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Enterocytozoon hepatopenaei sp. nov. (Microsporida: Enterocytozoonidae), a parasite of the black tiger shrimp *Penaeus monodon* (Decapoda: Penaeidae): Fine structure and phylogenetic relationships

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ABSTRACT

A new microsporidian species, *Enterocytozoon hepatopenaei* sp. nov., is described from the hepatopancreas of the black tiger shrimp *Penaeus monodon* (Crustacea: Decapoda). Different stages of the parasite are described, from early sporogonal plasmodia to mature spores in the cytoplasm of host-cells. The multinucleate sporogonal plasmodia existed in direct contact with the host-cell cytoplasm and contained numerous small blebs at the surface. Binary fission of the plasmodial nuclei occurred during early plasmodial development and numerous pre-sporoblasts were formed within the plasmodium. Electron-dense disks and precursors of the polar tubule developed in the cytoplasm of the plasmodium prior to budding of early sporoblasts from the plasmodial surface. Mature spores were oval, measuring 0.7 × 1.1 μm and contained a single nucleus, 5–6 coils of the polar filament, a posterior vacuole, an anchoring disk attached to the polar filament, and a thick electron-dense wall. The wall was composed of a plasmalemma, an electron-lucent endospore (10 nm) and an electron-dense exospore (2 nm). DNA primers designed from microsporidian SSU rRNA were used to amplify an 848 bp product from the parasite genome (GenBank FJ496356). The sequenced product had 84% identity to the matching region of SSU rRNA from *Enterocytozoon bieneusi*. Based upon ultrastructural features unique to the family Enterocytozoonidae, cytoplasmic location of the plasmodia and SSU rRNA sequence identity 16% different from *E. bieneusi*, the parasite was considered to be a new species, *E. hepatopenaei*, within the genus Enterocytozoon.

1. Introduction

The culture industry for black tiger shrimp *Penaeus monodon* in Thailand has been affected by monodon slow growth syndrome (MSGS) since 2003 (Chayaburakul et al., 2004; Flegel et al., 2004). MSGS-affected shrimp reared in earthen ponds grow at a greatly reduced rate compared to their unaffected counterparts, leading to significant financial losses, comparable to those caused by white-spot syndrome virus (WSSV) outbreaks (Flegel, 2001). The aetiology of MSGS has not been fully elucidated, although multiple infectious agents have been observed in several organs by histopathology, transmission electron microscopy (TEM), and polymerase chain reaction (PCR) methods (Chayaburakul et al., 2004; Anantasomboon et al., 2006). Most significantly, multiple spheroids were observed in the lymphoid organs and shown to contain several viruses of known and unknown types (Anantasomboon et al., 2006; Sritunyalucksana et al., 2006). Hepatopancreatic cells of affected shrimp sometimes also showed the presence of inclusions of monodon baculovirus (MBV), hepatopancreatic parvovirus (HPV) or a currently unidentified microsporidian.

Microsporidia are obligate intracellular parasites known to infect a wide range of eukaryotic hosts. Development of the parasite generally occurs within the cytoplasm of the host-cell via nuclear proliferation, and spore formation (sporogony), though certain genera are known to undergo similar development within the host nucleoplasm (Lom and Dyková, 2002; Stentiford and Bateman, 2007; Stentiford et al., 2007). Several genera of microsporidia have been reported to infect crustacean hosts. These include *Agmasoma*, *Ameson*, *Nosema*, *Pleistophora*, *Tuzetia*, *Thelohania*, *Flabelliforma*,

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Glugoides, *Vavraia*, *Ordospora, Nadelspora* and *Enterospora* (Landgdon, 1991; Larsson et al., 1996, 1997, 1998; Lightner, 1996; Canning et al., 2002; Refardt et al., 2002; Moodie et al., 2003; Amogan et al., 2006; Stentiford and Bateman, 2007). Various crustacean organs and tissues have been reported to be infected (Sprague et al., 1992; Anderson et al., 1989; Landgdon, 1991; Larsson et al., 1996, 1997, 1998; Lom et al., 2001). For example, Pasharawipas and Flegel (1994) reported infections of *Agmasoma penaei* in the muscles and connective tissue of *Penaeus merguensis* and *P. monodon*. Later, Canning et al. (2002) reported an infection by *Tuzetia weidneri* in the muscles of *Penaeus setiferus* and *Penaeus aztecus*. Pathogenesis involved progression of multifocal lesions to whole muscle blocks and abdominal segments of the shrimp, leading to a condition termed 'cotton tail' disease. Lightner (1996) has also reported microsporidian infections in muscle tissue of penaeid shrimp.

Few microsporidians have been shown to infect only the tubule epithelial cells of the crustacean hepatopancreas (Anderson et al., 1989; Hudson et al., 2001; Wang and Chen, 2007; Stentiford and Bateman, 2007; Stentiford et al., 2007). The publications by Stentiford et al. describe such infections of two European crab species by a microsporidians placed in a new genus of microsporidia (*Enterospora*) within the family Enterocytozoonidae because of the intranuclear location of the plasmodia. Placement within the family Enterocytozoonidae was based upon the formation of precursors to the spore extrusion apparatus (e.g. polar filament, anchoring disk) and other organelles within the parasite plasmodia (i.e. prior to isolation of sporoblasts). This discovery opened the possibility of other representatives of the Enterocytozoonidae within crustaceans.

The relatively frequent occurrence of a new cytoplamic microsporidian that infected only hepatopancreatic tubule epithelial cells of MSGS-affected *P. monodon* (Chayaburakul et al., 2004) provided an opportunity to study its development and taxonomy. This study focused mostly on histopathological and ultrastructural features of the parasite, but also included analysis of a portion of its SSU rRNA gene and comparison the sequence to those of other microsporidians in public databases.

