Hyperspora aquatica n.gn., n.sp. (Microsporidia), hyperparasitic in *Marteilia cochillia* (Paramyxida), is closely related to crustacean-infecting microspordian taxa

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SUMMARY

The Paramyxida, closely related to haplosporidians, paradinids, and mikrocytids, is an obscure order of parasitic protists within the class Ascetosporea. All characterized ascetosporeans are parasites of invertebrate hosts, including molluscs, crustaceans and polychaetes. Representatives of the genus *Marteilia* are the best studied paramyxids, largely due to their impact on cultured oyster stocks, and their listing in international legislative frameworks. Although several examples of microsporidian hyperparasitism of paramyxids have been reported, phylogenetic data for these taxa are lacking. Recently, a microsporidian parasite was described infecting the paramyxid *Marteilia cochillia*, a serious pathogen of European cockles. In the current study, we investigated the phylogeny of the microsporidian hyperparasite infecting *M. cochillia* in cockles and, a further hyperparasite, *Unikaryon legeri* infecting the digenean *Meiogymnophallus minutus*, also in cockles. We show that rather than representing basally branching taxa in the increasingly replete Cryptomycota/Rozellomycota outgroup (containing taxa such as *Mitosporidium* and *Paramicrosoridium*), these hyperparasites instead group with other known microsporidian parasites infecting aquatic crustaceans. In doing so, we erect a new genus and species (*Hyperspora aquatica* n. gn., n.sp.) to contain the hyperparasite of *M. cochillia* and clarify the phylogenetic position of *U. legeri*. We propose that in both cases, hyperparasitism may provide a strategy for the vectoring of microsporidians between hosts of different trophic status (e.g. molluscs to crustaceans) within aquatic systems. In particular, we propose that the paramyxid hyperparasite *H. aquatica* may eventually be detected as a parasite of marine crustaceans. The potential route of transmission of the microsporidian between the paramyxid (in its host cockle) to crustaceans, and, the 'hitch-hiking' strategy employed by *H. aquatica* is discussed.

Key words: *Marteilia*, Paramyxea, Cercozoa, Digenea, Phylogeny, Fungi, Cryptomycota.

INTRODUCTION

Microsporidia are widespread in aquatic environments. Almost half of the 190 genera described to date infect aquatic hosts, with over 20 in fish, 50 in aquatic arthropods and more than 20 in non-arthropod invertebrates, protists, and as hyperparasites of other taxa infecting aquatic animals (Stentiford *et al*. 2013). Microsporidian hyperparasites infect myxozoans (myxosporean and actinosporean stages) and acanthocephalans in fish (Diamant and Paperna, 1985; de Buron *et al*. 1990; Morris and Freeman, 2010), digeneans in fish, crustaceans and molluscs (Canning and Nicholas, 1974; Levron *et al*. 2004, 2005), monogeneans in toads (Cable and Tinsley, 1992), cestodes in snails and fish (Canning *et al*. 1974; Sene *et al*. 1997; Ba *et al*. 2007), dicyemidans in squid (Czaker, 1997) and gregarines in polychaetes and nemerteans (Caullery and Mesnil, 1897, 1914; Vinckier, 1975). Their reported ability to infect parasitic and free-living protists in the Gregarinasina (see above and Larsson, 2000; Larsson and Koie, 2006), Amoebozoa (Scheid, 2007) and Ciliophora (Foissner and Foissner, 1995; Fokin *et al*. 2008) probably vastly under represents their true potential to interact with protistan hosts within aquatic and terrestrial habitats (Stentiford *et al*. 2013).

The Paramyxida is an order of parasitic protists within the class Ascetosporea (supergroup Rhizaria) (Ward *et al*. 2016). Paramyxids are related to

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haplosporidians, paradinids and mikrocytids (Bass *et al*. 2009; Hartikainen *et al*. 2014*a*, *b*), although the evolutionary relationships among the five ascetosporean orders is currently unresolved. All characterized ascetosporeans are parasites of invertebrate hosts (Chatton, 1911; Haskin *et al*. 1966; Grizel *et al*. 1974; Larsson, 1987; Feist *et al*. 2009). Recent work utilizing genome sequence data from representative lineages within the Rhizaria have revealed how certain groups (e.g. the mikrocytids) are more diverse and wide spread in aquatic habitats than previously thought (Burki *et al*. 2013; Hartikainen *et al*. 2014*b*). Despite this, the rhizarians, and their constitutive taxa, remain one of the most understudied groups of eukaryotes. Representatives of the genus *Marteilia* are perhaps the best-studied paramyxids, largely due to their impact on cultured oyster stocks, and their listing in international legislative frameworks (OIE, 2014).

Several examples of microsporidian hyperparasitism of paramyxean hosts are reported in the literature. Based upon morphological data, Comps *et al*. (1979) described *Nosema ormieresi* infecting *Marteilia refringens*, in turn parasitizing the oyster *Ostrea edulis*. The taxonomic placement of the hyperparasitic microsporidian within *Nosema* was based upon the presence of a diplokaryon in all life stages; now considered a plesiomorphic trait (see Stentiford *et al*. 2010) and therefore likely to be erroneous. Furthermore, a microsporidian hyperparasite was also reported infecting *M. refringens* in mussel *Mytilus galloprovincialis* (Villalba *et al*. 1997) and recently, infecting the sister paramyxid *Marteilia cochillia* in cockles (Villalba *et al*. 2014). In the latter case, the authors proposed that the microsporidian hyperparasite was likely synonymous with that previously described infecting the cockle digestive gland by Comps *et al*. (1975). An additional complication in this case, however, is the co-infection of cockles by digenean trematodes which can also play host to a microsporidian hyperparasite, *Unikaryon legeri* (Canning and Nicholas, 1974).

Whilst paramyxid parasites are best known for their devastating effects on cultured and wild molluscan populations, they can also parasitize crustacean hosts. There is strong evidence that *M. refringens* may require passage through crustacean hosts to complete its lifecycle (Audemard *et al*. 2002; Arzul *et al*. 2014). Furthermore, representatives of the understudied genus *Paramarteilia*, a sister taxon to *Marteilia*, are known to infect amphipod (Ginsburger-Vogel and Desportes, 1979) and decapod (Feist *et al*. 2009) hosts. In amphipods, *Paramarteilia orchestiae* infections commonly co-occur with microsporidian pathogens (again, incorrectly attributed to the genus *Nosema*) (Ginsburger-Vogel and Desportes, 1979). Recent work has shown that paramyxean pathogens may at least be partly responsible for the elevated rates of feminization and other sexual disruption noted in some amphipod populations – a consequence previously assigned only to infection by microsporidians (Ginsburger-Vogel, 1991; Short *et al*. 2012). These paramyxeans, and co-infecting microsporidians of the genus *Dictyocoela* can be vertically transmitted to amphipod eggs and larvae (Short *et al*. 2012), possibly offering a route of re-infection to molluscan hosts. Taken together, these observations provide an intriguing insight in to potential for microsporidian pathogens to exploit the invasion and pathogenic strategies of paramyxean pathogens, and their potential to 'hitch-hike' between hosts of differing trophic positions (Lefèvre *et al*. 2008; Thomas *et al*. 2008). Furthermore, the observations pose interesting questions about the taxonomic status of microsporidian pathogens infecting paramyxean protist hosts. Are they primitive associations or, perhaps more recent acquisitions, thus taking advantage of an existing parasitic strategy of their protistan hosts? (Stentiford *et al*. 2013).

We have previously stated that of the known microsporidian genera infecting aquatic organisms, phylogenetic data pertaining to the ssuRNA gene are available for approximately 35 arthropod-infecting taxa, 17 fish-infecting taxa, and six taxa associated with non-arthropod invertebrates. To date, little sequence data exist for microsporidian parasites infecting protists or hyperparasites (Stentiford *et al*. 2013). In particular, the absence of data from apparently primitive genera, such as *Metchnikovella* which infect gregarine hyperparasites of polychaetes (Sokolova *et al*. 2013) provides a notable example. In the current study, we investigated the phylogeny of two microsporidian hyperparasites infecting the paramyxean protist *M. cochillia* and the digenean trematode *Meiogymnophallus minutus*, both occurring as co-infections in cockles. We show that rather than representing basally branching taxa, these hyperparasites align with other known microsporidian parasites infecting marine crustaceans. In doing so, we erect a new genus and species (*Hyperspora aquatica* n. gn., n.sp.) to contain the hyperparasite of *Marteilia* spp. and propose that such hyperparasitism may provide a strategy for the vectoring of microsporidian pathogens between hosts of different trophic status in aquatic systems.

MATERIALS AND METHODS

Sampling, histology and electron microscopy

Cockles (*Cerastoderma edule*) were hand-collected from shellfish beds of O Sarrido and Lombos do Ulla in the Ría de Arousa, Galicia, NW Spain in June 2012. For histology, a transverse section of each individual containing gill, visceral mass, mantle lobe and foot muscle, plus portions of the adductor muscle and siphon were fixed in Davidson's

sea water fixative (Hopwood, 1996) for 24 h. Following fixation, samples were dehydrated in an ethanol series and embedded in paraffin according to standard protocols. Histological sections (c. 5 μM) were mounted to glass slides before staining with Harris' haematoxylin and eosin (H&E) prior to examination under light microscopy for detection of two microsporidian hyperparasites of *M. cochillia* and metacercariae of the digenean *M. minutus*, infecting cockles. Smears of cockle digestive glands infected with *M. cochillia* were fixed in ethanol and stained with Panoptic rapid stain (a modified Romanowsky stain). Digital images were obtained using a Nikon E800 light microscope and the Lucia™ Screen Measurement System (Nikon). For electron microscopy, 2 mm^3 blocks of cockle digestive gland were fixed in a solution containing 2·5% glutaraldehyde in filtered seawater, post-fixed in 1% osmium tetroxide in 0·2 M sodium cacodylate solution and embedded in Epon resin. Ultrathin sections (50–70 nm) were stained with uranyl acetate and lead citrate and examined in a JEM-1010 transmission electron microscope (TEM, JEOL).

