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Ultrastructural study on a novel microsporidian, *Endoreticulatus eriocheir* sp. nov. (Microsporidia, Encephalitozoonidae), parasite of Chinese mitten crab, *Eriocheir sinensis* (Crustacea, Decapoda)

Wen Wang [a,b,*], Jianxiu Chen [a]

[a] *Department of Biological Science and Technology, Nanjing University, Nanjing 210093, P.R. China*
[b] *Jiangsu Key Lab for Biodiversity and Biotechnology, College of Biological Sciences, Nanjing Normal University, Nanjing 210097, P.R. China*

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Abstract

A microsporidian pathogen, infecting the epithelial cells of the hepatopancreas of Chinese mitten crab, *Eriocheir sinensis*, was studied by electron microscopy. The detailed ultrastructure of life cycle of the pathogen including proliferative and sporogonic developmental stages are provided. All stages of the parasite are haplokaryotic and develop in a vacuole bounded by a single membrane in contact with host cell cytoplasm. Sporogenesis is synchronous with the same developmental stage in one vacuole. Sporogony shows a characteristic of multinucleate sporogonial plasmodia divided by rosette-like division, producing 4 or 8 sporoblasts. The mature spore is ellipsoidal, length (mean) 1.7 μm, width 1.0 μm, with a uninucleate in the center of the sporoplasm, 7 turns of the polar filament, a bell-like polaroplast of compact membranes and obliquely positioned posterior vacuole. The morphological characteristics of this novel microsporidian pathogen have led us to assign the parasite to a new species of *Endoreticulatus*, *E. eriocheir* sp. nov., that has not been reported previously from crab.

Keywords: Microsporidia; Ultrastructure; *Eriocheir sinensis*; Hepatopancreas; *Endoreticulatus eriocheir* sp. nov

1. Introduction

Microsporidia were initially believed to be protozoan parasites but now are recognized as fungi (Peer et al., 2000; Keeling and Fast, 2002). They are an extremely large and diverse group of microbial eukaryotes composed exclusively of obligate intracellular parasites of other eukaryotes. There are ubiquitous in nature, with currently approximately 150 described genera of microsporidia with over 1200 individual species (Patrick and Naomi, 2002). The majority of described species are from insect and fish hosts (Cali and Takvorian, 2003; Lom and Nilsen, 2003). A few described microsporidia, *Ameson* (Sprague, 1970), *Nadelspora* (Olson et al., 1994, 1997), and *Abelspora* (Azevedo, 1987), are known genera that are pathogenic to crabs. Previous studies on the tremor disease (TD)[1] of Chinese mitten crab, *Eriocheir sinensis*, revealed three different pathogens (Wang et al., 2002; Wang and Gu, 2002; Wang and Chen, 2004) including a microsporidian in the crab from Sihong county of Jiangsu province in southeastern China. Although the organism is not the primary agent and the direct cause of TD, this is a serious pathogen of the digestive system of the crab, infecting the hepatopancreatic epithelial cells. Furthermore, unique morphological characteristics distinguish this pathogen from previously described parasitic genera in crabs. Transmission electron microscopy (TEM) provided the identification of the species of microsporidia involved and was crucial for the identification of this new species (Patrick and Naomi, 2002). TEM is essential for the characterization of microsporidia

* Corresponding author. Fax: +86 25 83598723.
E-mail address: njnuwang@263.net (W. Wang).

[1] *Abbreviations used:* tremor disease (TD)/tremor disease agent (TDA).

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from unusual or new locations (Weiss, 2001). So the ultrastructural studies on the different developing stages of the microsporidian from the mitten crab have been delineated by TEM in order to make a primary confirmation of the taxonomic position of this novel pathogen.

2. Materials and methods

2.1. Samples collection

From May to September 1999, 43 diseased crabs with typical TD signs, weighing 30–90 mg, were taken from 6 affected freshwater ponds with (O_2 = 5–7% and 19–28 °C) in Sihong and Hongze districts(east longitude 117°56′–118°46′, north latitude 33°08′–33°47′), Northern Jiangsu Province in China. 23 healthy crabs were taken from 2 unaffected ponds (O_2 = 7–8% and 20–28 °C) in Suzhou, Southeastern Jiangsu Province in China. During the same period in 2000 and 2001, the same 8 ponds, where the water temperature and dissolved oxygen were roughly the same as those in 1999, were again sampled. At the same time, 21 diseased crabs were sampled from other districts: Luhe, Yixin and Gehu in Jiangsu Province, and Tianchang in Anhui Province. Crabs with sign of obvious tremor were dissected and sampled.