2. Materials and methods

2.1. Samples and investigation process

P. monodon postlarvae (PL) and juveniles were obtained from hatcheries and commercial ponds experiencing MSGS in Thailand. The age and size of the juveniles varied from one to four months in culture and from 1 to 10 g body weight (BW). The PLs were mostly PL15 stage, with an approximate BW of 0.1 g. The hepatopancreas was dissected out and portions were smeared onto glass microscope slides and stained with haematoxylin and eosin (H&E). Other hepatopancreatic tissue specimens fixed, embedded, sectioned and stained for light (LM) and transmission electron (TEM) microscopy. Samples from individuals positively diagnosed with microsporidian infections via smears or histology were processed for ultrastructural investigation. The size of the microsporidian spores was determined from fresh spores prepared by differential centrifugation of homogenized, infected hepatopancreatic tissue ending with Percoll (GE Life Sciences) density gradient centrifugation. The spore band was removed from the gradient and examined directly in wet mounts. For LM and TEM, 10 juveniles and 10 PL15 were sampled. For molecular biology, DNA was extracted from more than 1000 juveniles and 10,000 PL15.

2.2. Histology and electron microscopy

For LM, a mid-sagittal cut was performed on infected shrimp which were then fixed in Davidsons' seawater fixative for 24 h, processed routinely for histology, sectioned at 5 μm and stained with haemotoxylin and eosin (H&E), Giemsa, and Chromotrope 2R modified trichrome (Weber et al., 1992). For TEM, small pieces (2 mm^3) of hepatopancreatic tissue from the same shrimp specimens were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde, or 6% glutaraldehyde, in Millonig buffer followed by washing in three changes of 0.1 M sodium cacodylate buffer and stained *en bloc* in 0.5% aqueous uranyl acetate for 1 h. The fixed tissues were embedded in epoxy resin 812 (Agar Scientific-pre-mix kit 812) following dehydration through a graded acetone series. Thick sections were stained with Toluidine Blue for viewing with a light microscope to identify sections with suitable target areas. Ultra thin sections (70–90 nm) of these samples were mounted on uncoated copper grids and stained with uranyl acetate and Reynolds' lead citrate. Sections were examined using a JEOL 1210 transmission electron microscope.

2.3. DNA extraction, PCR and sequencing

Total DNA was extracted from the hepatopancreas using a commercial tissue extraction kit (Qiagen, Germany). DNA was amplified using PCR primers designed from the alignment of 11 microsporidian SSU rRNA gene sequences at GenBank in such a manner that the primers were based in highly conserved regions of the gene but amplified a fragment from a highly variable region. The primers were MF1 (forward) 5′-CCG GAG AGG GAG CCT GAG A-3′ and MR1 (reverse) 5′-GAC GGG CGG TGT GTA CAA A-3′, relative to positions 242–260 and 1165–1183, respectively, of the small subunit (SSU) rRNA gene of *Enterocytozoon bieneusi* (Genbank accession No. AF024657). Based on the 11 sequences used, expected amplicons would be in the range of approximately 900–1000 bp. The PCR process was carried out in 50 μm reaction mixtures containing PCR buffer, 200 mM dNTP, 2 mM $MgCl_2$, 1.25 units *Taq* polymerase, 1 mM primers and 1 μl template. Reactions were followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C, and extension at 72 °C for 90 s, followed by a 5-min final extension at 72 °C. An SSU fragment of 1200 bp in length was amplified and inserted into a TOPO Cloning Kit (Invitrogen, USA). Plasmids were checked for inclusion of the desired insert and subsequently purified with NucleoSpin Extract Kit (Qiagen, Germany). Following hybridization with shrimp genomic DNA, clones that did not show signals were further processed for DNA sequence determination (Macrogen, South Korea). The insert from one of these clones (final length 848 bp minus the primer sequences) gave high sequence identity to microsporidian rRNA sequences in the GenBank database using a BLASTn (http://www.ncbi.nlm.nih.gov/BLAST) search and was sequenced from both strands. It was aligned with sequences of other microsporidians in the database using CLUSTAL W 1.7 multiple alignment software (Thompson et al., 1994). The phylogenic tree was constructed using CLUSTAL W 1.75 program with the microsporidian *Amblyospora canadensis* as an out-group since *Amblyospora* is from Clade 1 of the microsporidians (Vossbrinck and Debrunner-Vossbrinck, 2005). Tree topology was evaluated by bootstrap analysis using the neighbour joining method with the program parameter set for 1000 replicates.

3. Results

3.1. Histopathology and ultrastructure

Hepatopancreatic samples prepared by the smear method and viewed by light microscopy (LM) showed the presence of microsporidian spores (Fig. 1A) only in the cytoplasm of hepatopancreatic tubule epithelial cells. Using fresh spore preparations