DNA extraction, PCR amplification and sequencing

Corresponding pieces of digestive gland and mantle from individual cockles were preserved in 96% ethanol at the time of dissection. DNA extractions were performed using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol to isolate genomic DNA. DNA quality and quantity was checked using a Nanodrop® ND-1000 spectrophotometer (Nanodrop technologies, Inc.). A set of previously published primers and additional primer designs were utilized in this study (Table 1). Primers were designed to amplify overlapping fragments of the small subunit rRNA gene (SSU rRNA), the internal spacer transcribed (ITS) and the large subunit rRNA gene (LSU rRNA) of the microsporidian pathogens infecting *M. cochillia* and *M. minutus* in cockle hosts. The whole SSU region of the two microsporidian hyperparasites was amplified by PCR using the primers V1f/1342AC (Weiss *et al.* 1994; Yang *et al.* 2011). To amplify the end of the SSU, the ITS, and the end of the LSU rRNA gene of the microsporidian hyperparasites of *M. minutus*, HG4F/HG4R primers were used (Gatehouse and Malone, 1998). To amplify the end of the SSU, the ITS, and the end of the LSU rRNA gene of the hyperparasite of *M. cochillia*, a new primer set, defined as Micros_Mcochi_F/Micros_Mcochi_R was used. To do so, SSU rRNA sequences obtained for this microsporidian, and for another six microsporidian sequences deposited in GenBank were aligned using the programme Clustal W. These microsporidians were *Dictyocoela berillonum* (AJ438957), *Microsporidia* sp. (GQ246188), *Microsporidium* sp.

Table 1. Primer sets used for PCR and ISH detection of *Hyperspora aquatica* n. gen., n. sp. and *Unikaryon legeri*

Primer	Sequence 5′–3′	Location within GenBank sequence (TBC) of *H. aquatica*	Location within GenBank sequence (TBC) of *U. legeri* sequence	Purpose in this study	References
V1f	CACCAGGTTGATTCTGCCTGAC	1–22	1–22	Molecular characterization	Weiss *et al.* (1994)
1342AC	ACGGGCGGTGTGTACAAGGTACAG	1226–1249	1234–1257	Molecular characterization	Yang *et al.* (2011)
HG4F	GCGGCTTAATTTGACTCAAC		867–886	Molecular characterization	Gatehouse and Malone (1998)
HG4R	TCTCCTTGGTCCGTGTTTCAA		1891–1911	Molecular characterization	Gatehouse and Malone, (1998)
MicrosMcochi-F	CAGACCCGAGGACCATAAGA	899–918		Molecular characterization	Newly designed
MicrosMcochi-R	AGCTTTTCCTCGTTCACTCG	1580–1599		Molecular characterization	Newly designed
MicrosISH-F	CGAAGAAGGGCGATAATGAA	1429–1448		*In situ* hibridization assay	Newly designed
MicrosISH-R	ACTCGCCGCTACTACAGGAA	1565–1584		*In situ* hibridization assay	Newly designed
DictyISH-F	TCTTACGTCCCCGAATTTGA		1490–1509	*In situ* hibridization assay	Newly designed
DictyISH-R	CTTCTCTGTTCATTCGCCACT		1587–1607	*In situ* hibridization assay	Newly designed

(AJ438962), *Microsporidium* sp. (HM800853), *Potaspora morhaphis* (EU534408) and *Thelohania butleri* (DQ417114). Subsequently, new primers were designed using the Primer 3 program (Rozen and Skaletsky 2000).

All PCR assays were performed in a total volume of 25 μL containing 1 μL of genomic DNA (200 ng), PCR buffer at 1× concentration, 1·5 mM $MgCl_2$, 0·2 mM nucleotides (Roche Applied Science), 0·3 μM of each pair of primers and 0·025 U μL^{-1} Taq DNA polymerase (Roche Applied Science). A negative control (no DNA) was used in each PCR assay. The PCR assays were carried out in a T gradient thermocycler (Biometra), under the following reaction parameters: 94 °C for 5 min, 35 cycles at a melting temperature of 94 °C for 1 min, an annealing temperature of 62 °C for primers V1f/1342AC1 and 50 °C for primers HG4F/HG4R and Micros_Mcochi_F/Micros_Mcochi_R, for 1 min, an extension temperature of 72 °C for 2 min, followed by a final extension period of 72 °C for 10 min. After PCR, 10 μL of amplified DNA was separated by electrophoresis on 2% agarose gels, in 1% Tris acetate EDTA buffer, stained with ethidium bromide and scanned in a GelDoc XR documentation system (BioRad). PCR products were cleaned for sequencing using ExoSap-It (USB Corporation) for 15 min at 37 °C, followed by inactivation for 15 min at 80 °C. Sequencing was performed by the company Secugen (Madrid) and the chromatograms were analysed using ChromasPro v.1.41 (Technelysium Pty Ltd.). All generated sequences were assessed for similarity against known sequences using BLAST (Basic Local Alignment Search Tool) of the National Center for Biotechnology Information (NCBI, USA).

Phylogenetic analyses

Sequence alignments were made in mafft v.7 e-ins-i algorithm (Katoh and Standley, 2013). The alignment was refined by eye and analysed in RAxML BlackBox v.8 (Stamatakis, 2014) (GTR model with CAT approximation (all parameters estimated from the data); average of 10 000 bootstrap values was mapped onto the tree with the highest likelihood value). A Bayesian consensus tree was constructed using MrBayes v.3.2.5 (Ronquist *et al*. 2012). Two separate MC^3 runs with randomly generated starting trees were carried out for 2 M generations each with one cold and three heated chains. The evolutionary model applied included a GTR substitution matrix, a four-category autocorrelated gamma correction and the covarion model. All parameters were estimated from the data. Trees were sampled every 1000 generations. A total of 500 000 generations were discarded as 'burn-in' (trees sampled before the likelihood plots reached a plateau) and a consensus tree was constructed from the returning sample. All phylogenetic analyses were performed via the Cipres Science Gateway (Miller *et al*. 2010).

In situ hybridization (ISH) assays

ISH assays were performed in order to confirm that sequences obtained from PCR co-located to microsporidian hyperparasites of *M. cochillia* and *M. minutus* in cockles observed in histology sections. Two digoxigenin polynucleotide probes were used: (1) MicrosMarteiliaISH probe designed based on the LSU sequence of the microsporidian hyperparasites of *M. cochillia* and another *Microsporidium* spp. downloaded from the GenBank database; and (2) MicrosMetacercariaISH probe designed based on the LSU sequence of the microsporidian hyperparasite of *M. minutus*, and other *Dictyocoela* spp. obtained from GenBank. The PCR DIG Probe-Synthesis kit (Roche Applied Science) was used to amplify the fragment of LSU of the hyperparasite of *M. cochillia* and *M. minutus* using the primers MicrosISH_F/MicrosISH_R and DictyISH_F/DictyISH_R, respectively (Table 1). Both sets were labelled with digoxigenin by incorporation of DIG-dUTP during the PCR process. Three serial tissue sections (about 5 μM) were produced from each paraffin block to allow for testing of the MicrosMarteiliaISH and MicrosMetacercariaISH probes, and negative control (without probe), respectively. A further serial section was stained with Harris' H&E in order to identify and localize the paramyxean, digenean and hyperparasites of interest. Sections for ISH assays were placed on to silanized slides (Silane-prep slides; Sigma) following which assays were performed as detailed in Ramilo *et al*. (2014). Slides were viewed using light microscopy as above.

RESULTS

Light microscopy and ultrastructure of hyperparasitism

The microsporidian stages infect the cytoplasm of the primary cell of *M. cochillia* which itself initially located within the epithelial cells of the cockle digestive gland (Fig. 1A). Although early *M. cochillia* stages harbour the microsporidian parasite, the infection is most prominent in more advanced larger stages within which sporonts are also present (Fig. 1B). Sporonts and developing spores do not appear to be infected with the microsporidian hyperparasite and development of *M. cochillia* spores proceeds normally. Early development of the microsporidian can be visualized at the light microscope level in stained impression smears of infected cockle digestive gland. A sequence of merogonic development to mature spore formation is seen in Fig. 2. Released spores are also visible but we were unable to detect examples of *M. cochillia* in the

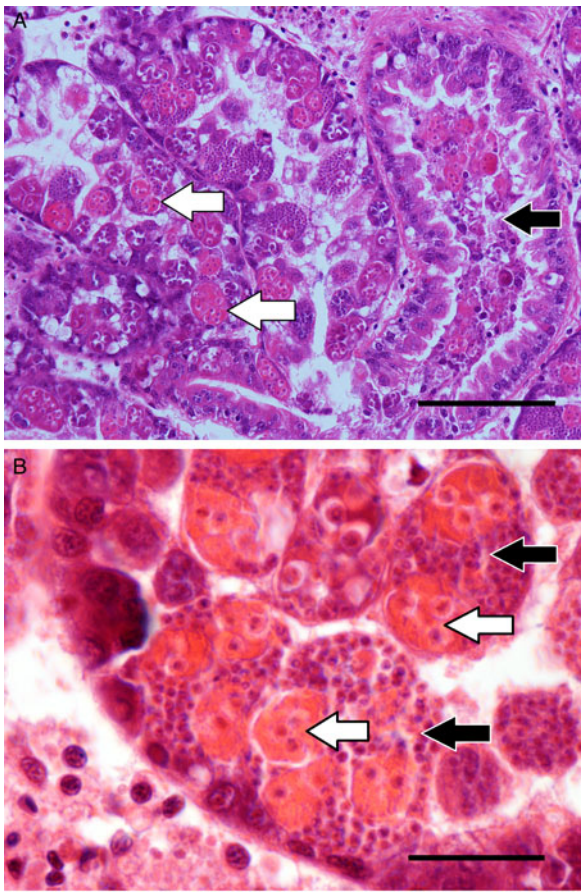

Fig.1. Microsporidian hyperparasitism of *Marteilia cochillia* infecting cockles (*Cerastoderma edule*). (A) The digestive gland tubules are significantly altered during advanced *M. cochillia* infection. *Marteilia* cells are clearly visible as large oeosinophilic bodies within tubule epithelial cells (white arrows). Hyperparasitized *M. cochilla* are shed to the lumen of infected tubules (black arrow). Scale 100 μM; (B) *M. cochilla* (white arrows) and microsporidian hyperparasite (black arrows) within tubule epithelial cells and tubule lumen. Scale 25 μM H&E histology.

process of spore release which is likely to be an uncontrolled event on rupture of primary cells within the lumen of the digestive tubule.

