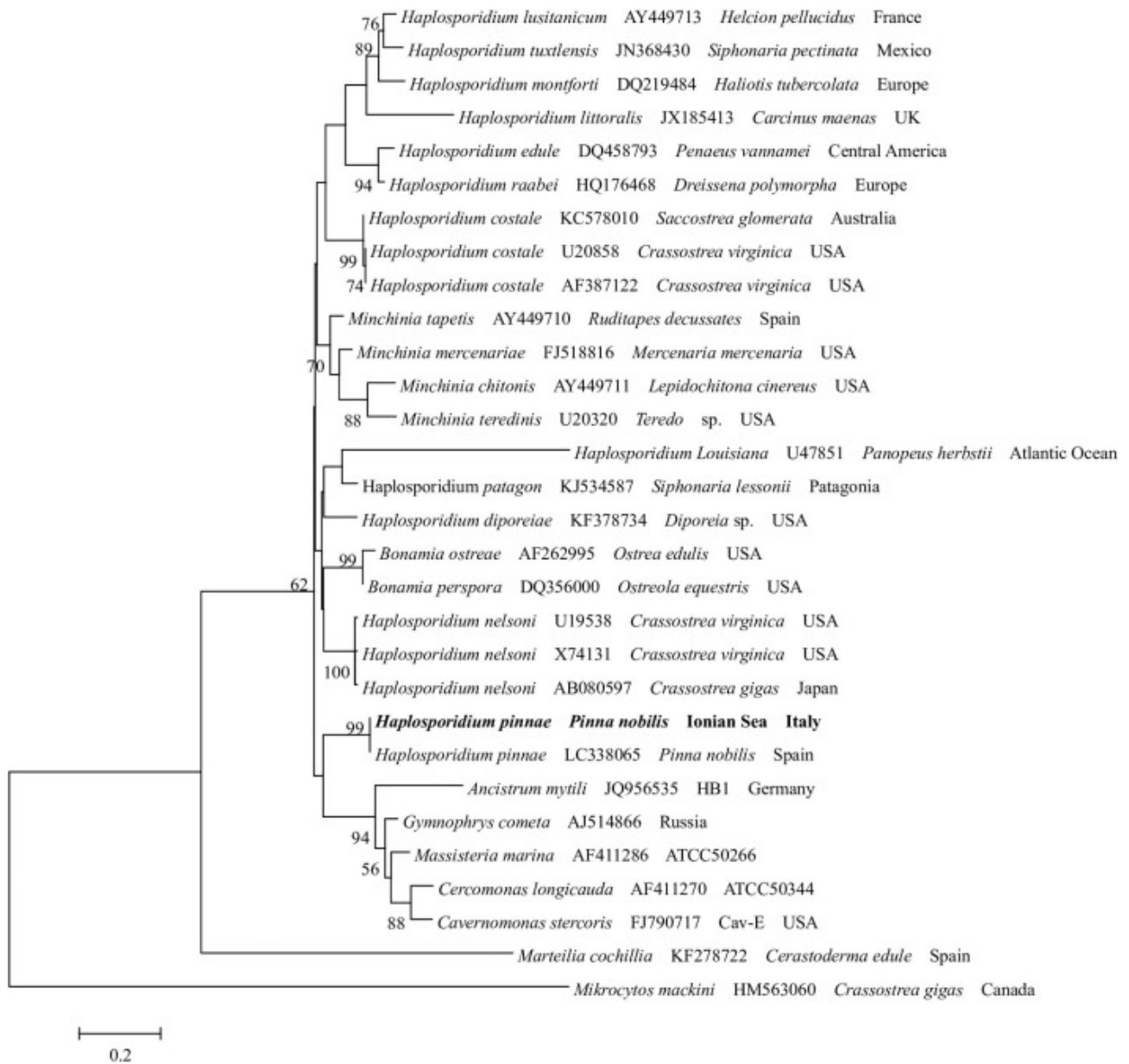


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 350 Fig. 2. Spores and sporocysts (arrow) of *Haplosporidium pinnae* in fresh preparation of a digestive gland.



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



360

361 Fig. 4. Maximum likelihood tree based on 18SrRNA sequences of *H. pinnae* generated with those of other
362 haplosporidians parasite available from GenBank. Bootstrap values are based on 4000 replicates and only
363 bootstraps >50% are indicated. Accession number, host and country of haplosporidians, *Mikrocytos mackini*
364 and *Marteilia cochillia* 18SrRNA sequences used as outgroups are reported.