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Parahepatospora carcini n. gen., n. sp., a parasite of invasive *Carcinus maenas* with intermediate features of sporogony between the *Enterocytozoon* clade and other microsporidia

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ABSTRACT

Parahepatospora carcini n. gen. n. sp., is a novel microsporidian parasite discovered infecting the cytoplasm of epithelial cells of the hepatopancreas of a single *Carcinus maenas* specimen. The crab was sampled from within its invasive range in Atlantic Canada (Nova Scotia). Histopathology and transmission electron microscopy were used to show the development of the parasite within a simple interfacial membrane, culminating in the formation of unikaryotic spores with 5–6 turns of an isofilar polar filament. Formation of a multinucleate meront (>12 nuclei observed) preceded thickening and invagination of the plasmodial membrane, and in many cases, formation of spore extrusion precursors (polar filaments, anchoring disk) prior to complete separation of pre-sporoblasts from the sporogonial plasmodium. This developmental feature is intermediate between the Enterocytozoonidae (formation of spore extrusion precursors within the sporont plasmodium) and all other Microsporidia (formation of spore extrusion precursors after separation of sporont from the sporont plasmodium). SSU rRNA-based gene phylogenies place *P. carcini* within microsporidian Clade IV, between the Enterocytozoonidae and the so-called *Enterocytospora*-clade, which includes *Enterocytospora artemiae* and *Globulispora mitoportans*. Both of these groups contain gut-infecting microsporidians of aquatic invertebrates, fish and humans. According to morphological and phylogenetic characters, we propose that *P. carcini* occupies a basal position to the Enterocytozoonidae. We discuss the discovery of this parasite from a taxonomic perspective and consider its origins and presence within a high profile invasive host on the Atlantic Canadian coastline.

1. Introduction

Microsporidia are a highly diverse group of obligate intracellular parasites, belonging to a sister clade to the Fungi Kingdom, which also includes the Aphelids and Cryptomycota (Haag et al., 2014; Corsaro et al., 2014; Karpov et al., 2015). Their diversity remains highly under-sampled, but known microsporidia infect a wide array of host taxa, many of which occur in aquatic habitats (Stentiford et al., 2013a,b). Molecular-phylogenetic approaches are not only clarifying the position of the Microsporidia amongst the eukaryotes, but are also increasingly defining within-phylum taxonomy (Stentiford et al., 2016).

Microsporidian phylogenies built upon ribosomal gene sequence data have led to proposals for five taxonomically distinctive microsporidian clades (I, II, III, IV, V), each of which can be

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further aligned to three broad ecological groupings; the Marinosporidia (V); Terresporidia (II, IV); and Aquasporidia (I, III) (Vossbrinck and Debrunner-Vossbrinck, 2005). Clade IV forms a particularly interesting group due to the fact that it contains the family Enterocytozoonidae, where all known taxa infect aquatic invertebrates or fish hosts; with the exception of a single species complex (*Enterocytozoon bieneusi*). *Enterocytozoon bieneusi* is the most common microsporidian pathogen infecting immune-suppressed humans (Stentiford et al., 2013a,b; Stentiford et al., 2016). Other genera within the Enterocytozoonidae include: *Desmozoon* (=*Paranucleospora*), *Obruspora, Nucleospora, and Enterospora*. Other species, such as *Enterocytozoon hepatopenaei*, which infect fish and shrimp, appear to have been assigned to the genus Enterocytozoon erroneously, using relatively low SSU sequence similarity (~88%) and similar development pattern contrary to a closer SSU sequence similarity to the Enterospora genus (~93%) (Tourtip et al., 2009). Based upon its phylogenetic position, *E. bieneusi* is almost certainly a zoonotic pathogen of humans, likely with origins in aquatic habitats (Stentiford et al., 2016). This makes the phylogeny of existing and novel microsporidians within, and related to, the family Enterocytozoonidae is an intriguing research topic. Aquatic crustaceans may offer a likely evolutionary origin to current day human infections by *E. bieneusi* (Stentiford et al., 2016).

The microsporidium *Hepatospora eriocheir* was recently discovered infecting the hepatopancreas of aquatic crustaceans (Stentiford et al., 2011; Bateman et al., 2016). Morphological characters and phylogenetic analysis found that *H. eriocheir* was related to the Enterocytozoonidae; grouping as a sister group to this family on SSU rRNA gene trees (Stentiford et al., 2011). *Hepatospora eriocheir* displayed somewhat intermediate characters between the Enterocytozoonidae and all other known taxa (e.g. potential to form spore extrusion precursors in bi-nucleate sporonts prior to their separation and, to uninucleate sporoblast and spore formation) even though the distinctive morphological characters of the Enterocytozoonidae were not observed (e.g. presence of spore extrusion precursors in multi-nucleate sporonts). Spore extrusion precursors develop after final separation of pre-sporoblasts from sporont plasmodia in all other microsporidians. The discovery of the genus *Hepatospora* led our laboratory to propose a sister family to the Enterocytozoonidae with intermediate traits between this family and other existing taxa. The family was tentatively assigned as the Hepatosporidae with *H. eriocheir* (and the newly erected genus *Hepatospora*), as its type member, pending discovery of further members (Stentiford et al., 2011).

This study describes a novel microsporidian infecting the hepatopancreas of *Carcinus maenas* (European shore crab, or invasive green crab), commonly referred to as the green crab in North America, collected from within its invasive range in Nova Scotia, Canada as part of ongoing studies to investigate diversity and origins of the families Enterocytozoonidae and Hepatosporidae in aquatic invertebrate hosts. We determined that this parasite falls at the base of the Enterocytozoonidae, Enterocytospora-like clade and the tentatively proposed Hepatosporidae based upon morphological, ultrastructural and phylogenetic evidence. The new parasite is distinct from *Abelspora portucalensis* (a previously described microsporidian infecting the hepatopancreas of *C. maenas,* but without available genetic data), and three other microsporidians, known to infect *C. maenas* from its native range in Europe (Sprague and Couch, 1971; Azevedo, 1987; Stentiford et al., 2013a,b). Given that the new parasite was not discovered within its host's native range, it is possible that it represents a case of parasite acquisition from the host community in which this non-native crab now resides. We erect the genus *Parahepatospora* n. gen. and species *Parahepatospora carcini* n. sp. to contain this novel parasite.

2. Materials and methods

2.1. Sample collection

Carcinus maenas were sampled from Malagash Harbour on the north shore of Nova Scotia, Canada (45.815154, −63.473768) on 26/08/2014 using a mackerel-baited Nickerson green crab trap. In total, 134 *C. maenas* were collected from this site and transported to the Dalhousie University Agricultural Campus where they were kept overnight in damp conditions. Animals were euthanized, then necropsied with muscle, hepatopancreas, heart, gonad and gill tissue, preserved for DNA extraction (100% ethanol), transmission electron microscopy (2.5% glutaraldehyde) and histopathology (Davidson's saltwater fixative) using protocols defined by the European Union Reference Laboratory for Crustacean Diseases (www.crustaceancrl.eu).