Uninfected *M. cochillia* primary cells show the characteristic development of secondary and tertiary cells with abundant ribosomes present as well as haplosporosomes and multivesicular bodies (Fig. 3A). Early stage infection with the microsporean results in merogonic proliferation within the primary cell with no involvement of the secondary cells (Fig. 3B). Subsequently, in late stage infections the primary cell of *M. cochillia* becomes replete with sporoblasts and mature spores (Fig. 3C). The earliest developmental stages of the microsporidian observed were thin-walled uninucleate meronts (Fig. 4A) with subsequent development into binucleate meronts (Fig. 4B). Maturation into sporonts and early sporoblasts is characterized by the formation of a thickened cell wall (Fig. 4C and D) and sporogony proceeds with the intracytoplasmic development of a membranous system and early polar vesicle formation (Fig. 4E). Further differentiation of the sporoblast can be seen in Fig. 4F where spore extrusion apparatus is under construction. Mature spores have dense osmiophilic cytoplasm, a single nucleus, four turns of the polar filament (Fig. 4G, Gi), Golgi apparatus and a polar vesicle (Fig. 4Gii).

Molecular characterization of microsporidian hyperparasites

The primer pair V1f/1342AC amplified SSU sequences of the microsporidians parasitizing *M. cochillia* and *M. minutus* in cockles. The end fragment of the SSU, ITS and LSU partial region of the microsporidian hyperparasite from *M. minutus* was amplified using the primer pair HG4F/HG4R. Since these primers did not amplify the same region for the hyperparasite of *M. cochillia*, a new primer set (Micros_Mcochi_F and Micros_Mcochi_R) was designed. Via these analyses we obtained the following: (1) a sequence of 1911 bp for the microsporidian hyperparasite of *M. minutus*, and (2) a consensus sequence of 1599 bp for the microsporidian hyperparasite of *M. cochillia*. The SSU–ITS–LSU sequence of the microsporidian hyperparasite of *M. cochillia* was 99% similar (over 96% of the compared sequence) to that of an unassigned '*Microsporidium* sp.' (HM800853), infecting the parasitic copepod *Lepeophtheirus hospitalis* on starry flounder *Platichthys stellatus* from British Columbia Canada. The hyperparasite from *M. cochillia* is also relatively closely related to numerous other microsporidian parasites described infecting marine and freshwater crustacean hosts and, is placed within clade 5 of the phylum according to the classification of Vossbrinck and Debrunner-Vossbrinck (2005). The SSU–ITS–LSU sequence of the microsporidian hyperparasite of *M. minutus* was 87% similar (over 99% of its sequence) to the crustacean amphipod pathogen *Dictyocoela diporeiae* (KF537632), similarly within clade 5 of the phylum. It is noteworthy that our results do not support a basal position of these hyperparasites within the phylum Microsporidia but rather, their close relationship to taxa described infecting aquatic crustacean and fish hosts (Fig. 5). We propose erection of a new genus and species to contain the hyperparasite infecting *M. cochillia*. The new taxon is named *H. aquatica* n. gen. n. sp.

ISH assay

The MicrosMarteiliaISH probe hybridized strongly, without background hybridization, to the microsporidian hyperparasite infecting *M. cochillia* (cockles). Examination of adjacent tissue sections stained with H&E confirmed that hybridization was localized to hyperparasites within the host

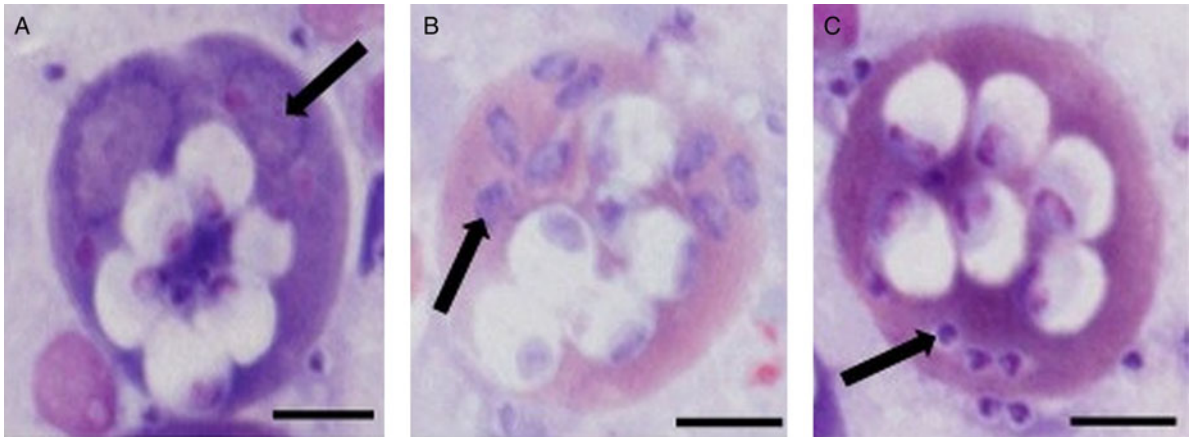

Fig. 2. Cockle digestive gland impression smears showing microsporidian hyperparasitism of *Marteilia cochillia* primary cells. (A) Infected primary cell containing six developing spores and putative *Hyperspora aquatica* meronts appearing as relatively pale staining regions (arrow); (B) Multiple *H. aquatica* sporonts with dark basophilic cytoplasm (arrow); (C) Intracellular spores of *H. aquatic*a within the *M. cochillia* primary cell (arrow) with several also visible externally. Note the absence of microsporean infection within the developing spores of *M. cochillia*. Panoptic Romanowsky stain. Scale 10 μM.

Marteilia primary cells. No hybridization signal was detected when the assay was made without probe (negative control, data not shown). The MicrosMarteiliaISH did not hybridize with the microsporidian hyperparasite of *M. minutus* in cockles. Instead, the MicrosMetacercariaISH probe hybridized strongly with *M. minutus* in cockles and cross-reactivity was not observed with the microsporidian hyperparasites infecting *M. cochillia* (Fig. 6).

Taxonomic summary

Hyperparasite of *Marteilia cochillia*. *Hyperspora aquatica* n. gen., n. sp., Genus *Hyperspora* n. gen.

Definition. Parasite life stages infecting primary cells of *M. cochillia* (Cercozoa: Marteiliidae) within the digestive epithelium of cockles (*C. edule*). Spores spherical to ellipsoid, approximately 1·2 × 1·0 μM with four polar filament coils in a single rank. Merogonic and sporogonic stages occur in direct contact with the cytoplasm of primary cells of *M. cochillia*. Observed life cycle is unikaryotic and progresses from uninucleate meronts to multinucleate meronts prior to initiation of sporogony. Sporogony is marked by thickening of the cell wall, budding of unincucleate sporonts and eventual development of the spore extrusion precursors in uninucleate sporoblasts. Sporoblasts mature to spores, which continue to occupy the cytoplasm of the primary cell of the host. Type species *H. aquatica* n. gen., n. sp.

Type Species: *Hyperspora aquatica* n. gn., n. sp.

Description: As for genus.

Diagnosis: Presence of a microsporidian parasite with descriptive features of the genus in the cytoplasm of primary cells of *M. cochillia* infecting the digestive gland of cockles (*C. edule*). Diagnosis of morphological features by histology/impression smear and TEM as described herein. Nucleic acid-based diagnosis via PCR amplification, analysis of the defined SSU–ITS–LSU gene sequence and comparison with GenBank Accession number (to be assigned).

Type host: *Marteilia cochillia* Carrasco *et al*. (2013)

Type locality: Ría de Arousa, Galicia, NW Spain

Site of infection: Cytoplasm of primary cells of *M. cochillia* infecting the digestive gland epithelial cells of cockles (*C. edule*).

Etymology: The generic name refers to the hyperparasitic habit of the parasite in the paramyxean *M. cochillia*. The specific epithet refers to its presence in aquatic habitats and its phylogenetic similarity to microsporidian pathogens infecting hosts from different trophic positions within such habitats.

Type material: Voucher specimens of histological sections stained with H&E and TEM resin blocks have been deposited in the Registry of Aquatic Pathology (RAP) at the Cefas Weymouth Laboratory, UK and in the Registry of Pathology at the *Centro de Investigacións Mariñas* (CIMA), Spain. *Hyperspora aquatica* SSU–ITS–LSU gene sequences have been deposited in GenBank under accession numbers (to be assigned).

Hyperparasite of of *M. minutus*. *Unikaryon legeri* (Canning and Nicholas, 1974).