2.2. Preparation for TEM and SEM

For TEM, small fragments (1–2 mm^3) of gill, guts, hepatopancreas, cardiac muscle, body muscle, thoracic ganglion and gonad of Chinese mitten crab *Eriocheir sinensis* were fixed in 4% glutaraldehyde in 0.3 M sodium phosphate buffer (pH 7.3) for 4 h at 4 °C. The samples were washed for 2 h at 4 °C in the same buffer and postfixed in buffered 2% osmium tetroxide for 2 h at 4 °C. The tissues were dehydrated through a graded series of acetone and embedded in Epon 812. Semithin sections for light microscopy (LM) were stained with 1% methylene blue. Ultrathin sections were double stained with uranyl acetate and lead citrate, and observed with a HITACHI H-600 TEM, oprerated at 75 kV.

For SEM, the tissues with mature spores were fixed in the same fixation for TEM and smeared on glass slides. After dried in air, the slides were coated with gold at 10 mA for 3 min and observed in JSM5610LV SEM (Gallagher and Rhoades, 1979).

3. Results

3.1. Epidemic and parasitic characteristics of microsporidian organism

All of the 64 diseased crabs from affected ponds were infected to varying degrees by the tremor disease agent (TDA) which has been last confirmed to be spiroplasma (Wang et al., 2004a,b). Twelve of sampled crabs were also infected by another kind of pathogen, a microsporidian. No parasite or disease agent was detected in 23 crabs examined from the two unaffected ponds.

Unlike TDA, which infects almost all connective tissues, muscles and nerve system but not epithelial cells (Wang and Gu, 2002), the microsporidia were only found in the epithelial cells of hepatopancreas although there were also some TDA infecting the connective tissues of the hepatopancreas. In the infected epithelial cells, microsporidian elicited host cell hypertrophy, producing parasite-hypertrophic host cells with large inclusions (Fig. 1).

3.2. Ultrastructure and life cycle of microsporidian organism

3.2.1. Spore structure

Spores were ellipsoidal in shape and measured 1.0 ± 0.2 μm in width and 1.7 ± 0.2 μm in length. The thick spore wall, with a thickness of 130 nm, comprised an inner electron-lucent endospore (100–120 nm) and an outer electron-dense exospore (20–30 nm). The spores are uninucleate and the nucleus occupied a central position of microsporidian, between the polaroplast and the posterior vacuole, surrounded by two or three layers of endoplasmic reticulum and a polar filament, or polar tube, arranged in one row of 7 coils (Figs. 2 and 3). Distinguishing features include a

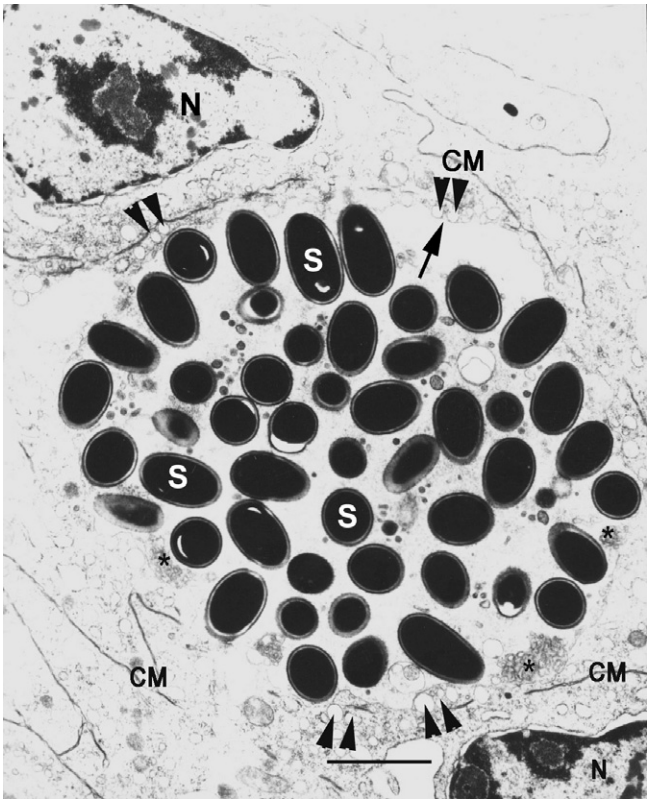

Fig. 1. TEM of a group of mature spores (S) in a vacuole of the hepatopancreas cell of *Eriocheir sinensis*. A nucleus (N) and cell membrane (CM) are seen in the host cell. Many small vesicles outside (double arrowheads) or inside (∗) the vacuole frequently lie in immediate proximity to the vacuole membrane (single arrow). Different dissections of the spores including straight-cut and crosscut are shown. Bar = 2 μm.