2.2. Histology

Tissues were submerged in Davidson's saltwater fixative (Hopwood, 1996) for 24–48 h then immersed in 70% ethanol prior to transportation to the Cefas Weymouth Laboratory, UK. Samples were prepared for histological analysis by wax infiltration using a robotic tissue processor (Peloris, Leica Microsystems, United Kingdom) before being embedded into wax blocks. Specimens were sectioned a single time at 3–4 μm (Finesse E/NE rotary microtome) and placed onto glass slides, prior to staining with haematoxylin and alcoholic eosin (H&E). Data collection and imaging took place on a Nikon-integrated Eclipse (E800) light microscope and digital imaging software at the Cefas laboratory (Weymouth).

2.3. Transmission electron microscopy (TEM)

Glutaraldehyde-fixed tissue biopsies were soaked in sodium cacodylate buffer twice (10 min) and placed into 1% Osmium tetroxide (OsO_4) solution for 1 h. Osmium stained material underwent an acetone dilution series as follows: 10% (10 min); 30% (10 min); 50% (10 min); 70% (10 min); 90% (10 min); 100% (×3) (10 min). Samples where then permeated with Agar100 Resin using a resin:acetone dilution series: 1:4; 1:1; 4:1; 100% resin (×2). Each sample was placed into a cylindrical mould (1 cm^3) along with fresh resin and polymerised in an oven (60 °C) for 16 h. The resulting blocks were cropped to expose the tissue using a razor blade and sectioned at 1 μm thickness (stain: Toluidine Blue) using a glass knife before being read on an Eclipse E800 light microscope to confirm infection. Ultra-thin sections were taken at ~80 nm thickness using a diamond knife, stained with uranyl acetate and Reynolds lead citrate (Reynolds, 1963), and read/annotated on a Jeol JEM 1400 transmission electron microscope (Jeol, UK).

2.4. PCR and sequencing

DNA was extracted from ethanol-fixed samples of hepatopancreas using an automatic EZ1 DNA extraction kit (Qiagen). Primers: MF1 (5′-CCGGAGAGGGAGCCTGAGA-3′) and MR1 (5′-GACGGGCGG TGTGTACAAA-3′) (Tourtip et al., 2009), were used to amplify a fragment of the microsporidian SSU rRNA gene using a GoTaq flexi PCR reaction [1.25U of Taq polymerase, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 100pMol of each primer and 2.5 μl of DNA template (10–30 ng/μl) in a 50 μl reaction volume]. Thermocycler settings were as follows: 94 °C (1 min) followed by 30 cycles of 94 °C (1 min), 55 °C (1 min), 72 °C (1 min) and then a final 72 °C (10 min) step. Electrophoresis through a 2% Agarose gel (120 V, 45 min) was used to separate and visualise a resulting 939 bp amplicon. Amplicons were purified from the gel and sent for

forward and reverse DNA sequencing (Eurofins genomics sequencing services: https://www.eurofinsgenomics.eu/).

2.5. Phylogenetic tree construction

Several microsporidian sequences were downloaded from NCBI (GenBank), biased towards clade IV (Vossbrinck and Debrunner-Vossbrinck, 2005), but also including members of clade III, and the genus *Glugea* (clade V) as an out-group. BLASTn searches were used to retrieve the closest related sequences to the *C. maenas* parasite. The consensus sequence of the SSU rRNA gene of the new parasite (939nt) was added and aligned with the aforementioned dataset using the E-ins-I algorithm within mafft version 7 (Katoh and Standley, 2013). The resulting alignment, (65 sequences, 1812 positions analysed) was refined manually and analysed firstly using Maximum Likelihood (ML) in RAxML BlackBox version 8 (Stamatakis, 2014) [Generalized time-reversible (GTR) model with CAT approximation (all parameters estimated from the data)]; an average of 10,000 bootstrap values was mapped onto the tree with the highest likelihood value. A Bayesian consensus tree was then constructed using MrBayes v3.2.5 for a secondary comparative tree (Ronquist et al., 2012). Two separate MC^3 runs with randomly generated starting trees were carried out for 5 million generations, each with one cold and three heated chains. The evolutionary model used by this study included a GTR substitution matrix, a four-category auto-correlated gamma correction, and the covarion model. All parameters were estimated from the data. Trees were sampled every 1000 generations. The first 1.25 M generations were discarded as burn-in (trees sampled before the likelihood plots reached stationarity) and a consensus tree was constructed from the remaining sample. The 18S rDNA sequence generated by this study is available from NCBI (accession number: KX757849).

3. Results

3.1. Histopathology

Of the 134 individuals sampled from the shoreline at Malagash, a single individual (trap-caught male) was found to be parasitized by a microsporidian parasite targeting the epithelial cells of the hepatopancreatic tubules (1/134; <1%). At the time of dissection, the hepatopancreas of the infected individual appeared to be healthy without clearly-visible clinical signs of infection at the time of necropsy. Histopathological analysis revealed the microsporidian infection to be contained within the cytoplasm of infected hepatopancreatocytes (Fig. 1a–c). Presumed early life stages of the parasites (meronts and sporont plasmodia) stained dark blue/purple under H&E whilst apparent later life stages (sporoblasts, spores) became eosinophilic and refractile (Fig. 1b). In general, early life-stages of the parasite were observed to develop at the periphery of the infected cell while spores generally occupied more central positions (Fig. 1b). In late stages of cellular colonisation, infected host cells appeared to lose contact with neighbour cells and the basement membrane for presumed expulsion to the tubule lumen (hepatopancreatic tubules empty to the intestine) (Fig. 1c). Infected hepatopancreatic tubules appeared heavily degraded during late stage infection due to the sloughing of infected cells from the basal membrane (Fig. 1a–c).

3.2. Microsporidian ultrastructure and proposed lifecycle

All stages of the microsporidian parasite occurred within a simple interfacial membrane, which separated parasite development stages from the host cell cytoplasm. Earliest observed life stages, apparent uninucleate meronts, contained a thin cell membrane and were present at the periphery of the interfacial membrane (Fig. 2a). Unikaryotic meronts appeared to undergo nuclear division without cytokinesis, leading to a diplokaryotic meront, again occurring predominantly at the periphery of the interfacial membrane (Fig. 2b). Darkening of the diplokaryotic cell cytoplasm and separation of the adjoined nuclei, possibly via nuclear dissociation, preceded further nuclear divisions to form multinucleate meronts, with the greatest number of (visible) nuclei observed being 12 (Fig. 2c and d). The multinucleate plasmodia appear to invaginate and elongate (Fig. 2d). Following thickening of the multinucleate plasmodial wall, primary spore organelle formation (polar filament and anchoring disk precursors) occurred prior to the separation of pre-sporoblasts from the sporont plasmodium in most cases (primary pathway); only in a few cases were spore pre-curser organelles not present (Fig. 2e and f). Other sporonts appeared to progress to sporoblasts by forming precursor spore organelles after separation from the multinucleate sporont plasmodium. Each sporoblast contained a single nucleus (Fig. 2f). Sporoblasts displayed noticeable thickening of the endospore and electron lucent zones of their walls (Fig. 3a). Mature spores contained an electron dense cytoplasm and were oval shaped with a length of 1.50 μm ± 0.107 μm (n = 10) and a width of 1.12 μm ± 0.028 μm (n = 16). Spores were unikaryotic, and possessed a relatively thin spore wall, consisting of a thin endospore [39.21 nm ± 8.674 (n = 30)], exospore [26.47 nm ± 2.301 nm (n = 30)] and internal cell membrane. The polar filament was layered with electron lucent and electron dense rings resulting in an overall diameter of 64.18 nm ± 5.495 nm (n = 22). The polar filament underwent 5–6 turns (Fig. 3b–d) and was terminated with an anchoring disk [width: 292.20 nm ± 19.169 nm (n = 5)]. The endospore appeared slightly thinner in the vicinity of the anchoring disk. A highly membranous polaroplast and electron lucent polar vacuole were observed at the anterior and posterior of the spore, respectively (Fig. 3b–d). A depiction of the full lifecycle is presented in Fig. 4.