The microsporidian hyperparasite infecting the digenean *M. minutus* in Spanish *C. edule* observed in the current study is presumed (via morphological similarity, data not shown) to be *U. legeri* Canning and Nicholas (1974). This hyperparasite was previously described infecting the same hosts on the south coast of England as far back as 1897 (Giard, 1897 in Canning and Nicholas, 1974). Although a full taxonomic re-description of *U. legeri* is not required

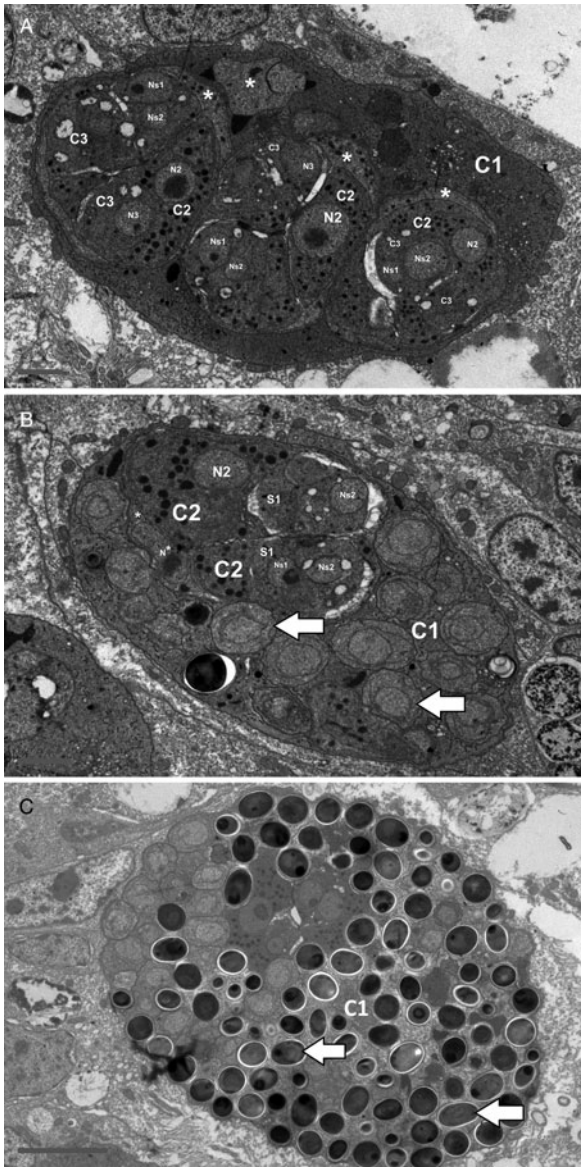

Fig. 3. Microsporidian hyperparasitism of *Marteilia cochillia* infecting cockles (*Cerastoderma edule*). (A). Uninfected *M. cochillia*. Primary cell (C1) contains three visible secondary cells (C2), each containing tertiary cells (C3). Key: *(cytoplasm of primary, secondary and tertiary cells), N1, N2, N3 (nucleus of primary, secondary and tertiary cells, respectively), Ns1, Ns2 (*M. cochilla* spore nuclei). Scale 1 μM. (B) Microsporidian-infected *M. cochilla*. Microsporidian hyperparasites in this case are predominantly sporonts (arrows) and are confined to the cytoplasm of the paramyxean primary cell (C1). Two secondary cells (C2) of *M. cochillia* are also visible but not apparently parasitized. Key: as above but with S1 (*M. cochillia* spore). Scale 1 μM. (C) Later stage infection of *M. cochillia* primary cell. The majority of microsporidian life stages are spores (arrows) but all appear to remain confined to the primary cell (C1) of *M. cochillia*. Scale = 2 μM. All TEMs.

here, it is important to note that for the first time, molecular phylogenetic data have placed the parasite within clade 5 of the phylum Microsporidia (see Vossbrinck and Debrunner-Vossbrinck, 2005) with relatively close relationship to parasites infecting crustacean and fish hosts [including numerous *Dictyocoela* spp. infecting amphipods and, *Triwangia caridinae* Wang *et al.* (2013) infecting caridinid shrimps] (Fig. 5). This is first such information relating to the molecular phylogeny of microspordian hyperparasites of digenean hosts. *Unikaryon legeri* gene sequences have been deposited in GenBank under accession numbers (to be assigned).

DISCUSSION

In this paper, we describe the type genus and species of a novel microsporidian hyperparasite, *H. aquatica* n.gn. n.sp. infecting the paramyxid protist *M. cochillia*, in cockles. Furthermore, we provide clarity on the phylogenetic placement of the hyperparasitic microsporidian *U. legeri* Canning and Nicholas (1974) infecting the digenean *M. minutus*, also within cockles. Both *Hyperspora* and *Unikaryon* form distinct lineages within clade 5 (Marinosporidia) (Vossbrinck and Debrunner-Vossbrinck, 2005), but both cluster phylogenetically with microsporidian pathogens previously described infecting aquatic arthropod and fish hosts (Stentiford *et al.* 2013). Based upon SSU rRNA sequence data, *H. aquatica* is most closely related (99% sequence similarity) to an undesignated microsporidian pathogen ('*Microsporidium* sp.', GenBank accession HM800853) infecting the marine parasitic copepod *L. hospitalis* on starry flounder (*P. stellatus*) (Jones *et al.* 2012). The SSU rRNA sequence HM800853 most closely resembles that derived from microsporidian pathogens infecting aquatic amphipod crustacean hosts. Jones *et al.* (2012) do not provide a detailed description of the nature of the association between the microsporidian and the host *L. hospitalis* (and thereby cannot discount the possibility that the host is simply a mechanical vector for the microsporidian from which sequence HM800853 was obtained). Observations relating to two other microsporidian pathogens infecting other *Lepeophtheirus* spp. within the same study (*Facilispora margolisi* and *Desmozoon lepeophtherii*) however, demonstrate susceptibility to microsporidian infection, and to disease, in this host genus (Freeman *et al.* 2003). Furthermore, Jones *et al.* (2012) demonstrate that detection of sequence relating to HM800853 was only found associated with the flounder ectoparasite *L. hospitalis* (suggesting that it was not a widespread contaminant in the environment). The current study, which reveals potential synonymy between *H. aquatica*, a microsporidian hyperparasite of paramyxid protists infecting bivalve molluscs, and one infecting ectoparasitic copepods on marine fish becomes the major focus for the remainder of this discussion. In particular, it is pertinent to consider whether *H. aquatica* from paramyxid hosts is also a pathogen of crustacean hosts and further,

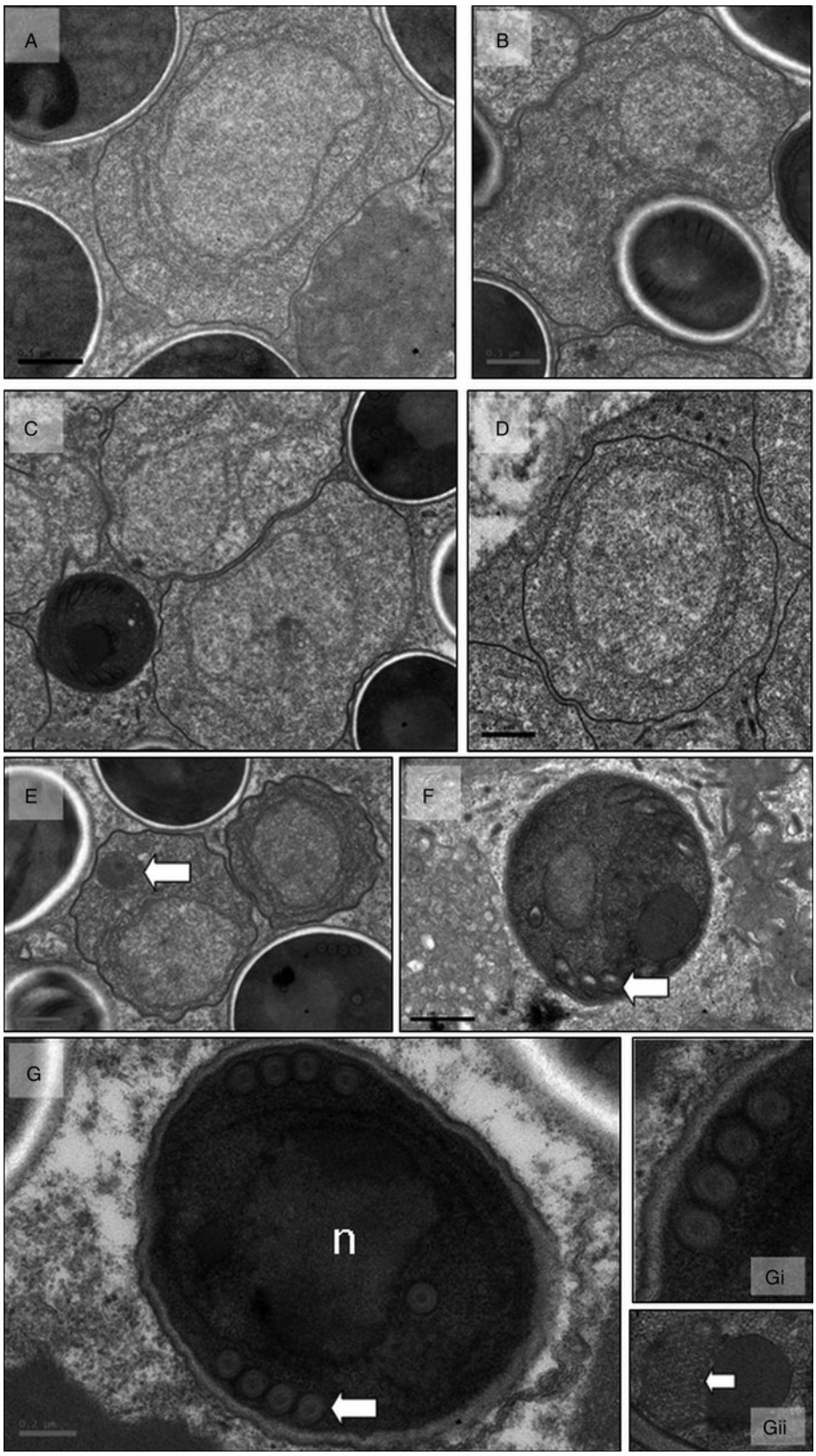

Fig. 4. Microsporidian hyperparasite of *Marteilia cochillia* infecting cockles (*Cerastoderma edule*). (A) Uninucleate meronts. Scale 0·5 μM; (B) Bi-nucleate (though not diplokaryotic) meronts. Scale 0·5 μM; (C) Transition to sporont (thickening of cell wall). Scale 0·5 μM; (D) Uninucleate sporont. Scale 0·5 μM. (E) Maturation of uninucleate sporont to early sporoblast. Formation of membranous system and early polar vesicle within cytoplasm (arrow). Scale 0·5 μM. (F) Sporoblast with precursors of spore extrusion apparatus (arrow). Scale 0·5 μM. (G) Mature spore with single nucleus (n) and four turns of the polar filament (arrow). Scale 0·2 μM. (Gi) Detail of polar filament. (Gii) Detail of Golgi apparatus (arrow) and polar vesicle. All TEMs.