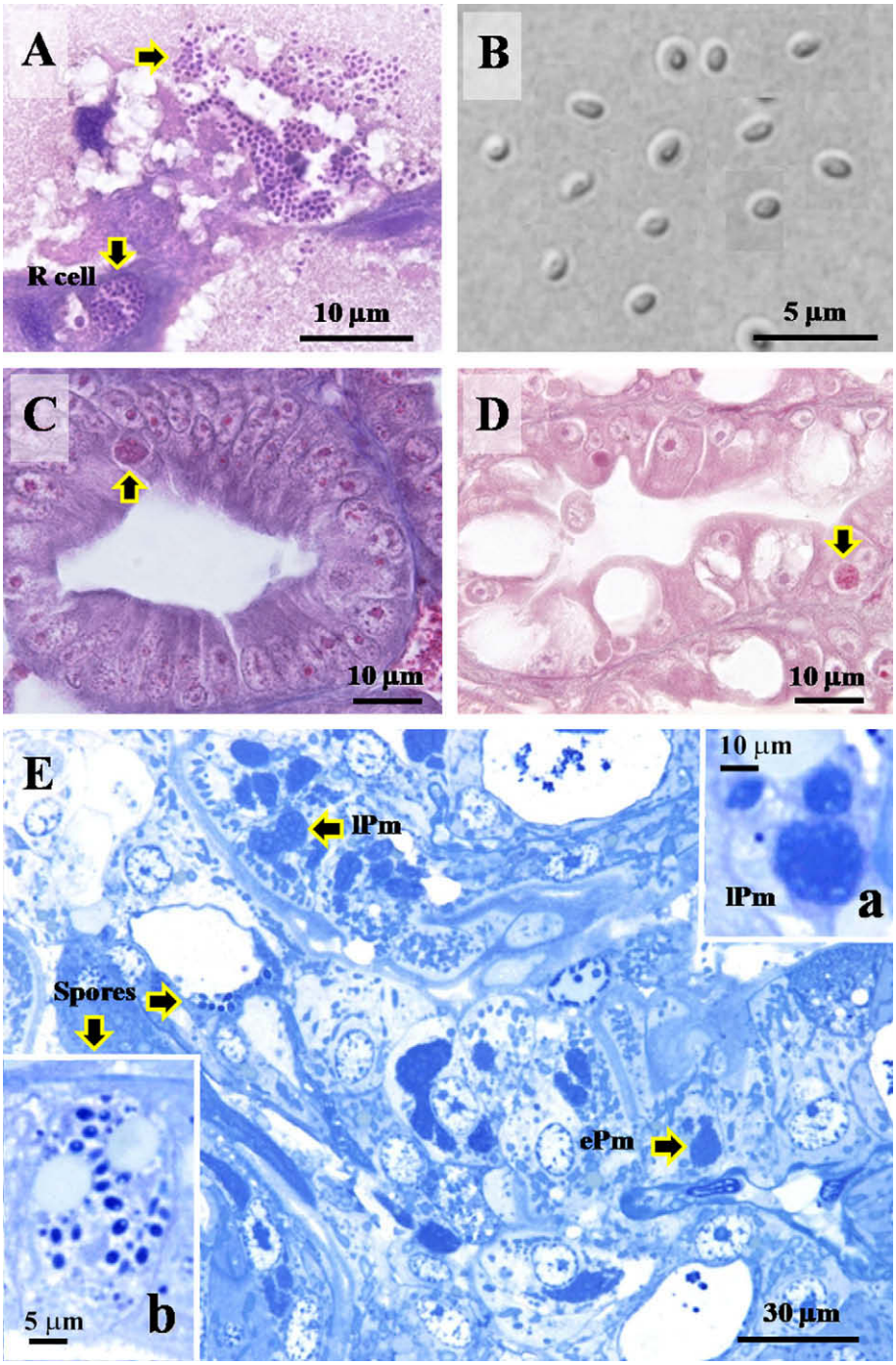

Fig. 1. Photomicrographs of *Enterocytozoon hepatopenaei* tubule epithelial cells of the hepatopancreas of *Penaeus monodon*. (A) H&E-stained smear of hepatopancreatic tissue showing numerous microsporidian spores (arrows). (B) Fresh preparation of microsporidian spores from a Percoll gradient. (C and D) Hepatopancreatic tissue sections showing acidophilic, granular inclusions in the cytoplasm of tubule epithelial cells (arrows). (E) Semi-thin section of hepatopancreatic tissue showing early and late plasmodia (inset a) and mature spores (inset b) in the cytoplasm of tubule epithelial cells. Some spores show unstained spots that represent their concave surfaces at one end (inset b). A, haematoxylin stain; C, Trichrome stain; D, H&E stain; E, Toluidine blue stain. ePm, early plasmodium; lPm, late plasmodium.

from Percoll gradient separations (Fig. 1B), spore size was 1.1 ± 0.2 µm × 0.7 ± 0.1 µm (N = 100), while that in HP smears was 1.1 ± 0.2 µm × 0.6 ± 0.2 µm (N = 10). Histologically, spores appeared as acidophilic structures confined within a vacuole within the cytoplasm of R (reserve), B (blister) and E (embryonic) cells, but F (fibrillar) cells (Fig. 1C and D). However, the midgut and hindgut epithelial cells, or cells of other tissues and organs were unaffected. Histological sections revealed several developmental stages of the microsporidian, depicted by differences in the staining of various cytoplasmic inclusions (Fig. 1E). Mature spores appeared to contain electron-lucent regions that likely coincided with the posterior vacuole observed by TEM (see below).

TEM revealed several stages of a microsporidian parasite in the cytoplasm of hepatopancreatic, tubule epithelial cells. Definitive early meront stages were not observed but early and late stage plasmodia were frequent and showed multiple nuclei and sporogony typical of members of the family Enterocytozoonidae (Fig. 2A and B). The plasma membrane of some plasmodia was