3.3. Phylogeny of the novel microsporidian infecting C. maenas

A single consensus DNA sequence (939 bp) from the microsporidian parasite was obtained and utilised to assess the phylogeny of the novel taxon. BLASTn results revealed the highest scored hit belonged to *Globulispora mitoportans* (KT762153.1; 83% identity; 99% coverage; total score = 815; e-value = 0.0). The closest overall identity match belonged to '*Microsporidium sp. BPAR2 TUB1*' (FJ756098.1; 85% identity; 57% coverage; total score = 527; e-value = 2e−145). This suggested that the new parasite belonged in Clade IV of the Microsporidia (Vossbrinck and Debrunner-Vossbrinck, 2005) but, with distinction from all described taxa to date.

Maximum Likelihood (ML) and Bayesian (Pp) analyses grouped the new parasite grouped within the Clade IV of the microsporidia and was positioned basally to the Enterocytozoonidae, *Enterocytospora*-like clade, putative Hepatosporidiae and other taxonomic families (indicated on Fig. 5), at weak confidence: 0.30 (ML) and 0.53 (Pp) (Fig. 5). This provides us with a rough estimate of its phylogeny but with little confidence as to its true position and association to the families represented in the tree.

A second tree representing microsporidian taxa that have been taxonomically described (including developmental, morphological and SSU rDNA sequence data) is presented in Fig. 6. This tree is annotated with developmental traits at the pre-sporoblastic (sporont) divisional level and identifies that *H. eriocheir* and *P. carcini* show intermediate development pathways between the Enterocytozoonidae and the Enterocytospora-like clade, supported weakly [0.38 (ML), 0.42 (Pp)] by the 18S phylogenetics. *Parahepatospora carcini* branched between the formally described *Agmasoma penaei*

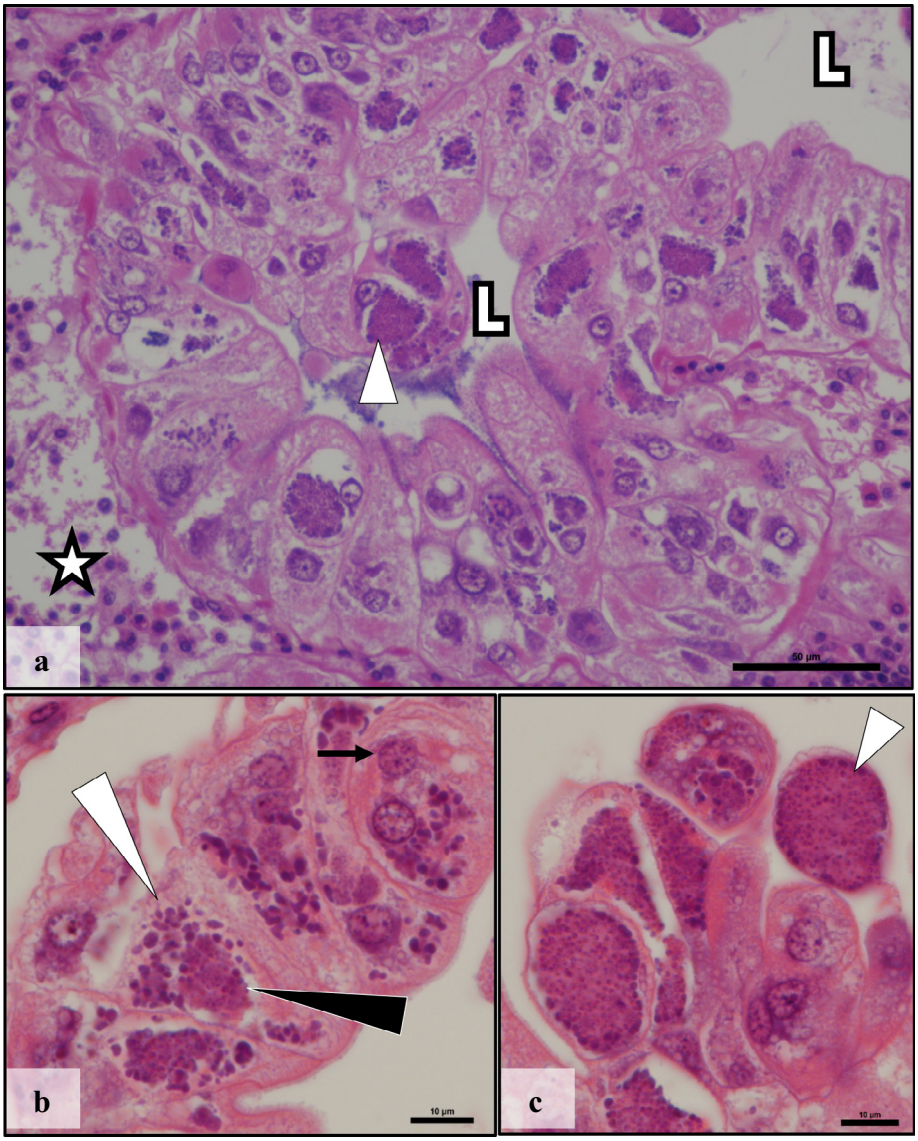

Fig. 1. Histology of a *Parahepatospora carcini* n. gen n. sp. infection in the hepatopancreas of *Carcinus maenas*. (a) A cross-section of an hepatopancreatic tubule infected with *P. carcini* (white arrow). The star indicates a blood vessel and 'L' represent the lumen of two tubules. Scale = 50 µm. (b) A high magnification image of early infected cells. Development of early sporonts occurs as the periphery of the cell cytoplasm (white arrow) and spores appear to aggregate in the centre (black arrow). Scale = 10 µm. (c) Cells can be seen sloughing from the basal membrane (white arrow) into the lumen, filled with microsporidian spores. Scale = 10 µm.

and *H. eriocheir*: both parasites of Crustacea but each with different developmental strategies at the pre-sporoblastic level (Fig. 6).