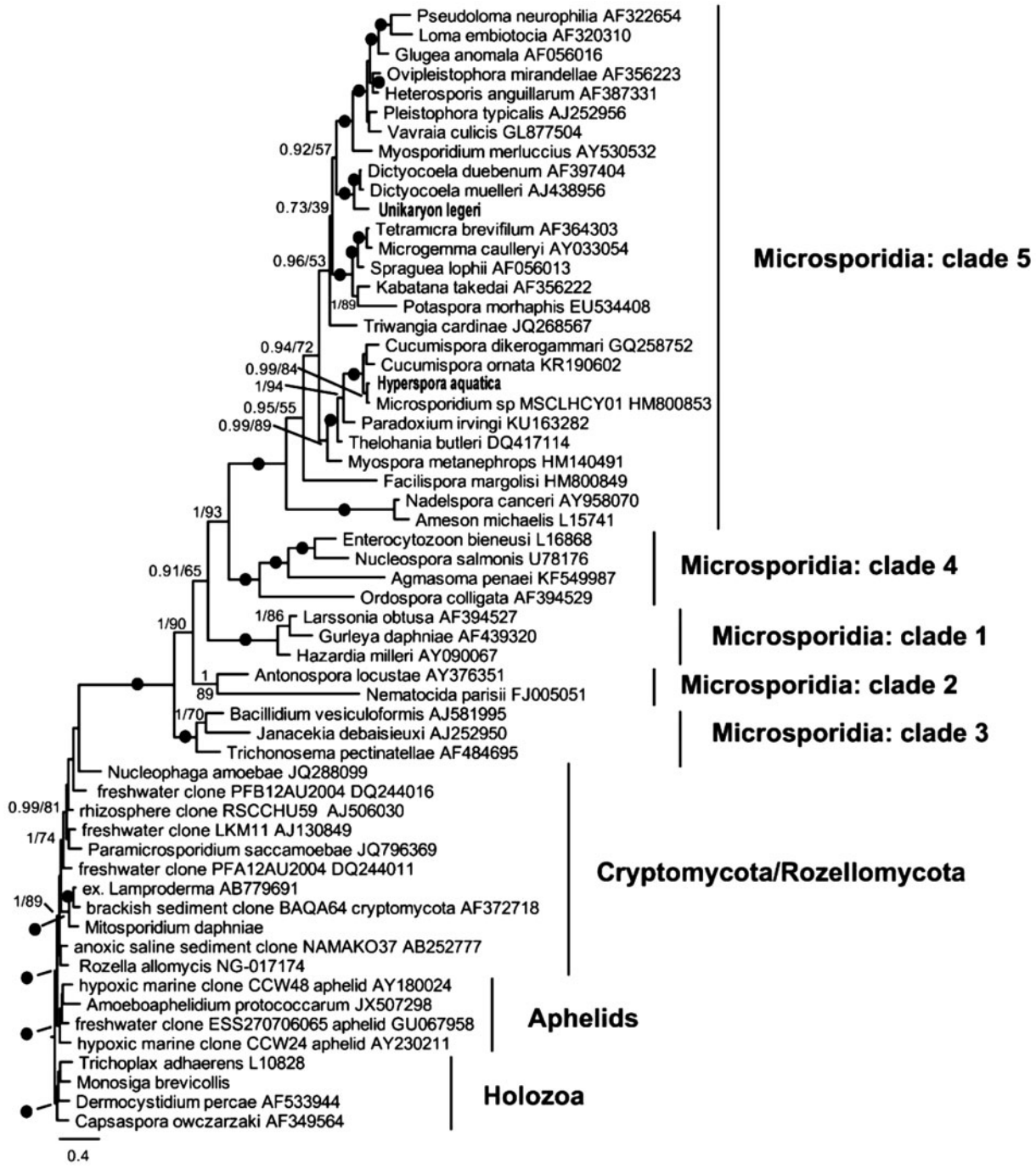

Fig. 5. Bayesian tree containing *Hyperspora aquatica* and *Unikaryon legeri* (this study) and 37 other microsporidian SSU sequences obtained from GenBank. Representative sequences from previously described Clades 1–5 are included. The tree is rooted to a subsection of Cryptomycota/Rozellomycota, Aphelid and Holozoan SSU sequences. Posterior probabilities and Bootstrap support appear next to each node.

whether it utilizes a strategy of 'hitch-hiking' within the paramyxid in order to transmit between marine molluscs and susceptible crustacean hosts (Lefèvre *et al*. 2008; Thomas *et al*. 2008).

Does H. aquatica hitch-hike to crustacean hosts within Marteilia?

Within the molluscan digestive gland epithelium, *H. aquatica* infects the primary cell cytoplasm of *M. cochillia*. In addition, sporogonic developmental stages and eventually spores of *Marteilia* are also produced by endogeny within this primary cell, which are not infected. The unique cell-within-cell development of paramyxids therefore allows separation of spore development of *H. aquatica* and *Marteilia*, such that infection of the primary cell cytoplasm with *H. aquatica* does not appear to hinder sporogenesis of the paramyxid (since normal development of sporonts and mature spores are

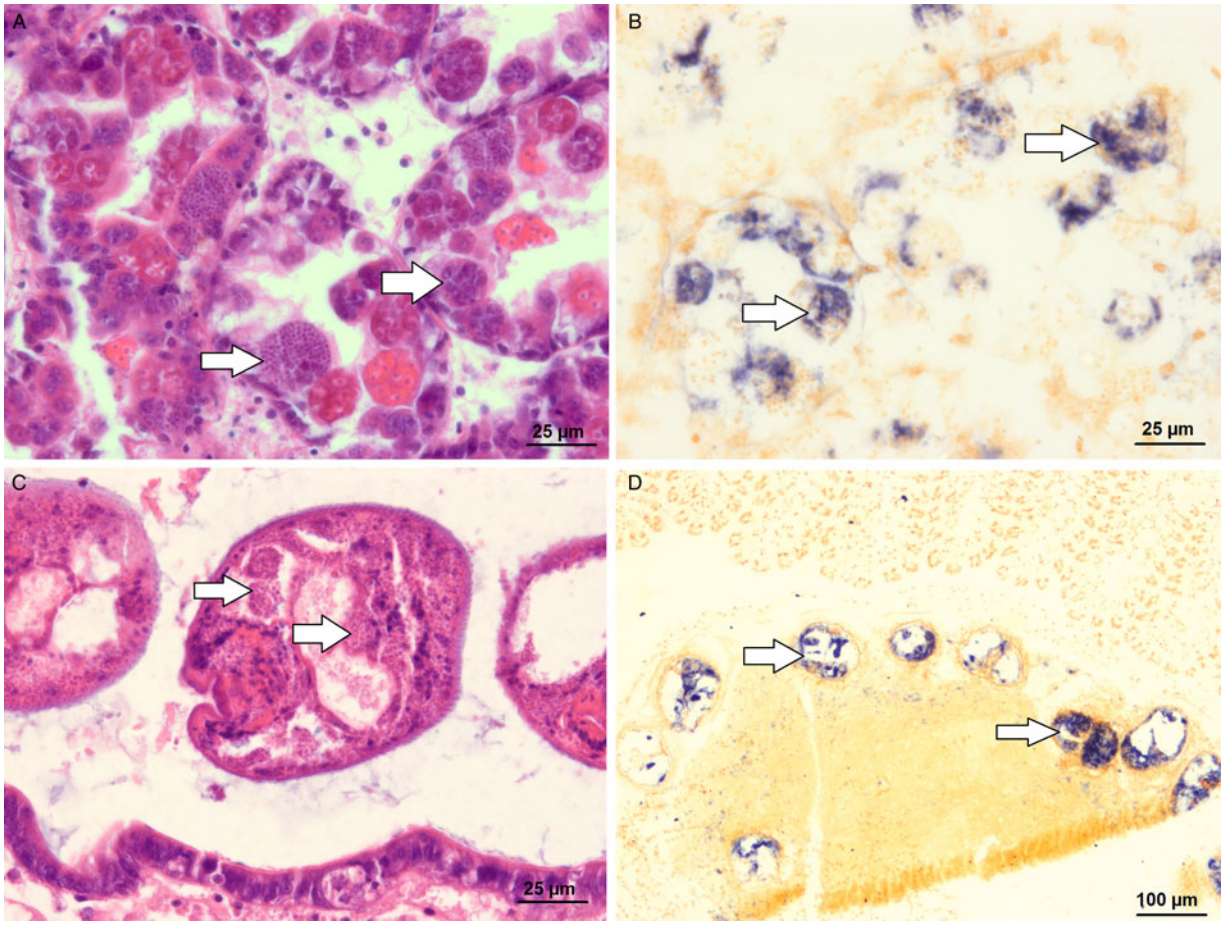

Fig. 6. ISH of *Hyperspora aquatica* n. gen., n. sp., and *Unikaryon legeri*. (A, B) *Hyperspora aquatica* n. gen., n. sp. infecting *Marteilia cochillia* in cockles. (A) H&E histology showing microsporidian infected paramyxeans in the digestive gland (arrows); (B) positive control ISH labelling of microsporidian hyperparasites (arrows) (unlabelled negative control images not shown). (C, D) *Unikaryon legeri* infecting *Meiogymnophallus minutus* in cockles; (C) H&E histology showing trematode parasites infected with *U. legeri* (arrows); (D) positive control ISH labelling of microsporidian parasites (arrows) (unlabelled negative control images not shown).

commonly observed) (see Fig. 3). The primary cell therefore contains the infective stages of two taxonomically distinct pathogens and conceivably, is a vehicle for co-transmission of paramyxid sporangia (containing sets of mature *Marteilia* spores), and of *H. aquatica*, via the digestive gland lumen, presumably for excretion via the gut, to the environment. There is an interesting parallel with the current case, of hyperparasitism with the microsporidian (*Neoflabelliforma aurantiae*) infecting another spore forming parasite, the myxozoan Aurantiactinomyxon-type actinosporean stage infecting the oligochaete *Tubifex tubifex* (Morris and Freeman, 2010). In this case the microsporidian infects both the actinosporean early stage bi-nucleate cells as well as oligochaete cells themselves. The infection progressively inhibits spore formation, unlike the case with *H. aquatica* where spore formation of the paramyxean host does not seems to be inhibited.