bell-like anchoring disc positioned in an anterior portion of the compact membranes with 2–3 vacuoles in posterior portion. The anterior end of the spore, in front of the anchoring disk, has a protuberant area (Fig. 4). All of these characteristics, including a polar filament, a polar sac-anchoring disk complex, a polaroplast, and posterior vacuoles forming the extrusion apparatus, are typical structures of a microsporidian.

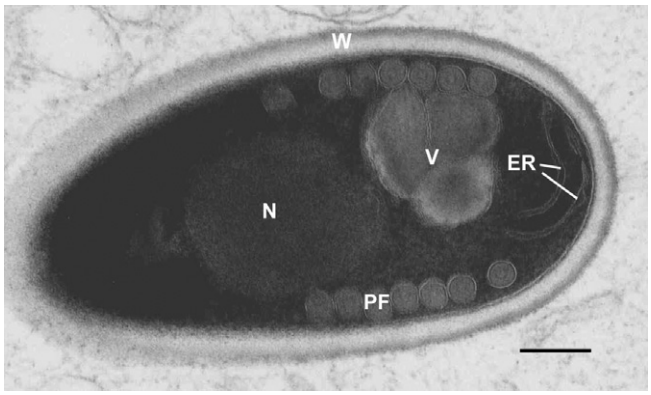

Fig. 2. TEM photograph of a longitudinal axial section of a mature spore containing a single nucleus (N), vesicular polaroplasts (V) and the polar filament (PF) that shows 7 coils. The wall (W) contains several layers that shown in detail in the following schematic drawing. Bar = 0.3 μm.

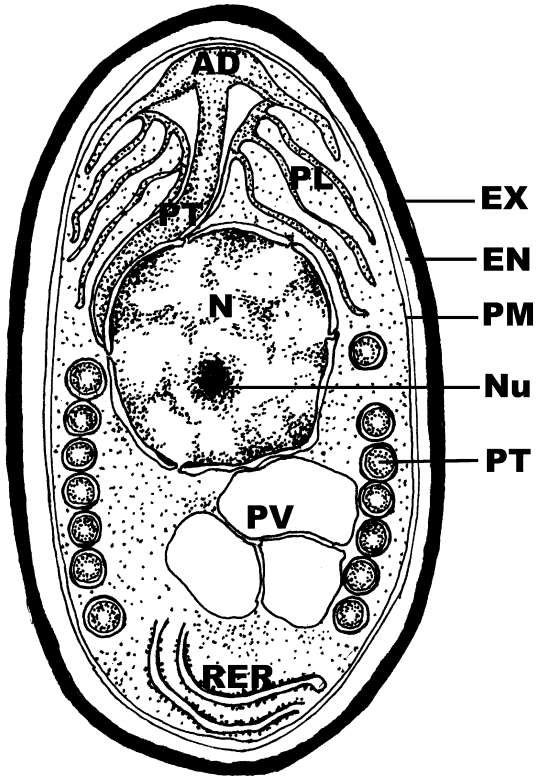

Fig. 3. Schematic drawing, longitudinal axial section of a mature microsporidian spore, showing the major structures including polar filament (PF) with anchoring disc (AD), polaroplast lamellar (PL), posterior vacuoles (PV), nucleus (N) with nucleolus (Nu); rough endoplasmic reticulum (RER), exospore (EX), endospore (EN), and plasma membrane (PM).

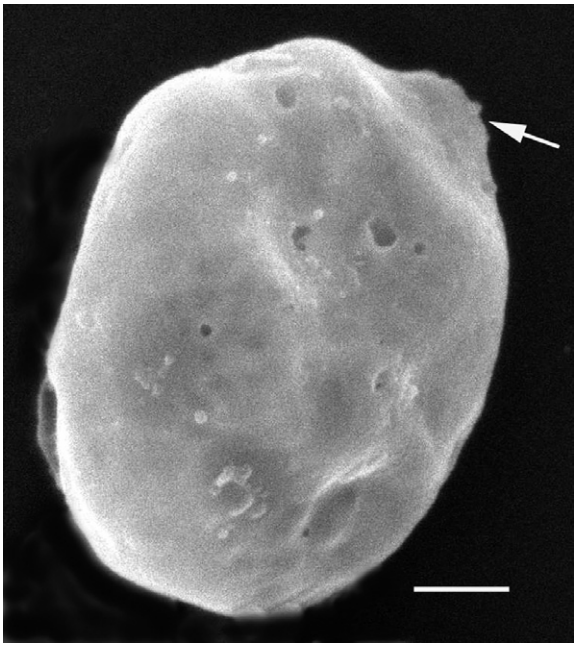

Fig. 4. SEM photograph of a mature spore showing the protuberant front of the anchoring disk (arrow). Bar = 0.3 μm.