4. Taxonomic description

4.1. Higher taxonomic rankings

Super-group: Opisthokonta
Super-Phylum: Opisthosporidia (Karpov et al., 2015)
Phylum: Microsporidia (Balbiani, 1882)
Class: Terresporidia (Clade IV) (nomina nuda) (Vossbrinck and Debrunner-Vossbrinck, 2005)

4.2. Novel taxonomic rankings

Genus: *Parahepatospora* (Bojko, Clark, Bass, Dunn, Stewart-Clark, Stebbing, Stentiford gen. nov.)

Genus description: Morphological features are yet to be truly defined as this is currently a monotypic genus. Developmental characteristics may include: polar-filament development prior to budding from the multinucleate plasmodium; multinucleate cell formation; nuclear division without cytokinesis at the meront stage; and budding from a plasmodial filament, would increase the confidence of correct taxonomic placement. Importantly, sporonts (pre-sporoblasts) have the capacity to develop precursors of the spore extrusion apparatus prior to their separation from the sporont plasmodium. Novel taxa placed within this genus will likely have affinity to infect the hepatopancreas (gut) of their host and clade closely to the type species *P. carcini* (accession number: KX757849 serves as a reference sequence for this genus).

Type species: Parahepatospora carcini (Bojko, Clark, Bass, Dunn, Stewart-Clark, Stebbing, Stentiford sp. nov.)

Description: All life stages develop within a simple interfacial membrane in the cytoplasm of host cells. Spores appear oval shaped (L: 1.5 µm ± 0.107 µm, W: 1.1 µm ± 0.028 µm), and have an electron lucent endospore (thickness: 39.21 nm ± 8.674 nm) coupled with an electron dense exospore (thickness: 26.47 nm ± 2.3 nm) by TEM. The polar filament turns 5–6 times and the polaroplast of the spore is highly membranous. The spores

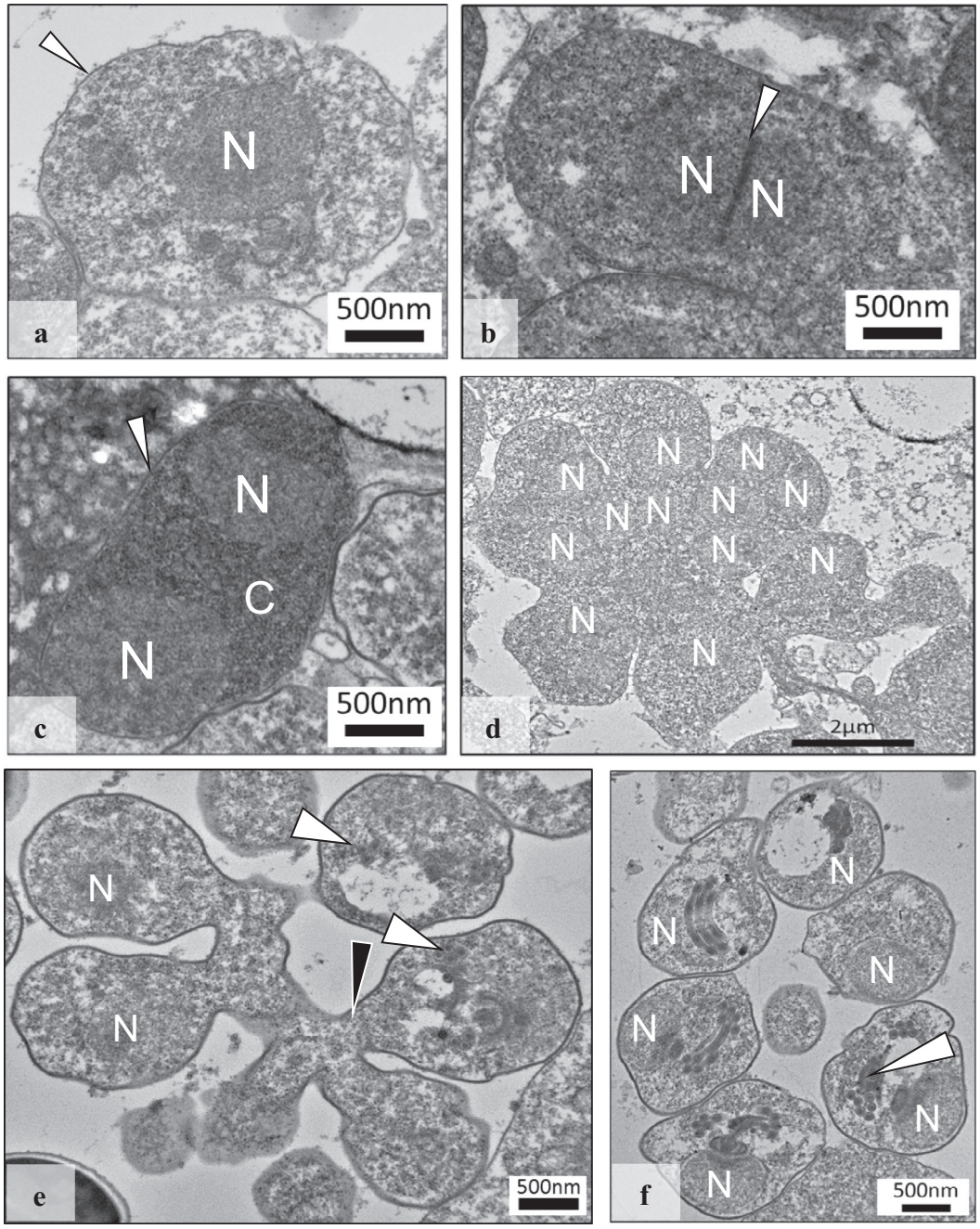

Fig. 2. Transmission electron micrograph of the early developmental stages of *Parahepatospora carcini* n. gen. n. sp. (a) Unikaryotic meront with thin cell membrane (white arrow) and single nucleus (N). Scale = 500 nm. (b) Diplokaryotic meront with connected nuclei (N/N). Scale = 500 nm. (c) Separation of the nuclei (N) within the diplokaryotic cell in preparation for multinucleate cell formation. Note the darkening of cytoplasm (C) and thickening cell membrane (white arrow). Scale = 500 nm. (d) Multinucleate plasmodium containing 12 nuclei (N). Scale = 2 µm. (e) Plasmodium cell division. Individual pre-sporoblasts bud from the main plasmodium (black arrow). Early polar filament and anchoring disks can be seen (white arrow) alongside further cell membrane thickening. Scale = 500 nm. (f) Sporoblast formation after multinucleate cell division. Each sporoblast contains a single nucleus (N) and polar filament with an anchoring disk (white arrows). Scale = 500 nm.

are unikaryotic with unikaryotic merogonic stages during early development, which progress through a diplokaryotic meront stage to a multinucleate plasmodium stage in which spore extrusion precursors primarily form prior to the separation of sporonts (pre-sporoblasts). Sporonts bud from the plasmodium via an elongation of the cytoplasm. *Parahepatospora carcini* SSU rDNA sequence data is represented by accession number: KX757849.