Numerous studies have proposed a role for copepods in the life cycle of sister parasite *M. refringens*. Audemard *et al.* (2002) and later Boyer *et al.* (2013) demonstrated that the pathogen could be detected in the calanoid copepod *Paracartia grani* via PCR. Furthermore, the pathogen appeared to be localized to ovarian tissues using ISH. Carrasco *et al.* (2007) extended the potential range of intermediate hosts to the orders Cyclopoida and Harpaticoida in a region where *Marteilia* is endemic within natural stocks of molluscs. Boyer *et al.* (2013) demonstrated that infection prevalence peaks in copepods corresponded to peaks in prevalence in molluscs from the same locations and further, that up to 90% of the planktonic stages of copepods (including adults, nauplii and eggs) were retained during filter-feeding in mussels. Finally, Carrasco *et al.* (2008) showed using ISH that the presence of initial infective stages of *M. refringens* within the gill and mantle epithelia is strong evidence for acquisition of paramyxean parasite infection via filter feeding (see also Kleeman *et al.* 2002). The combined evidence from these studies implies that in addition to potential for direct transmission, *Marteilia* can infect intermediate hosts (such as

copepods) and that the parasite may be released from, or contained within, intermediate host life stages (including eggs and larvae) prior to re-infection of susceptible molluscan hosts.

Given that *H. aquatica* is also likely to be liberated to the environment via the cockle gut, along with infective life stages of *M. cochillia*, it is conceivable that the microsporidian could be similarly transmitted to copepod (or other crustacean) hosts via the aforementioned route. Supporting evidence for the co-transmission theory arises from studies on the sister paramyxid *Paramarteilia* sp. in amphipod hosts. Here, although molluscan hosts have not yet been implicated in transmission cycles of *Paramarteilia*, high co-occurrence of microsporidian and paramyxean pathogens have been reported in *Echinogammarus marinus* (Short *et al*. 2012). In this case, unpublished work from our laboratory has shown that the microsporidian does not hyperparasitize the paramyxid but rather, the parasites form two separate infections at different locations within the same individual. In this instance, if both pathogens did enter the amphipod simultaneously (as proposed for the *M. cochillia*/*H. aquatica* model), the pathogens form separate infections thereafter. In other cases (e.g. *Paramarteilia canceri* infecting the European edible crab *Cancer pagurus*), although no consistent associations between paramyxid and microsporidian infection have been reported (Feist *et al*. 2009), several microsporidian genera are known to co-infect this crab host (Stentiford *et al*. 2007, 2008, 2011).

Based upon SSU rRNA sequence data, *H. aquatica* is most closely related (99% sequence similarity) to an undesignated microsporidian infecting a marine parasitic copepod *L. hospitalis* (Jones *et al*. 2012). Interestingly, cross-taxa transmission has already been demonstrated for another microsporidian parasite (*D. lepeophtherii* (=*Paranucleospora theridion*) from its ectoparasitic copepod host (*Lepeophtheirus salmonis*) to fish (Nylund *et al*. 2011). Although it is beyond the remit of the current study, apparent synonymy between *H. aquatica* and the '*Microsporidium* sp.' infecting *Lepeophtheirus* sp. on flounder raises the potential for further cross-taxa transmission of microsporidians such as *H. aquatica* from protists (within bivalves) to crustaceans. Extending this concept to the study of three host transmission (protist, crustacean, fish) is worthy of additional consideration, particularly at sites where *H. aquatica* is prevalent and where co-habiting fauna (crustaceans, fish) can be collected during environmental surveys.

Hyperspora does not group with basal microsporidians

In recent years, a consensus molecular phylogenetic-based viewpoint has emerged which affiliates the phylum Microsporidia with the Fungi (Lee *et al*. 2008; Capella-Gutiérrez *et al*. 2012). Whether they branch within (e.g. Tanabe *et al*. 2002) or outside of the group (Jones *et al*. 2011*a*, *b*; Capella-Gutierrez *et al*. 2012), however, has been a topic of debate. More recent multi-gene evidence places Cryptomycota (=Rozellomycota) as sister to fungi (James *et al*. 2013), and rRNA gene phylogenies show a relationship between this clade, 'core' fungi, and aphelids (intracellular parasitoids of algae) although their relative branching orders are unresolved (Karpov *et al*. 2013, 2014; Corsaro *et al*. 2014). Several new lineages have been characterized branching within or between Cryptomycota and Microsporidia: *Mitosporidium* (Haag *et al*. 2014), *Paramicrosporidium* (Corsaro *et al*. 2014) and *Nucleophaga* (Corsaro *et al*. 2014). *Mitosporidium* and *Paramicrosporidium* are morphologically intermediate between Cryptomycota and Microsporidia, whereas *Nucleophaga*, although similar to *Paramicrosporidium*, has fewer microsporidian characters, and are more *Rozella*-like (Corsaro *et al*. 2014). Interestingly, our rDNA phylogenetic analyses (Fig. 5), the first to simultaneously analyse a broad representation of environmental cryptomycotans together with all available characterized lineages, show that the evolution of (albeit rudimentary) infection machinery (e.g. polar filament, anchoring disc) in taxa such as *Mitosporidium* occurred prior to the more extreme genome reduction events typical of many (sequenced) members of the phylum Microsporidia *per se*, and that there is no clear phylogenetic pattern to the development of these characters within the cryptomycotan radiation. Clearly more sampling and characterization of lineages within Cryptomycota as currently circumscribed and those that might branch between these and the longer-branch microsporidians will be very important to understand the evolution of highly specialized modes of parasitism seen in the latter.

An interesting group, the so-called 'metchnikovellids' are conspicuously absent from modern phylogenies of Microsporidia and their basal lineages. These gregarine-infecting parasites are morphologically similar to both the intranuclear parasite of amoeba, *Paramicrosporidium* (Rotari *et al*. 2015) and, to the gut-infecting parasite of *Daphnia*, *Mitosporidium* (see Haag *et al*. 2014). Their affiliation with Microsporidia, and their proposed 'basal' position was stated early by Vivier (1965) and later, by Sprague (1977), who suggested in fact that they may represent rudimentary forms (at the time, erecting the class Rudimicrosporea, consist of a single order, the Metchnikovellida). Whilst the literature tentatively reports SSU rDNA sequence data to support an affiliation of the metchnikovellids with the aforementioned basal groups (Simdianov *et al*. 2009), primary data are absent. Nevertheless, morphological similarities between representatives (such as *Metchnikovella incurvata* infecting

gregarines, Sokolova *et al*. 2013) and other members of this basal group infecting other single-celled eukaryotes (e.g. amoebae and algae), including *Mitosporidium* and *Paramicrosporidium* suggest that metchnikovellids branch within the cryptomycota radiation as defined by Karpov *et al*. (2014). These cases clearly contrast with the phylogenetic position and description of *H. aquatica* provided in this paper. In the latter, not only does meront, sporont and spore morphology fall within the standard variation observed across the phylum Microsporidia, but also, rRNA-based phylogenies place it well within a previously defined microsporidian group (Clade 5).

In summary, we demonstrate the presence of non-basal lineages of Microsporidia, morphologically and phylogenetically similar to taxa infecting aquatic crustaceans and fish, infecting paramyxid and digenean parasites of molluscs. Our results propose that these hyperparasites may have exploited the parasitic lifestyle of their hosts to 'hitch hike' between hosts at different trophic levels. Further, it is proposed that those cases of paramyxean and digenean hyperparasites reported herein represent more recent acquisitions than those infecting other hosts within the developing 'basal' clade of the Cryptomycota.

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REFERENCES

Arzul, I., Chollet, B., Boyer, S., Bonnet, D., Gaillard, J., Baldi, Y., Robert, M., Joly, Y., Garcia, J.P. and Bouchoucha, M. (2014). Contribution to the understanding of the cycle of the protozoan parasite *Marteilia refringens*. *Parasitology* **141**, 227–240.

Audemard, C., Le, R.F., Barnaud, A., Collins, C., Sautour, B., Sauria, P.G., de, M.X., Coustau, C., Combes, C. and Berthe, F. (2002). Needle in a haystack: involvement of the copepod *Paracartia grani* in the life-cycle of the oyster pathogen *Marteilia refringens*. *Parasitology* **124**, 315–323.

Ba, C.T., Toguebaye, B.S. and Marchand, B. (2007). Ultrastructure of *Nosema* sp., microsporidia hyperparasite of *Bothriocephalus clavipes* (Cestoda, Bothriocephalidae) parasite of intestine of *Anguilla anguilla* (Pisces, Teleostei) from Corse, France. *Journal of Eukaryotic Microbiology* **54**, 24S.

Bass, D., Chao, E.E.Y., Nikolaev, S., Yabuki, A., Ishida, K-I., Berney, C., Pakzad, U., Wylezich, C. and Cavalier-Smith, T. (2009). Phylogeny of novel naked filose and reticulose Cercozoa: Granofilosea cl. n. and Proteomyxidea revised. *Protist* **160**, 75–109.

Boyer, S., Chollet, B., Bonnet, D. and Arzul, I. (2013). New evidence for the involvement of *Paracartia grani* (Copepoda, Calanoida) in the life cycle of *Marteilia refringens* (Paramyxea). *International Journal for Parasitology* **43**, 1089–1099.

Burki, F., Corradi, N., Sierra, R., Pawlowski, J., Meyer, G.R., Abbott, C.L. and Keeling, P.J. (2013). Phylogenomics of the intracellular parasite *Mikrocytos mackini* reveals evidence for a mitosome in Rhizaria. *Current Biology* **23**, 1541–1547.

Cable, J. and Tinsley, R.C. (1992). Microsporidian hyperparasites and bacteria associated with *Pseudodiplorchis americanus* (Monogenea, Polystomatidae). *Canadian Journal of Zoology* **70**, 523–529.