3.2.2. Life cycle stages

The life cycle of the microsporidian pathogens in the hepatopancreas cells of the mitten crab included four stages: proliferation (merogony), sporogony, sporoblast spores and liberation. The first stage is initiated by piercing the host cell with the polar tube of the spore and extruding infective sporoplasm into the cytoplasm of the host cell. The sporoplasm develops and forms rounded to oval merozoites within the host cell cytoplasm. The next distinct phase in the life cycle is a proliferative phase (merogony), responsible for the massive increase in numbers inside the host cell (Fig. 5). The sporogonic phase (sporogony) follows, in which sporonts produce sporoblasts that mature into spores. All these stages of the parasite are haplokaryotic and develop in a vacuole bounded by a single membrane in contact with host cell cytoplasm (Figs. 5 and 6). Sporogenesis is synchronous, showing the same development stage of the generative parasites in one host cell (Figs. 1, 5–8). Spores are released from the host cells when they are mature and infect new host cells.

3.2.2.1. Merogony. The merogony (proliferative phase) occurs once the sporoplasm enters the host cell. The proliferative cells, referred to as meronts, are roundish cells encircled by a typical unit membrane. They have a large nuclear region with a single nucleus and then form rounded plasmodial multinuclear cells that divide by plasmotomy. Cells repeat their division cycles one to several times in this phase. Further nuclear fissions yield a plurinuclear plasmodium, which split in a rosette-like manner (divided by rosette-like division), producing 4 or 8, usually 8 sporoblasts (Fig. 5). The meronts develop in a parasitophorous-like vacuole (PV) surrounded by single membrane and they

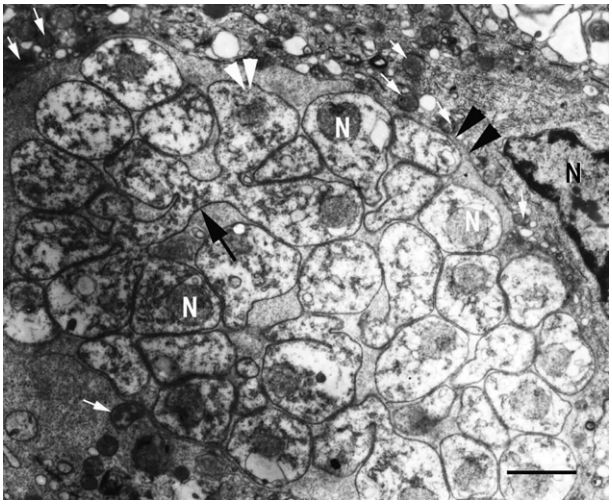

Fig. 5. TEM photograph of merogony (proliferative phase) in a host cell with a nucleus (N). Many mitochondria (white arrows) are seen in close proximity to the membrane of the parasitophorous-like vacuole (double black arrowheads). Merozoites divide by rosette-like division producing 4 or 8 sporoblasts (a single black arrow). The cytoplasm of the meronts is rich in poly-ribosome (double white arrowheads) and nuclei (white N) are uniformly granular. Bar = 1.6 μm.