Type host: Carcinus maenas, Family: Portunidae. Common names include: European shore crab and invasive green crab.

Type locality: Malagash (invasive range) (Canada, Nova Scotia) (45.815154, −63.473768).

Site of infection: Cytoplasm of hepatopancreatocytes.

Etymology: "*Parahepatospora*" is named in accordance to the genus "*Hepatospora*" based upon a similar tissue tropism (hepatopancreas) and certain shared morphological characters. The specific epithet "*carcini*" refers to the type host (*Carcinus maenas*) in which the parasite was detected.

Type material: Histological sections and TEM resin blocks from the infected Canadian specimen is deposited in the Registry of Aquatic Pathology (RAP) at the Cefas Weymouth Laboratory, UK. The SSU rRNA gene sequence belonging to *P. carcini* has been deposited in Gen-Bank (NCBI) (accession number: KX757849).

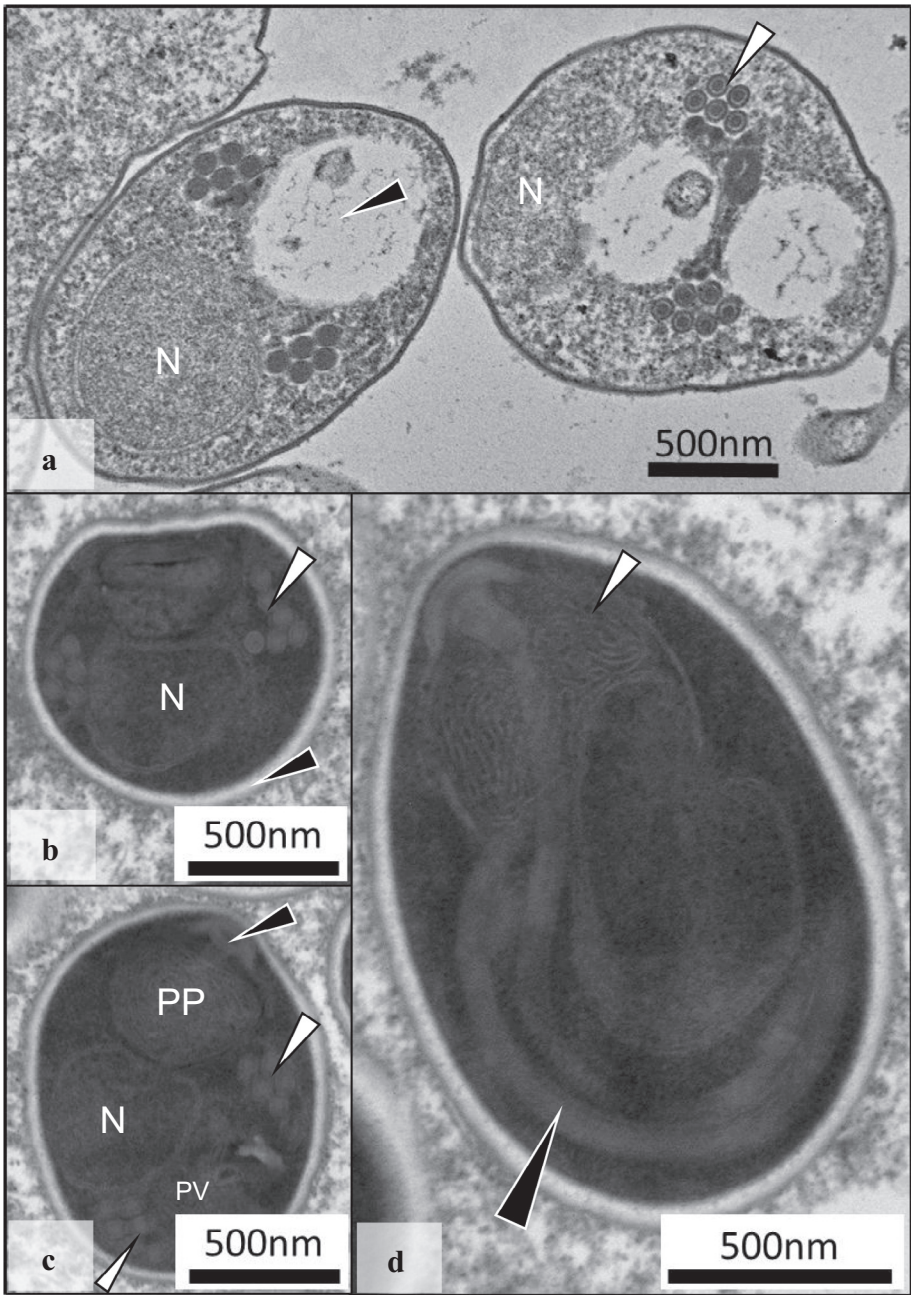

Fig. 3. Final spore development of *Parahepatospora carcini* n. gen. n. sp. (a) Sporoblasts of *P. carcini* hold 5–6 turns of the polar filament, a single nucleus and an electron lucent organelle, suspected to develop into the polaroplast (black arrow). Scale = 500 nm. (b) Cross section of a fully developed spore displaying a single nucleus (N) and 5–6 turns of the polar filament (white arrow). Note the fully thickened, electron lucent endospore (black arrow). Scale = 500 nm. (c) Cross section of a fully formed spore depicting a single nucleus (N), polaroplast (PP), polar vacuole (PV), cross sections of the polar filament (white arrow) and anchoring disk (black arrow). Scale = 500 nm. (d) The final spore of *P. carcini* with a membranous polaroplast (white arrow) and curving, right-leaning, polar filament with anchoring disk (black arrows). Note the thinner endospore at the point closest to the anchoring disk. Scale = 500 nm.

5. Discussion

This study describes a novel microsporidian parasite infecting the hepatopancreas of a European shore crab (*Carcinus maenas*), from an invasive population in Atlantic Canada (Malagash, Nova Scotia). Our SSU rRNA phylogenies place *Parahepatospora carcini* within Clade IV of the Microsporidia, and specifically at the base of the Enterocytozoonidae (containing *Enterocytozoon bieneusi*) and recently-described *Enterocytospora*-like clade (infecting aquatic invertebrates) (Vávra et al., 2016). Its appearance at the base of these clades coupled with its host pathology and development, suggest that this species falls within the Hepatosporidae. However, this cannot be confirmed with current genetic and morphological data. Collection of further genetic data in the form of more genes from both this novel species and other closely related species, will help to infer a more confident placement in future. *Parahepatospora carcini* is morphologically distinct from the microsporidian *Abelspora portucalensis*, which parasitizes the hepatopancreas of *C. maenas* from its native range in Europe (Azevedo, 1987). It is important here to consider whether *P. carcini* has been acquired