Canning, E.U. and Nicholas, J.P. (1974). Light and electron microscopy observations on *Unikaryon legeri* (Microsporida, Nosematidae), a parasite of the metacercaria of *Meigymnophallus minutus* in *Cardium edule*. *Journal of Invertebrate Pathology* **23**, 92–100.

Canning, E.U., Lai, P.F. and Lie, K.J. (1974). Microsporidian parasite of trematode larvae from aquatic snails in West Malaya. *Journal of Protozoology* **21**, 19–25.

Capella-Gutiérrez, S., Marcet-Houben, M., Gabaldón, T. (2012). Phylogenomics supports microsporidia as the earliest diverging clade of sequenced fungi. *BMC Biology* **10**, 47.

Carrasco, N., López-Flores, I., Alcaraz, M., Furones, M.D., Berthe, F.C.J. and Arzul, I. (2007). First record of a *Marteilia* parasite (Paramyxea) in zooplankton populations from a natural estuarine environment. *Aquaculture* **269**, 63–70.

Carrasco, N., Arzul, I., Berthe, F.C.J. and Furones, M.D. (2008). *In situ* hybridization detection of initial infective stages of *Marteilia refringens* (Paramyxea) in its host *Mytilus galloprovincialis*. *Journal of Fish Diseases* **31**, 153–157.

Carrasco, N., Hine, M., Durfort, M., Andree, K.B., Malchus, N., Lacuesta, B., González, M., Roque, A., Rodgers, C. and Furones, M.D. (2013). *Marteilia cochillia* sp. nov., a new *Marteilia* species affecting the edible cockle *Cerastoderma edule* in European waters. *Aquaculture* **413–414**, 223–230.

Caullery, M. and Mesnil, F. (1897). Sur un type nouveau (*Metchnikovella* n.g.) d'organismes parasites des grégarines. *Comptes Rendus de Seances de la Societe de Biologie, Paris* **49**, 960–962.

Caullery, M. and Mesnil, F. (1914). Sur les Metchnikovellidae et autre protistes des grégarines d'annélides. *Comptes Rendus de Seances de la Societe de Biologie, Paris* **77**, 527–532.

Chatton, E. (1911). Sur une Cnidosporidie sans cnidoblaste (*Paramyxa paradoxa*, n.g., n.sp.). *Comptes Rendus de Seances de la Societe de Biologie, Paris* **152**, 631–633.

Comps, M., Grizel, H., Tigé, G. and Duthoit, J.L. (1975). Parasites nouveaux de la glande digestive des mollusques marins *Mytilus edulis* L. et *Cardium edule*. *Comptes Rendus de Seances de la Societe de Biologie, Paris* **281**, 179–181.

Comps, M., Pichot, Y. and Deltreil, Y-P. (1979). Mise en evidence d'une microsporidie parasite de *Marteilia refringens* agent de la maladie de La glande digestive de *Ostrea edulis* L. *Rev. Trav. Inst. Pêches marit.* **43**, 409–412.

Corsaro, D., Walochnik, J., Venditti, D., Steinmann, J., Müller, K-D. and Michel, R. (2014). Microsporidia-like parasites of amoebae belong to the early fungal lineage Rozellomycota. *Parasitology Research* **113**, 1909–1918.

Czaker, R. (1997). *Wittmannia antarctica* n.g., n.sp. (Nosematidae), a new hyperparasite in the Antarctic dicyemid mesozoan *Kantharella antarctica*. *Journal of Eukaryotic Microbiology* **44**, 438–446.

de Buron, I., Loubès, C. and Maurand, J. (1990). Infection and pathological alterations within the acanthocephalan *Acanthocephaloides propinquus* attributable to the microsporidian hyperparasite *Microsporidium acanthocephali*. *Transactions of the American Microscopical Society* **109**, 91–97.

Diamant, A. and Paperna, I. (1985). The development and ultrastructure of *Nosema ceratomyxae* sp. nov., a microsporidian hyperparasite of the myxosporean *Ceratomyxa* sp. from Red Sea rabbitfish (Siganidae). *Protistologica* **21**, 249–258.

Feist, S.W., Hine, P.M., Bateman, K.S., Stentiford, G.D. and Longshaw, M. (2009). *Paramarteilia canceri* sp. n. (Cercozoa) in the European edible crab (*Cancer pagurus*) with a proposal for the revision of the order Paramyxida Chatton, 1911. *Folia Parasitologia* **56**, 73–85.

Foissner, I. and Foissner, W. (1995). *Ciliatosporidium platyophryae* nov. gen., nov. spec. (Microspora, Encephalitozoonidae), a parasite of *Platyophrya terricola* (Ciliophora, Colpodea). *European Journal of Protistology* **31**, 248–259.

Fokin, S.I., Di Giuseppe, G., Erra, F. and Dini, F. (2008). *Euplotespora binucleata* n. gen., n. sp. (Protozoa: Microsporidia), a parasite infecting the hypotrichous ciliate *Euplotes woodruffi*, with observations on microsporidian infections in ciliophora. *Journal of Eukaryotic Microbiology* **55**, 214–228.

Freeman, M. A., Bell, A. S. and Sommerville, C. (2003). A hyperparasitic microsporidian infecting the salmon louse, *Lepeophtheirus salmonis*: an rDNA-based molecular phylogenetic study. *Journal of Fish Diseases* **26**, 667–676.

Gatehouse, H. S. and Malone, L. A. (1998). The ribosomal RNA gene region of *Nosema apis* (Microspora): DNA sequence for small and large subunit rRNA genes and evidence of a large tandem repeat unit size. *Journal of Invertebrate Pathology* **71**, 97–105.

Ginsburger-Vogel, T. (1991). Intersexuality in Orchestia mediterranea Costa, 1853, and Orchestia aestuarensis Wildish, 1987 (Amphipoda): a consequence of hybridization or parasitic infestation? *Journal of Crustacean Biology* **11**, 530–539.

Ginsburger-Vogel, T. and Desportes, I. (1979). Structure and biology of *Marteilia* sp. in the amphipod *Orchestia gammarellus*. *Marine Fisheries Review* **41**, 3–7.

Grizel, H., Comps, M., Bonami, J. R., Cousserans, F., Duthoit, J. L. and Le Pennec, M. A. (1974). Recherche sur l'agent de la maladie de la glande digestive de *Ostrea edulis* Linné. *Sciences Pêche* **240**, 7–29.

Haag, K. L., James, T. Y., Pombert, J-F., Larsson, R., Schaer, T. M. M., Refardt, D. and Ebert, D. (2014). Evolution of a morphological novelty occurred before genome compaction in a lineage of extreme parasites. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 15480–15485.

Hartikainen, H., Ashford, O. S., Berney, C., Okamura, B., Feist, S. W., Baker-Austin, C., Stentiford, G. D. and Bass, D. (2014*a*). Lineage-specific molecular probing reveals novel diversity and ecological partitioning of haplosporidians. *ISME Journal* **8**, 177–186.

Hartikainen, H., Stentiford, G. D., Bateman, K. S., Berney, C., Feist, S. W., Longshaw, M., Okamura, B., Stone, D., Ward, G., Wood, C. and Bass, D. (2014*b*). Mikrocytids are a broadly distributed and divergent radiation of parasites in aquatic invertebrates. *Current Biology* **24**, 807–812.

Haskin, H. H., Stauber, L. A. and Mackin, J. A. (1966). *Minchinia nelsoni* n. sp. (Haplosporida, Haplosporidiidae): causative agent of the Delaware Bay oyster epizootic. *Science* **153**, 1414–1416.

Hopwood, D. (1996). Theory and practice of histopathological techniques. In *Fixation and Fixatives*, 4th Edn (ed. Bamcroft, J. D. and Stevens, A.), pp. 23–46. Churchill Livingstone, Hong Kong.

James, T. Y., Pelin, A., Bonen, L., Ahrendt, S., Sain, D., Corradi, N. and Stajich, J. E. (2013). Shared signatures of parasitism and phylogenomics unite Cryptomycota and Microsporidia. *Current Biology* **23**, 1548–1553.

Jones, M. D. M., Forn, I., Gadelha, C., Egan, M. J., Bass, D., Massana, R. and Richards, T. A. (2011*a*). Discovery of novel intermediate forms redefines the fungal tree of life. *Nature* **474**, 200–203.

Jones, M. D., Richards, T. A., Hawksworth, D. L. and Bass, D. (2011*b*). Validation and justification of the phylum name Cryptomycota phyl. nov. *IMA Fungus* **2**, 173–175.

Jones, S. R., Prosperi-Porta, G. and Kim, E. (2012). The diversity of Microsporidia in parasitic copepods (Caligidae: Siphonostomatoida) in the Northeast Pacific Ocean with description of *Facilispora margolisi* n. g., n. sp and a new family Facilisporidae n. fam. *Journal of Eukaryotic Microbiology* **59**, 206–217.

Karpov, S. A., Mikhailov, K. V., Mirzaeva, G. S., Mirabdullaev, I. M., Mamkaeva, K. A., Titova, N. N. and Aleoshin, V. V. (2013). Obligately phagotrophic aphelids turned out to branch with the earliest-diverging Fungi. *Protist* **164**, 195–205.

Karpov, S. A., Mamkaeva, M. A., Aleoshin, V. V., Nassonova, E., Lilje, O. and Gleason, F. H. (2014). Morphology, phylogeny, and ecology of the aphelids (Aphelidea, Opisthokonta) and proposal for the new superphylum Opisthosporidia. *Frontiers in Microbiology* **5**, 112.

Katoh, K. and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30**, 772–780.

Kleeman, S. N., Adlard, R. D. and Lester, R. J. G. (2002). Detection of the initial infective stages of the protozoan parasite *Marteilia sydneyi* in *Saccostrea glomerata* and their development through to sporogenesis. *International Journal for Parasitology* **32**, 767–784.

Larsson, J. I. R. (1987). On *Haplosporidium gammari*, a parasite of the amphipod *Rivulogammarus pulex*, and its relationships with the phylum Ascetospora. *Journal of Invertebrate Pathology* **49**, 159–169.