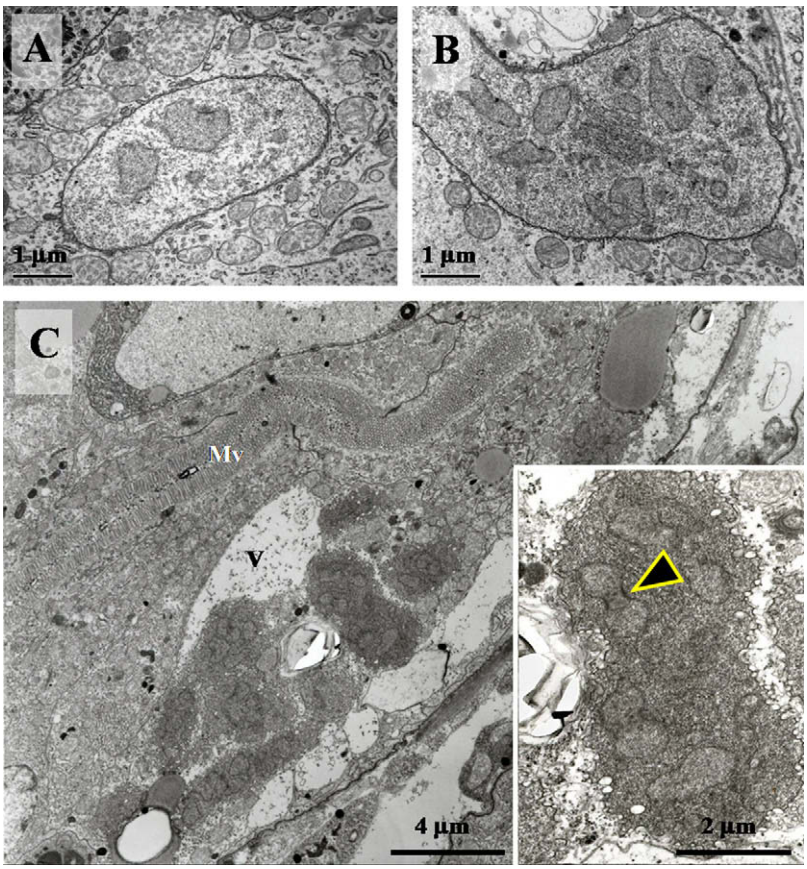

Fig. 2. Trasmission electron micrographs (TEM) of early stages of *E. hepatopenaei* development in hepatopancreatic tubule epithelial cells of *P. monodon*. (A) Early plasmodium stage with two nuclei. (B) Early plasmodium stage with multiple nuclei. (C) A group of plasmodia surrounded by a vacuole within the cytoplasm of the host-cell and showing nuclear division (inset, arrowhead). Mv, microvilli; V, vacuole.

in direct contact with the host-cell cytoplasm (Fig. 2A and B), whereas some were apparently surrounded by cytoplasmic vacuoles, often encompassing multiple, discrete plasmodia (Fig. 2C). Plasmodia were delimited by an electron dense membrane that separated plasmodial components from the host-cell cytoplasm (Fig. 2A and B). Evidence of nuclear division, or binary fission was observed in some plasmodia, characterized by the presence of spindle plaques on the outer surface of the nuclear envelope (Fig. 2C, inset).

Sporogonal plasmodia were characterized by the appearance of round vesicles (100–200 nm) with electron-dense, surrounding membranes and short polar filament precursors within the plasmodial cytoplasm (Fig. 3A, inset a). Circular disks with outer electron dense layers and inner electron-lucent cores were also observed (Fig. 3A, inset b). The tubules and the circular disks were presumably precursors of the polar filaments of the spore. As plasmodia matured, the tubules and the disks appeared to increase in number, concomitant with a decrease in the number of vesicles (Fig. 3B). Polar filament precursors increased in length and in curvature to form several distinct coils. At this stage, the parasite nuclei were oriented toward the periphery of the plasmodium, with polar filament coils located on the inner side (Fig. 3C).

The sporogonal, plasmodial membrane, which was in direct contact with the host-cell cytoplasm, displayed a microvillous appearance with characteristic surface blebs (Fig. 3D and E). The membrane on which these blebs occurred was externally reinforced by a dense coat and was composed of two inner membranous layers and an electron-dense outer layer of 10 nm thickness. The membrane of the microvillous structure was also thickened, but it was somewhat thinner than that in the region of the blebs. Surrounding the plasmodium was host-cell cytoplasm, with abundant rough and smooth endoplasmic reticula (RER and SER), clear vesicles and mitochondria.

As development progressed, pre-sporoblast units (containing a single nucleus and elements of the spore extrusion apparatus) were located towards the periphery of the plasmodium, with up to 20 pre-sporoblasts visible within a single plasmodial section (Fig. 3F). A space was formed between the coils of the polar filament and the nucleus, which was occupied by the early posterior vacuole (Fig. 4A). A dense cap, a precursor of the anchoring disk, was also formed at the anterior end of the pre-sporoblast. The posterior vacuole apparently became larger as the sporoblast matured (Fig. 4B). The coiled polar filaments (5–6 coils) also appeared to increase in diameter to approximately 75 nm with the innermost electron-dense core surrounded by an electron-lucent layer and an outer electron-dense coat. A double-layered nuclear membrane was clearly observed at this stage.

Pre-sporoblasts protruded to form buds from the plasmodial membrane that apparently separated to form electron-dense sporoblasts that lay in direct contact with the host cytoplasm (Fig. 5A). Sporoblasts underwent direct development to mature, electron dense and oval spores. The spores contained a single nucleus, with a polar filament coiled 5–6 times at the posterior end and terminating at an anchoring disk at the anterior end (Fig. 5B–D). The posterior vacuole was occasionally visible within mature spores. The wall of the spore was composed of a cell membrane within an electron-lucent endospore (10 nm) and an electron-dense exospore (2 nm).