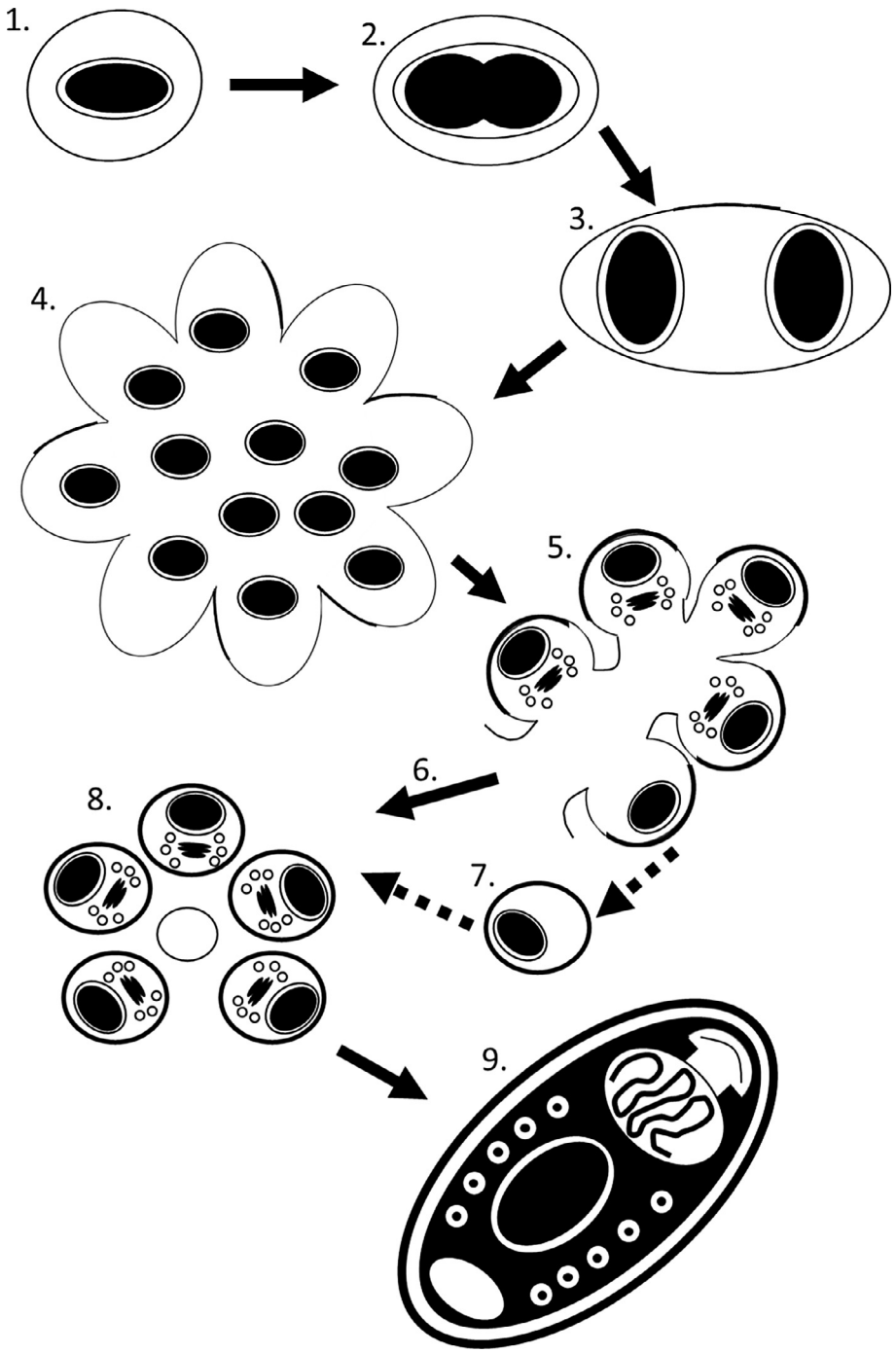

Fig. 4. Predicted lifecycle of *Parahepatospora carcini* n. gen. n. sp. (1) The lifecycle begins with a uninucleate meront. (2) The nucleus of the meront divides to form a diplokaryotic meront. (3) The diplokaryotic nucleus divides, eventually forming a large meront plasmodium. (4) The meront plasmodium shows cytoplasmic invagination before early sporont formation. (5) A cytoplasmic elongation from a sporogonial plasmodium coupled with budding sporonts; most with early spore-organelle formation following the primary development pathway. (6) Sporonts equipped with early spore-organelles mature to sporoblasts. (7) Sporonts without early spore-organelles now develop these organelles to become sporoblasts; a secondary, uncommon pathway of development. (8) Sporoblasts mature with further thickening of the cell wall and completely separate from the sporogonial plasmodium. (9) The final, infective, uninucleate spore is formed, completing the lifecycle.

in the invasive range of the host, or whether this novel microsporidian is an invasive pathogen carried by its host from its native range.

5.1. Could Parahepatospora carcini n. gen. n. sp. be Abelspora portucalensis (Azevedo, 1987)?

Abelspora portucalensis was initially described as a common microsporidian parasite of *C. maenas* native to the Portuguese coast (Azevedo, 1987). While *A. portucalensis* and *P. carcini* infect the same organ (hepatopancreas), and both develop within interfacial membranes separating them from the cytoplasm of infected cells, the two parasites do not resemble one another morphologically. No visible pathology was noted for *P. carcini* whereas *A. portucalensis* leads to the development of 'white cysts' on the surface of the hepatopancreas, visible upon dissection. In contrast to the high prevalence of *A. portucalensis* in crabs collected from the Portuguese coast, *P. carcini* infection