Larsson, J. I. R. (2000). The hyperparasitic microsporidium *Amphiacantha longa* Caullery et Mesnil, 1914 (Microspora: Metchnikovellidae) – description of the cytology, redescription of the species, emended diagnosis of the genus Amphiacantha and establishment of the new family Amphiacanthidae. *Folia Parasitologia* **47**, 241–256.

Larsson, J. I. R. and Koie, M. (2006). The ultrastructure and reproduction of *Amphiamblys capitellides* (Microspora, Metchnikovellidae), a parasite of the gregarine *Ancora sagittata* (Apicomplexa, Lecudinidae), with redescription of the species and comments on the taxonomy. *European Journal of Protistology* **42**, 233–248.

Lee, S. C., Corradi, N., Byrnes, E. J., Torres-Martinez, S., Dietrich, F. S., Keeling, P. J. and Heitmand, J. (2008). Microsporidia evolved from ancestral sexual fungi. *Current Biology* **18**, 1675–1679.

Lefèvre, T., Lebarbenchon, C., Gauthier-Clerc, M., Missé, D., Poulin, R. and Thomas, F. (2008) The ecological significance of manipulative parasites. *Trends in Ecology and Evolution* **24**, 41–48.

Levron, C., Ternengo, S., Toguebaye, B. S. and Marchand, B. (2004). Ultrastructural description of the life cycle of *Nosema diphterostomi* sp. n., a microsporidia hyperparasite of *Diphterostomum brusinae* (Digenea: Zoogonidae), intestinal parasite of *Diplodus annularis* (Pisces: Teleostei). *Acta Protozoologica* **43**, 329–336.

Levron, C., Ternengo, S., Toguebaye, B. S. and Marchand, B. (2005). Ultrastructural description of the life cycle of *Nosema monorchis* n. sp. (Microspora, Nosematidae), hyperparasite of *Monorchis parvus* (Digenea, Monorchiidae), intestinal parasite of *Diplodus annularis* (Pisces, Teleostei). *European Journal of Protistology* **41**, 251–256.

Miller, M. A., Pfeiffer, W., and Schwartz, T. (2010). Creating the CIPRES science gateway for inference of large phylogenetic trees. In *Proceedings of the Gateway Computing Environments Workshop (GCE)*, 14 November 2010, New Orleans, LA, pp. 1–8.

Morris, D. J. and Freeman, M. A. (2010). Hyperparasitism has wide-ranging implications for studies on the invertebrate phase of myxosporean (Myxozoa) life cycles. *International Journal for Parasitology* **40**, 357–369.

Nylund, S., Andersen, L., Saevareid, I., Plarre, H., Watanabe, K., Arnesen, C. E., Karlsbakk, E. and Nylund, A. (2011). Diseases of farmed Atlantic salmon Salmo salar associated with infections by the microsporidian *Paranucleospora theridion*. *Diseases of Aquatic Organisms* **94**, 41–57.

OIE (2014). Diagnostic Manual for Aquatic Animal Diseases. http://www.oie.int/international-standard-setting/aquatic-manual/access-online/.

Ramilo, A., Abollo, E. and Villalba, A. (2014). Species-specific oligonucleotide probe for detection of *Bonamia exitiosa* (Haplosporidia) using *in situ* hybridisation assay. *Diseases of Aquatic Organisms* **110**, 81–91.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A. and Huelsenbeck, J. P. (2012). MrBayes 3·2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systems Biology* **61**, 539–542.

Rotari, Y. M., Paskerova, G. G. and Sokolova, Y. Y. (2015). Diversity of metchnikovellids (Metchnikovellidae, Rudimicrosporea), hyperparasites of bristle worms (Annelida, Polychaeta) from the White Sea. *Protistology* **9**, 50–59.

Rozen, S. and Skaletsky, H. J. (2000). Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (ed. Krawetz, S. and Misener, S.), pp. 365–386. Humana Press, Totowa, NJ.

Scheid, P. (2007). Mechanism of intrusion of a microspordian-like organism into the nucleus of host amoebae (*Vannella* sp.) isolated from a keratitis patient. *Parasitology Research* **101**, 1097–1102.

Sene, A., Ba, C. T., Marchand, B. and Toguebaye, B. S. (1997). Ultrastructure of *Unikaryon nomimoscolexi* n. sp. (Microsporida, Unikaryonidae), a parasite of *Nomimoscolex* sp. (Cestoda, Proteocephalidea) from the gut of *Clarotes laticeps* (Pisces, Teleoste, Bagridae). *Diseases of Aquatic Organisms* **29**, 35–40.

Short, S., Guler, Y., Yang, G., Kille, P. and Ford, A. T. (2012). Paramyxean microsporidian co-infection in amphipods: is the consensus that Microsporidia can feminise their hosts presumptive? *International Journal for Parasitology* **42**, 683–691.

Simdianov, T., Yudina, V. and Aleoshin, V. (2009). First data on molecular phylogeny of microsporidian family Metchnikovellidae. In *Proceedings of the XIII International Congress of Protistology*, Armacao dos Buzios, Brasil, p. 144.

Sokolova, Y. Y., Paskerova, G. G., Rotari, Y. M., Nassonova, E. S. and Smirnov, A. V. (2013). Fine structure of *Metchnikovella incurvata* Caullery and Mesnil 1914 (microsporidia), a hyperparasite of gregarines *Polyrhabdina* sp. from the polychaete *Pygospio elegans*. *Parasitology* **140**, 855–867.

Sprague, V. (1977). Annotated list of species of microsporidia. In *Comparative Pathobiology*, Vol. 2 (ed. Bulla, L. E. and Cheng, T. C.), pp. 31–334. Plenum Press, New York and London.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**, 1312–1313.

Stentiford, G. D. (2008). Diseases of the European edible crab (*Cancer pagurus*): a review. *ICES Journal of Marine Science* **65**, 1578–1592.

Stentiford, G. D., Bateman, K. S., Longshaw, M. and Feist, S. W. (2007). *Enterospora canceri* n. gen., n. sp., intranuclear within the hepatopancreatocytes of the European edible crab *Cancer pagurus*. *Diseases of Aquatic Organisms* **75**, 61–72.

Stentiford, G. D., Bateman, K. S., Small, H. J., Moss, J., Shields, J. D., Reece, K. S. and Tuck, I. (2010). *Myospora metanephrops* (n. g., n. sp.) from marine lobsters and a proposal for erection of a new order and family (Crustaceacida; Myosporidae) in the class Marinosporidia (Phylum Microsporidia). *International Journal for Parasitology* **40**, 1433–1446.

Stentiford, G. D., Bateman, K. S., Dubuffett, A. and Stone, D. (2011). *Hepatospora eriocheir* (Wang and Chen, 2007) gen. et comb. nov infecting invasive Chinese mitten crabs (*Eriocheir sinensis*) in Europe. *Journal of Invertebrate Pathology* **108**, 156–166.

Stentiford, G. D., Bateman, K. S., Feist, S. W., Stone, D. M. and Dunn, A. M. (2013). Microsporidia: diverse, dynamic and emergent pathogens in aquatic systems. *Trends in Parasitology* **29**, 567–578.

Tanabe, Y., Watanabe, M. M. and Sugiyamaaff, J. (2002). Are Microsporidia really related to Fungi?: a reappraisal based on additional gene sequences from basal fungi. *Mycology Research* **106**, 1380–1391.

Thomas, F., Renaud, F. and Poulin, R. (2008). Exploitation of manipulators: 'hitch-hiking' as a parasite transmission strategy. *Animal Behaviour* **56**, 199–206.

Villalba, A., Mourelle, S. G., Carballal, M. J. and Lopez, C. (1997). Symbionts and diseases of farmed mussels *Mytilus galloprovincialis* throughout the culture process in the Rias of Galicia (NW Spain). *Diseases of Aquatic Organisms* **19**, 127–139.

Villalba, A., Iglesias, D., Ramilo, A., Darriba, S., Parada, J. M., No, E., Abollo, E., Molares, J. and Carballal, M. J. (2014). Cockle *Cerastoderma edule* fishery collapse in the Ría de Arousa (Galicia, NW Spain) associated with the protistan parasite *Marteilia cochillia*. *Diseases of Aquatic Organisms* **109**, 55–80.

Vinckier, D. (1975). *Nosemoides* gen. n., *N. vivieri* (Vinckier, Devauchelle & Prensier, 1970) comb. nov. (Microsporidie); etude de la différentiation sporoblastique et genèse des différentes structures de la spore. *Journal of Protozoology* **22**, 170–184.

Vivier, E. (1965). Étude, au microscope électronique, de la spore de *Metchnikovella hovassei* n. sp.: appartenance des Metchnikovellidae aux Microsporidies. *Comptes Rendus de Seances de la Societe de Biologie, Paris* **260**, 6982–6984.

Vossbrinck, C. R. and Debrunner-Vossbrinck, B. A. (2005) Molecular phylogeny of the Microsporidia: ecological, ultrastructural and taxonomic considerations. *Folia Parasitologia* **52**, 131–142.

Wang, T. C., Nai, Y. S., Wang, C. Y., Solter, L. F., Hsu, H. C., Wang, C. H. and Lo, C. F. (2013). A new microsporidium, *Triwangia caridinae* gen. nov., sp. nov. parasitizing fresh water shrimp, *Caridina formosae* (Decapoda: Atyidae) in Taiwan. *Journal of Invertebrate Pathology* **112**, 281–293.

Ward, G., Bennett, M., Bateman, K. S., Stentiford, G. D., Feist, S. W., Williams, S., Berney, C. and Bass, D. (2016). A new phylogeny and eDNA insight into paramyxids: an increasingly important but enigmatic clade of protistan parasites of marine invertebrates. *International Journal for Parasitology* **10**, 605–619.

Weiss, L. M., Zhu, X., Cali, A., Tanowitz, H. B. and Wittner, M. (1994). Utility of microsporidian rRNA in diagnosis and phylogeny: a review. *Folia Parasitologia* **41**, 81–90.

Yang, G., Short, S., Kille, P. and Ford, A. T. (2011). Microsporidia infections in the amphipod, *Echinogammarus marinus* (Leach): suggestions of varying causal mechanisms to intersexuality. *Marine Biology* **158**, 461–470.