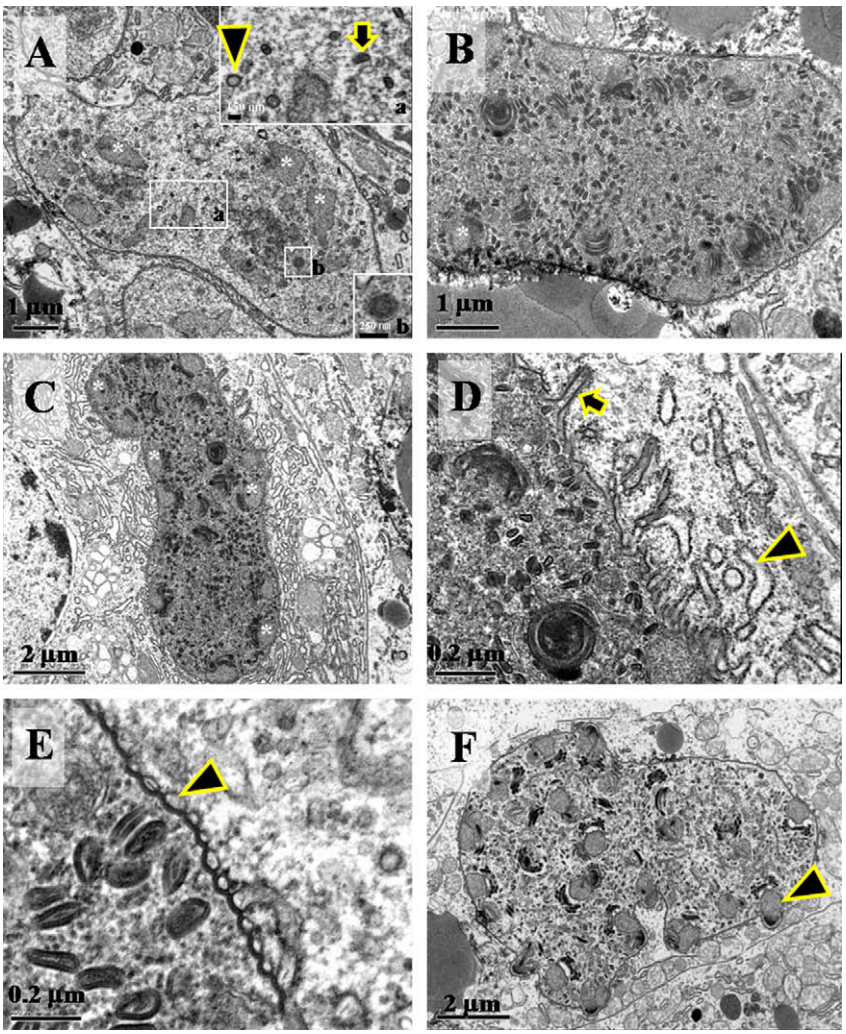

Fig. 3. TEM of sporogonial stages of *E. hepatopenaei* development in hepatopancreatic tubule epithelial cells of *P. monodon*. (A) Early sporogonial plasmodium containing nuclei (asterisks), clear vesicles (inset a, arrowhead), and precursors of polar filaments (inset a, arrows; and inset b). (B) Sporogonial plasmodium with peripherally located sporoplasm (asterisk). (C) Sporogonial plasmodium with several peripherally located pre-sporoblasts (asterisks). (D) Plasmodial membrane showing a microvillus-like structure surrounded by host-cell cytoplasm (arrow) containing extensive RER (arrowhead). (E) Thickened plasmodial plasmalemma showing vesicular structures at the interface with host cytoplasm (arrowhead). (F) Late sporogonial plasmodium showing assembled pre-sporoblasts, polar filaments and a dense cap (arrowhead).

3.2. SSU rRNA gene fragment analysis

PCR using primers based on conserved regions of microsporidian SSU rRNA sequences listed at GenBank and DNA template from microsporidian-infected *P. monodon* hepatopancreatic tissue yielded an amplicon of 886 bp which was in the expected amplicon range of approximately 900–1000 bp. After cloning and sequencing, a fragment of 848 bp (excluding the primer sequences) was subjected to a general BLASTn search that yielded hits only for microsporidian sequence records. Top hits included *Nucleospora salmonis* (AF185998) at 87% identity and *E. bieneusi* (GenBank AF023245) at 86% identity. By contrast, sequence identity for *A. penaei* (the causative agent of 'cotton shrimp' reported from *P. monodon* in Thailand) was only 71%. In addition, the sequence identity match between *N. salmonis* and *E. bieneusi* (from different genera in the family Enterocytozoonidae) was 85%. These results indicated that the 848 bp sequence from *P. monodon* was novel and approximately equidistant from *N. salmonis* and *E. bieneusi* in terms of identity difference. A subsequent CLUSTAL W alignment was carried out and a phylogenetic tree was constructed with the optimal criteria set for distance and using SSU rRNA gene sequences of available microsporidians in the public database. The un-rooted tree constructed using this data (Fig. 6) revealed that the sequence of the microsporidian from *P. monodon* most closely grouped with *N. salmonis* and *E. bieneusi*.

4. Discussion

This is the first report on ultrastructural features and a partial SSU rRNA gene sequence of a microsporidian exclusively infecting hepatopancreatic tubule epithelial cells of the black tiger shrimp *P. monodon*. Its unique ultrastructural features linked it to the family Enterocytozoonidae. Based on its distinction from the microsporidian genera *Nucleospora* and *Enterospora* in the family and on its similarity to the single species in the genus *Enterocytozoon*, we assigned the new microsporidian infecting *P. monodon* to the genus *Enterocytozoon*. We believe that its occurrence in an invertebrate host and its relatively low SSU rRNA sequence identity with *E. bieneusi* justify its assignment to a new species within the genus.