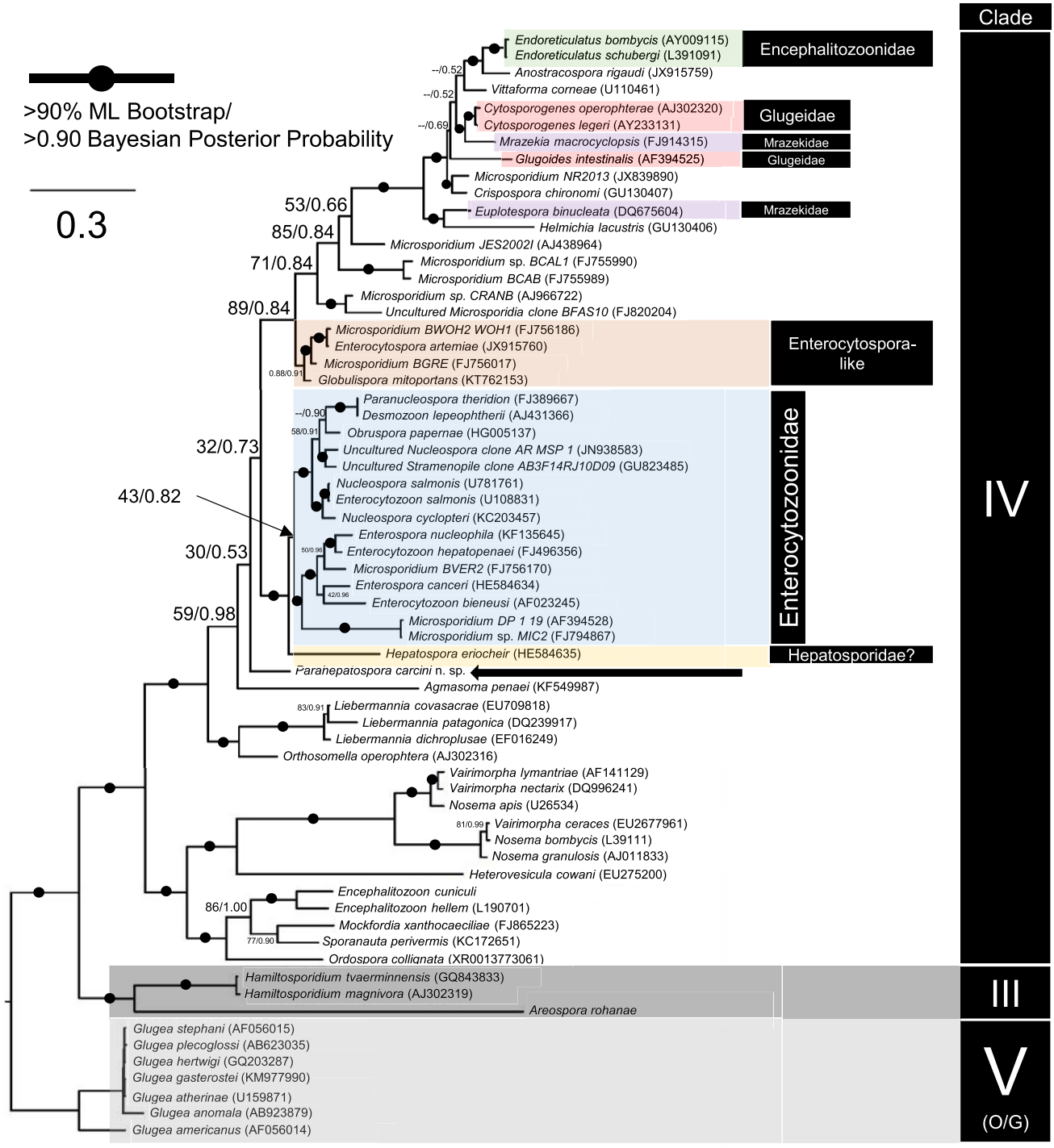

Fig. 5. *Parahepatospora carcini* n. gen. n. sp. phylogenetics based on Bayesian and maximum likelihood tree analyses. This tree is formed from the Bayesian analysis and provided with both maximum likelihood and Bayesian confidence indices (ML/Pp). Branches supported by >90% are represented by a black circle on the branch. The relevant clade to each group of microsporidia is highlighted at the right of the page. Important microsporidian families and groups are also highlighted with accompanying colours (Enterocytozoonidae, Enterocytospora-like, Hepatosporidae, etc.). Members of the Glugea genus (Clade V) are utilised as an out-group (O/G). Scale = 0.3 Units.

was rare (<1%) in crabs collected from the Malagash site (this study).

The parasites share some ultrastructural characteristics, such as: a uninucleate spore with 5–6 turns of a polar filament and a thin endospore. However, the ellipsoid spore of each species shows dissimilar dimensions [*A. portucalensis* (L: "3.1–3.2 μm", W: "1.2–1.4 μm") Azevedo, 1987] [*P. carcini* (L: 1.5 μm ± 0.107 μm, W: 1.1 μm ± 0.028 μm)]. In addition, *A. portucalensis* spores were observed to develop in pairs, within a sporophorous vesicle whilst life stages of *P. carcini* develop asynchronously within an interfacial membrane (Figs. 2 and 3). *Parahepatospora carcini* undergoes nuclear division to form a diplokaryotic meront without cytokinesis (Fig. 2b) where both *A. portucalensis* and *H. eriocheir* undergo nuclear division with cytokinesis at this developmental step;