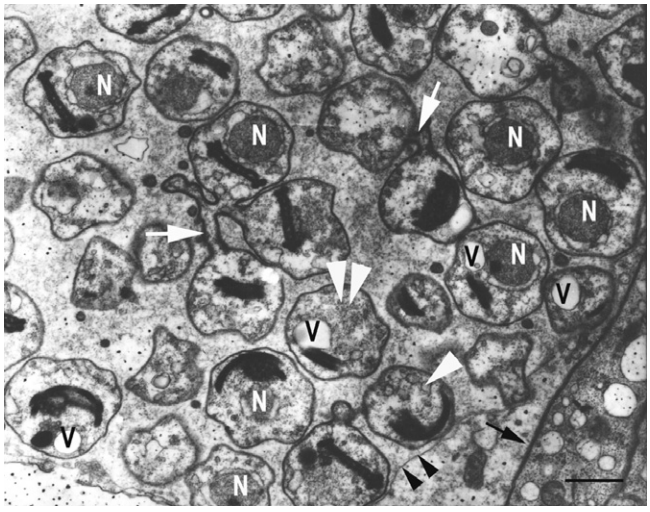

Fig. 6. TEM photograph of spore morphogenesis in sporogony. The meronts are dividing into multi-sporonts and showing some lobed plasmodia (white arrows). Many early sporoblasts are transforming into spores in a vacuole. The membrane of the vacuole is indicated by double arrowheads and the host cell membrane is indicated by a single arrow. Within the spore, uninucleate (N) including nucleolus, posterior vacuoles (V) and polar filament (PF) could be distinguished clearly, but ribosome (double white arrowheads) and endoplasmic reticulum (a single white arrow) are appear to be forming. Bar = 1 μm.

are synchronized within each vacuole. The cytoplasm of meronts is rich in free ribosomes and the nuclei are uniformly granular. Many mitochondria are seen near the plasma membrane (Fig. 5).

3.2.2.2. Sporogony. This process essentially involves conversion of a meront into a sporont, the cell that produces the sporoblasts. Sporoblasts are cells that mature and

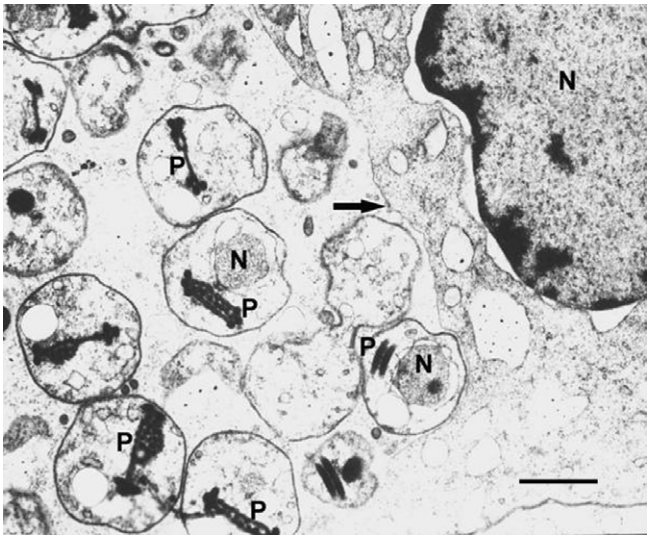

Fig. 7. TEM photograph of the early stage of spores forming in a membrane-packed (arrow) vacuole. The nucleus of the host cell is at the top right corner. The spores forming are shown with some organelles developed including nucleolus (N), posterior vacuoles (V) and polar filament (P). Bar = 1 μm.

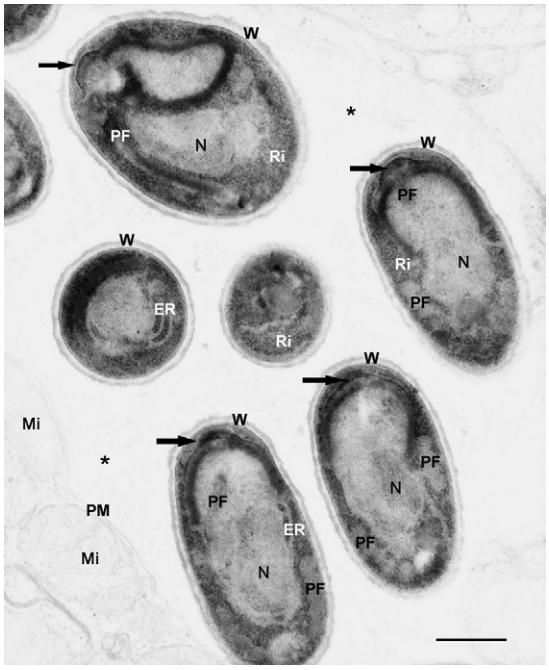

Fig. 8. TEM micrograph showing ultrastructural features of mature microsporidian spores showing coils of the polar filament (PF) near the constriction of the body and an anchoring disc at one pole (arrows). Ribosome (R) and endoplasmic reticulum (ER) and wall are well developed. Plasma membrane (PM) and mitochondria (Mi) of the host cell are shown. Bar = 0.5 μm.

transform into spores without further division. Ultrastructural evidence of patches of electron-dense material deposited on the outer surface of the plasmalemma, indicates that the meronts evolve into spores (Figs. 6 and 7). There is also a general, progressive increase in cytoplasmic density as more ribosomes and endoplasmic reticulum are formed (Fig. 8).