The name *E. hepatopenaei* is proposed for the new species with justification based on ultrastructural characters that conform to the unique features of the family Enterocytozoonidae but distinguish it from other species in the family, i.e., *E. beiniusi* that also develops in the host-cell cytoplasm and species in the genera *Ent-*

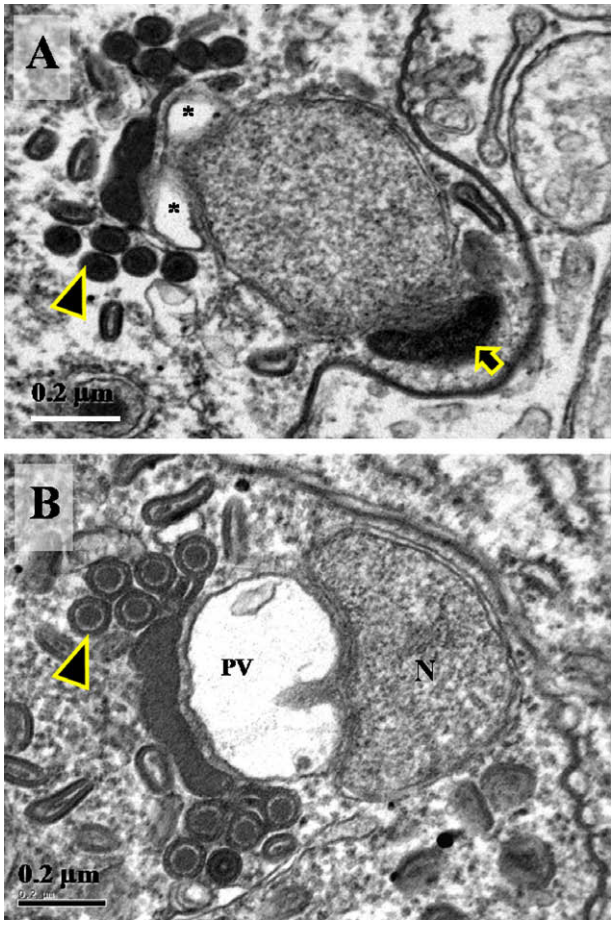

Fig. 4. TEM of *E. hepatopenaei* sporoblasts in hepatopancreatic tubule epithelial cells of *P. monodon*. (A) Sporoblast at the periphery of a sporogonal plasmodium showing the nucleus, early posterior vacuole (asterisks), immature polar filaments with five cross sections (arrowhead) and a dense cap or anchoring disk (arrow). (B) Late stage sporoblast showing mature polar filaments (arrowhead). N, nucleus; PV, posterior vacuole.

erospora and *Nucleospora* that develop within the host-cell nucleus. Distinction from *Enterospora* and *Nucleospora* is clear based their intranuclear rather than cytoplasmic development. However, distinction from *E. bieniusi* is not initially so clear.

E. hepatopenaei has several features similar to *E. bieneusi*, the only current member in the genus *Enterocytozoon* (Franzen and Müller, 1999). For both species, the sporogonal plasmodium is localized in the host-cell cytoplasm and it generates pre-cursors of the spore extrusion apparatus prior to the formation and budding of sporoblasts. For both, sporoblasts are liberated to the host cytoplasm following which they develop directly into oval spores. Other similar morphological features included spores with a posterior vacuole and 5–7 coils of polar filaments arranged in two rows (Franzen and Müller, 1999; Wasson and Peper, 2000; Visvesvara, 2002; Didier, 2005). However, the mean spore size of *E. hepatopenaei* (1.1×0.7 µm in fresh preparations) was somewhat smaller than that of *E. bieneusi* (1.5×0.5–1.0 µm) (Franzen and Müller, 1999; Didier et al., 2000; Wasson and Peper, 2000), although the difference was not great, and it might be argued that it was not sufficient to warrant designation of two separate species.

Since only monokaryotic nuclei have been reported from infections of *E. bieneusi* in vertebrates, it is conceivable that other life cycle stages such as diplokaryotic forms and gametes might occur in an alternative host, and that shrimp could constitute such a host. However, the fact that the spores in *P. mondon* are also monokaryotic and that they develop in a manner similar to those of *E. bieneusi* rather than developing from diplokaryotic or meiotic nuclei (Flegel and Pasharawipas, 1995) argues against this possibility. In addition, work on possible sources of *E. bieneusi* in human infections has implicated birds and mammals rather than shrimp, and fresh water sources rather than marine (Dowd et al., 1998; Didier et al., 2004; Lobo et al., 2006). All this information argues in favor of our contention that *E. hepatopenaei* is distinct from *E. bieneusi*.

The argument for distinction of the two species is further supported by the 16% difference in identity for compared fragments of their SSU rRNA genes. A rough estimation of the sequence identity cutoff for a species is approximately 97% (Stackebrandt and Goebel, 1994) so that 84% identity is well outside the range of what would be considered reasonable for con-specific isolates. Indeed, the fact that *N. salmonis* and *E. bieneusi* are in different genera and differ by 85%, might suggest that the hepatopancreatic microsporidian in *P. monodon* should also be placed in a new genus. Thus, our proposal for a new species within the genus *Enterocytozoon* rather than a new genus may be viewed as conservative.

With respect to the phylogenetic tree, it may be argued that comparison using 848 bp of a single gene does not cover a sufficient proportion of the target genomes to prepare a robust tree. Despite this caveat, the relationships among the database microsporidians in our phylogenetic tree (Fig. 6) are similar to those reported for them in more comprehensive comparisons (Vossbrinck and Debrunner-Vossbrinck, 2005). In any case, our preliminary analysis based solely on this region indicates that *E. hepatopenaei* tends to group with other microsporidians associated with aquatic host species. This intriguing relationship may be followed up using longer sequences and more genes whenever this information becomes available.

For other members of the family Enterocytozoonidae previously described from crab hosts (i.e., genus *Enterospora*, Stentiford, 2007) there is no available genetic information. However, they differ from *E. hepatopenaei* greatly in that they develop within the host nucleus rather than the cytoplasm. In addition, *E. hepatopenaei* infected all cell types of the hepatopancreatic tubule epithelium except F-cells while *Enterospora canceri* infected all cell types except B-cells (Stentiford et al., 2007; Stentiford and Bateman, 2007).