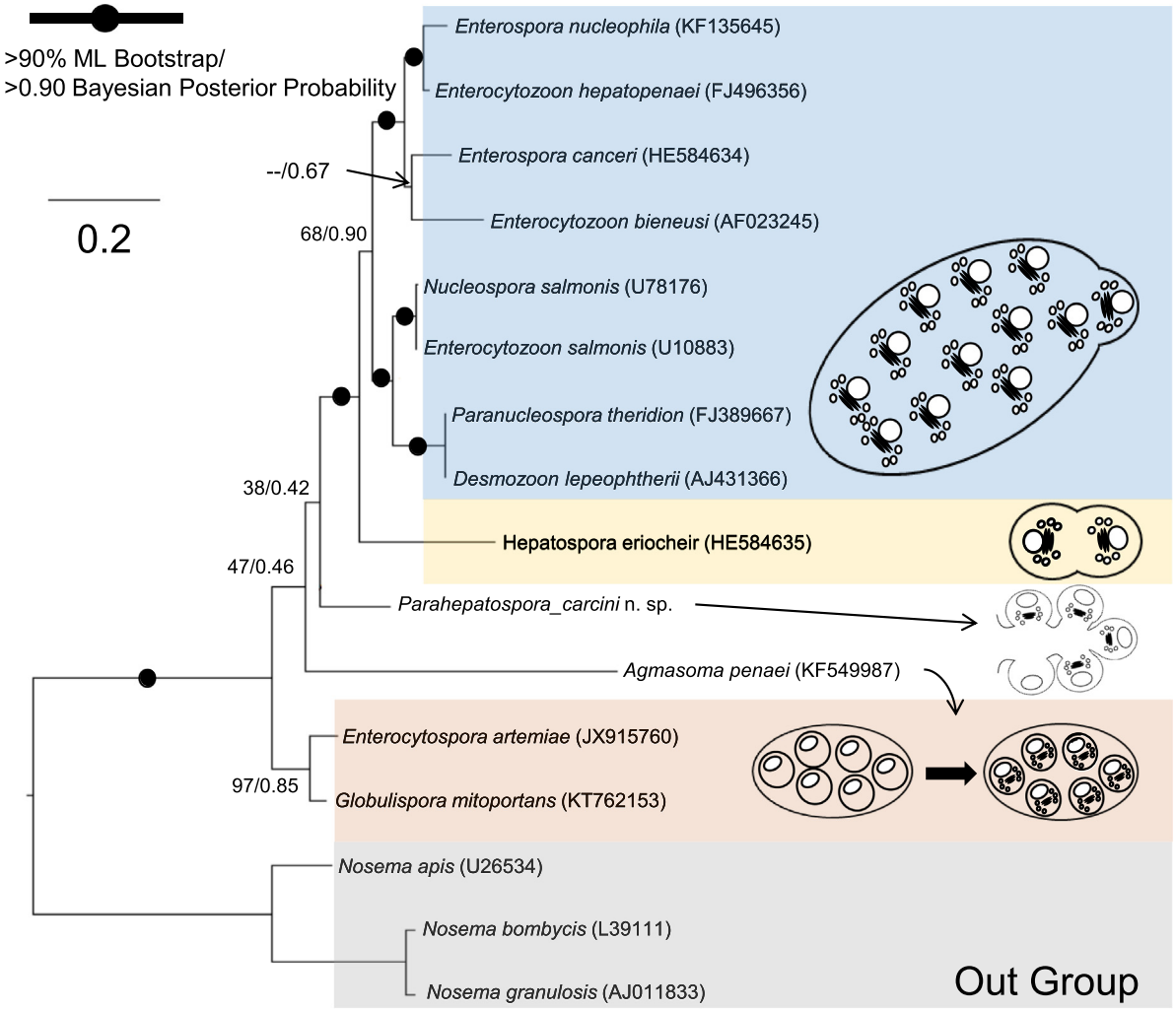

Fig. 6. *Parahepatospora carcini* n. gen. n. sp., tying together of development pathways and phylogenetic data. This tree is formed from a Bayesian (tree presented here) and maximum likelihood analyses and represents all the microsporidia with accompanying developmental and genetic information from three families within Clade IV. The blue group (Enterocytozoonidae) all utilise large plasmodia with polar-filament development at the pre-sporoblastic divisional level. The yellow group (Hepatosporidae) show precursor development to the aforementioned trait. The orange group (Enterocytospora-like clade) develop the polar filament post-sporoblastic division; considered a conventional microsporidian development method. *Parahepatospora carcini* development is included alongside as an intermediate feature. *Nosema spp.* act as an out-group. Scale = 0.2 Units.

further distinguishing these two species from *P. carcini*. *Parahepatospora carcini* also possesses a characteristically distinctive development stage in which multinucleate plasmodia lead to the production of early sporoblasts. These sporoblasts develop spore extrusion organelles prior to their separation from the plasmodium (Fig. 2e and f). This critical developmental step, characteristic of all known members of the Enterocytozoonidae (Stentiford et al., 2007) has also been observed (albeit in reduced form) in *H. eriocheir*, the type species of the Hepatosporidae (Stentiford et al., 2011). This feature was not reported by Azevedo (1987) for *A. portucalensis*, providing further support that *P. carcini* and *A. portucalensis* are separate.

Because of these differences, and in the absence of DNA sequence data for *A. portucalensis*, we propose that *P. carcini* is the type species of a novel genus (*Parahepatospora*) with affinities to both *Hepatospora* (Hepatosporidae) and members of the Enterocytozoonidae. However, given the propensity for significant morphological plasticity in some microsporidian taxa (Stentiford et al., 2013b), we note that this interpretation may change in light of comparative DNA sequence data becoming available for *A. portucalensis*.

5.2. Could Parahepatospora carcini n. gen n. sp belong within the Hepatosporidae (Stentiford et al., 2011)?

The Hepatosporidae was tentatively proposed to contain parasites infecting the hepatopancreas of crustacean hosts (Stentiford et al., 2011). To date, it contains a single taxon, *H. eriocheir*, infecting Chinese mitten crabs (*Eriocheir sinensis*) from the UK (Stentiford et al., 2011), and from China (Wang and Chen, 2007). The Hepatosporidae (labelled within Fig. 5) is apparently a close sister to the Enterocytozoonidae. As outlined above, *P. carcini*, *H. eriocheir* and all members of the Enterocytozoonidae share the developmental characteristic of early spore organelle formation (such as the polar filament and anchoring disk) within the pre-divisional sporont plasmodium. In contrast, members of the Enterocytospora-like clade display developmental features consistent with all other known microsporidian taxa (i.e. spore precursor

organelles form after the separation of the sporont from the plasmodium, Rode et al., 2013). Like *H. eriocheir*, *P. carcini* displays early spore-organelle formation both pre- and post- sporont separation from the sporont plasmodium. It is tempting to propose that this characteristic is an intermediate trait between the Enterocytozoonidae and all other Microsporidia and, that this trait is possibly definitive for members of the Hepatosporidae; but further SSU rRNA gene phylogeny data is required to further confirm this, and to link these observations. Intriguingly, *Agmasoma penaei* (branching below *P. carcini*), a pathogen of the muscle and gonad (only gonad in type host), which is closely associated to *P. carcini* phylogenetically (Figs. 5 and 6), shows tubular inclusions at the plasmodium developmental stage; however polar filament precursors do not fully develop until after sporont division (Sokolova et al., 2015); this could indicate a further remnant of the developmental pathways seen in *P. carcini*, *H. eriocheir* and members of the Enterocytozoonidae.

The shared developmental and pathological characteristics of *P. carcini* and *H. eriocheir* suggest a taxonomic link; however this is not clearly supported by the SSU rRNA gene phylogenies (Figs. 5 and 6). Confidence intervals supporting the placement of *P. carcini* outside of both the Enterocytozoonidae, the Enterocytospora-like clade and the Hepatosporidae are low (Figs. 5 and 6) forcing us to suggest that additional data in the form of further gene sequencing of this novel parasite, or possibly from others more closely related through diversity studies, is required before confirming a familial taxonomic rank for this new taxon.

5.3. Is Parahepatospora carcini n. gen. n. sp. an invasive pathogen or novel acquisition?

The 'enemy release' concept proposes that invasive hosts may benefit from escaping their natural enemies (including parasites) (Colautti et al., 2004). Invasive species may also introduce pathogens to the newly invaded range, as illustrated by spill-over of crayfish plague (Jussila et al., 2015) to endangered native crayfish in Europe. Invaders can also provide new hosts for endemic parasites through parasite acquisition (e.g. Dunn and Hatcher, 2015).

Invasive populations of *C. maenas* in Canada are thought to have originated from donor populations in Northern Europe, specifically: Scandinavia, the Faroe Islands and Iceland, based on microsatellite analysis (Darling et al., 2008). *Carcinus maenas* are yet to be screened for microsporidian parasites within these ancestor populations and they may prove to be a good geographic starting point for studies to screen for *P. carcini*. Alternatively, the recent discovery of *P. carcini* at low prevalence in *C. maenas* from the invasive range in Canada could indicate that the parasite has been acquired from the Canadian environment via transfer from an unknown sympatric host. The low prevalence (a single infected specimen) of infection could suggest the single *C. maenas* in this study was infected opportunistically, however the potential remains for *P. carcini* to be present at low prevalence, with gross pathology, as a mortality driver and emerging disease in *C. maenas* on the Canadian coastline. Currently, no evidence is available to confirm whether *P. carcini* is non-native or endemic.

For future studies it is important to consider whether *P. carcini* may be a risk to native wildlife (Roy et al., 2016), or, if the parasite has been acquired from the invasive range (pathogen acquisition), how it was acquired. If invasive, important questions about the invasion pathway of *P. carcini* would help to indicate its risk and invasive pathogen status (Roy et al., 2016). Finally, assessing the behavioural and life-span implications of infection could address whether *P. carcini* has the potential to be used to control invasive *C. maenas* on the Canadian coastline (potential biological control agent).

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