The only other microsporidian described from penaeid shrimp in Thailand is *Agmasoma penaei* (Pasharawipas and Flegel, 1994) that causes infections in muscle and connective tissue. The nuclei in the plasmodia are diplokaryotic and give rise to monokaryotic nuclei by reductive sporogony (Flegel and Pasharawipas, 1995). Further, the SSU rRNA sequence of *A. penaei* (Pasharawipas et al., 1994; GenBank) shared only 71% identity with that of *E. hepatopenaei* reported herein. They are obviously only distantly related.

Several microsporidian species develop directly within the host-cell cytoplasm and not within a vacuole. However, *Encephalitozoon* spp. and *Glugoides intestinalis* possess a parasitophorous vacuole at an early stage of infection that probably acts as a protection against destruction within the host-cell cytoplasm (Cali and Kolter, 1993; Cali and Takvorian, 1999; Vavra and Larsson, 1999). In *E. hepatopenei*, it was observed that some plasmodia were contained within a vacuole while some were not. However, blebs and microvilli-like structures were present in the thick membrane surrounding plasmodia that were not contained in vacuoles. It is possible that plasmodia begin as small structures in vacuoles and then expand until regions of the plasmodial membrane come into direct contact with the vacuolar membrane to form what appears to be a thick, double-layered membrane. If so, lack of complete

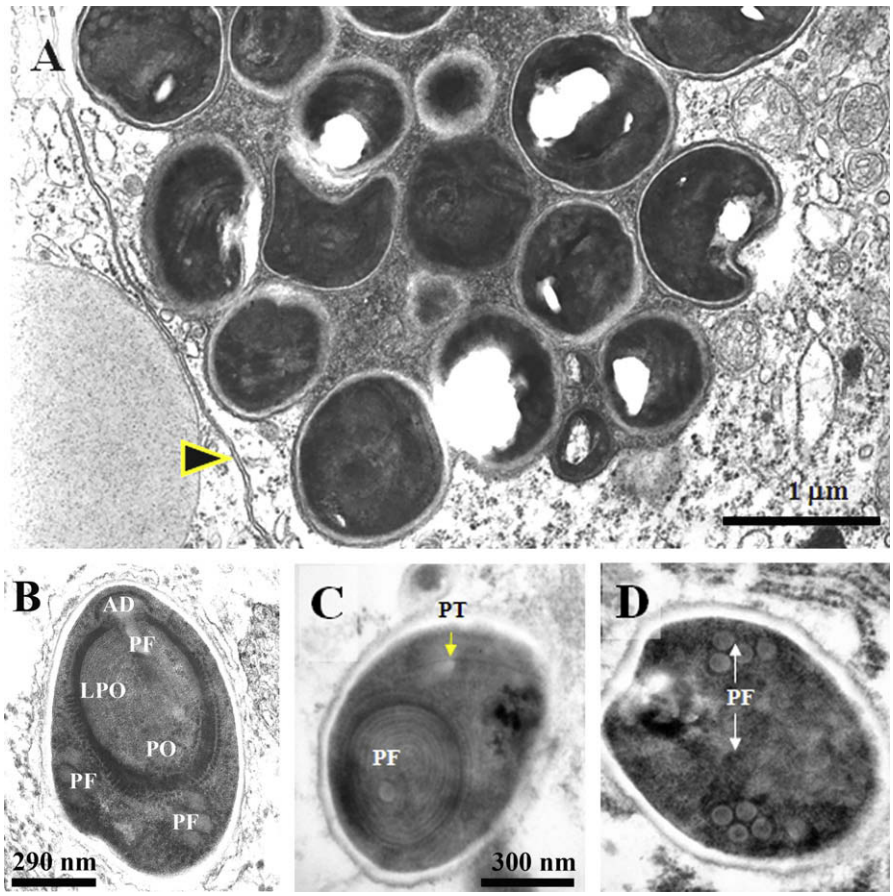

Fig. 5. TEM of *E. hepatopenaei* spore development. (A) Immature spores covered with the plasmodial membrane (arrow). (B) Immature spore showing the anchoring disk (AD), the polar filament (PF) and the polaroplast (PO) including its lamellar portion (LPO). (C) Cross section of a mature spore just below the anchoring disk showing a cross section of the polar filament (PF) surrounded by the lamellar portion of the polaroplast (LPO) and a longitudinal section of the polar filament outside the polaroplast. (D) Mature spore showing a section of the coiled portion of the polar filament. The scale bar on (c) is common for (d).

opposition of the membranes could lead to the observed blebs and microvillus-like structures. A similar thin, electron-dense amorphous layer on the external surface of the plasmodial membrane has also been observed in the microsporidian *Vavraia mediterranica* infecting the muscle of the shrimp *Crangon crangon* (Azevedo, 2001). Unfortunately, no rRNA gene sequence information is available for this species.

Abundant RER and mitochondria were found in the host cytoplasm surrounding *E. hepatopenaei*, but not within the cytoplasm of plasmodia. This is normal for microsporidia. Many now believe them to constitute a Phylum in the fungal kingdom and to represent reduced forms that have lost their mitochondria and other organelles through adaptation to their intracellular parasitic life-style (Lee et al., 2008; Keeling et al., 2005; Keeling and Fast, 2002; Fischer and Palmer, 2005; James et al., 2006; Hibbett et al., 2007). This is supported not only by gene sequence homology but also by similarity in steps of the meiotic division cycle and in possession of paired nuclei in diplokaryons (microsporidia) and dikaryons (fungi) (Flegel and Pasharawipas, 1995).

The hepatopancreatic microsporidian infections we studied occurred most frequently in MSGS *P. monodon* that were concomitantly infected with several viruses (Chayaburakul et al., 2004) or with pathogens in the lymphoid organ (Anantasomboon et al., 2006) or hematopoietic tissue. They were less frequent in normal shrimp. Thus, it is possible that the microsporidian infections were opportunistic in nature, with the pathogen exploiting a weakened immune status of the host. For example, the occurrence of human and mammalian infections of *E. bieneusi* in enterocytes of the duodenum and ileum of AIDS patients is dependent on chronic immunosuppression (Desportes et al., 1985).

5. Taxonomic summary

Phylum: Microspora (Sprague, 1977).
Class: Microsporea (Delphy, 1963).
Order: Microsporida (Balbiani, 1882).
Family: Enterocytozoonidae (Cali and Owen, 1990).
Genus: *Enterocytozoon* (Desportes et al., 1985).

5.1. E. hepatopenaei n. sp.

Specific diagnosis: Spores ovoid, measuring 0.7 × 1.1 µm in fresh preparation (shorter than those of *E. bienneusi*) and containing 5–6 visible coils of the polar filament. Polar filament precursors and other spore organelles formed within the sporogonal plasmodium and packaged into pre-sporoblast units prior to budding of sporoblasts to the host-cell cytoplasm.

Type host: P. monodon L. (Decapoda: Penaeidae)

Type locality: Chantoburi Province, eastern coast of Thailand

Site of infection: Cytoplasm of the tubule epithelial cells of the hepatopancreas

Etymology: The specific epithet relates to the location of the infection in hepatopancreatic tissue and to the generic name (*Penaeus*) of the host shrimp, thus *hepatopenaei*.

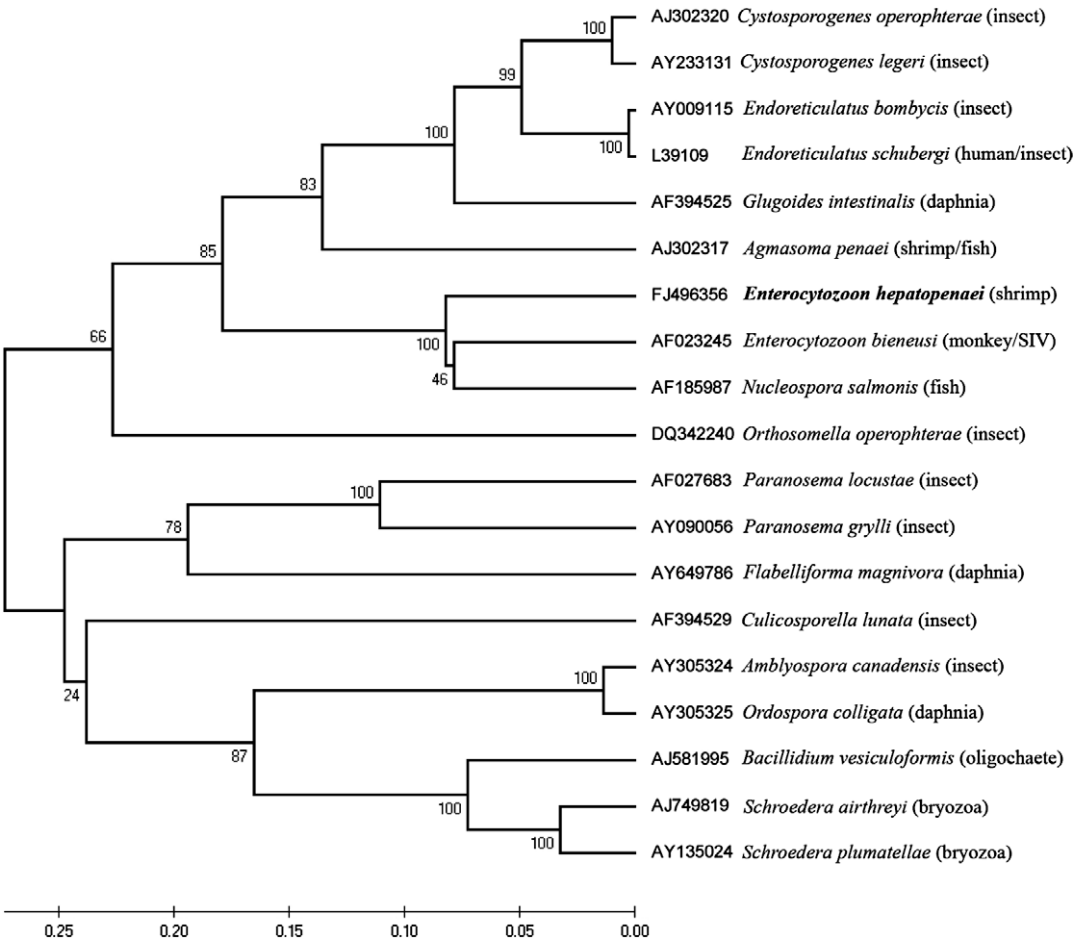

Fig. 6. Phylogenetic tree of *E. hepatopenaei* and selected microsporidians derived by using a CLUSTAL W alignment of matched SSU rRNA gene sequences with the optimality criterion set for distance and with *Amblyospora canadensis* as an out-group. Numbers at branch points indicate bootstrap values for 1000 replicates.

Type material: Paraffin and plastic blocks containing infected material have been deposited in the Center of Excellence for Shrimp Molecular and Biotechnology (Centex shrimp), Mahidol University, Thailand.

Gene sequence: The partial SSU rDNA sequence of the microsporidian, *E. hepatopenaei*, has been deposited in the GenBank/EMBL database under Accession No. FJ496356.

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