Sporogony is probably multi-sporoblastic. The sporoplasm is uninucleate and formed from rounded plasmodial multinucleate cells that divide by plasmotomy. All of the developing sporogony states occur in a vacuole. Many small vesicles frequently lie in immediate proximity to the vacuole membrane (Fig. 1). Rosette-like multi-sporonts, two or four in number, are observed in ultrathin section (Fig. 6). The widest sectioned nucleus of lobed plasmodia is 1.5 μm wide. Within the spore, well-organized rows of ribosome are arranged around the uninucleate and form a cytoplasmic groove (Figs. 6 and 7). The apical anchoring disc, polar cap, overlying the manubrium of the polar filament, and two regions of the polaroplasm can be distinguished (Fig. 8): the anterior part is the laminar region and the posterior is the saccate membrane system region. A posterior vacuole can be found at the posterior pole of the spore (Fig. 6).

4. Discussion

4.1. Microsporidian in crabs

There have been reports of microsporidian infections in crab. Sprague (1970) reported *Ameson michaelis* in the muscle tissue of an ocean crab in the Atlantic, Gulf of Mexico, causing severe muscle lysis that resulted in a condition known as "cotton crab". The crab meat infected with the microsporidian was cottony in texture, and poorly flavored. The agent was highly pathogenic, but could not be associated with outbreaks (Sprague, 1970; Overstreet, 1988). Azevedo (1987) found a new species of a microsporidian, *Abelspora portucalensis*, in the hepatopancreas of *Carcinus maenas* (Crustacea, Decapoda). This pathogen was characterized by one uninucleate schizont, that gave rise to two sporonts, each giving rise to two sporoblasts within a sporophorous vacuole circumscribed by a persistent pansporoblastic membrane. A novel microsporidian parasite of Dungeness crab (*Cancer magister*), *Nadelspora canceri* was found and studied by Olson et al. (1994, 1997). All of these reported microsporidians in crabs are different from what was found in the mitten crab. The microsporidian discussed in this paper possessed a single nucleus in all stages of development, characteristic of multinucleate sporogonial plasmodia that divided by rosette-like division, producing 4 or 8 sporoblasts and spore that developed in a parasitophorous-like vacuole (PV). These features most closely fit the characteristics of *Endoreticulatus* and this genus has not been reported previously from decapod crustaceans. So a new species of *Endoreticulatus*, *Endoreticulatus eriocheir* sp. nov., has been erected.

Studies on microsporidian in China are mainly focused on the insect hosts, especially silkworms (Wan et al., 1995; Gao and Huang, 1999) and bees (Li et al., 2004; Wan et al., 2005). There are few reports about microsporidian that have imperilled aquaculture in freshwater animals in China in recent years. Most of reported hosts are fish (Wu et al., 2005). There have been only two reported crustacean hosts of microsporidian in China, *Penaeus chinesis* (Hao and Mou, 1984; Gou et al., 1995) and *Penaeus monodon* (Wang et al., 2001), although there are many fish hosts of microsporidian in China. The ultrastructural characteristics of the microsporidian we found in the mitten crab are unique and different from that of all the aquatic animals. So the finding of this microsporidian pathogen in Chinese mitten crab may present a great potential as a novel disease agent in the freshwater environment in China.

4.2. Special characteristics of E. eriocheir sp. nov.

There are some special characteristics in the new *Endoreticulatus* species, *E. eriocheir* sp. nov. compared with described *Endoreticulatus* species such as *E. fidelis* (Brooks et al., 1988), *E. schuberg* (Cali and Garhy, 1991), *E. bombycis* (Wan et al., 1995; Zhang et al., 1995) and *Endoreticulatus*. sp. Taiwan (Wang et al., 2005). First, the mature spore's size is smaller, $1.0 \pm 0.2\,\mu m \times 1.7 \pm 0.2\,\mu m$. Second, sporogenesis is synchronous showing the same development stage of the generative parasites in one host cell, whereas the *Endoreticulatus* species from *Ocinara lida* in Taiwan showed different developmental stages found in parasitophorous vacuoles (Wang et al., 2005). Third, the polar filament, nuclear membrane and nucleolus are distinctly visualized at the early stage of spores forming, which are hardly seen in described *Endoreticulatus* species.

Molecular methods such as subunit rDNA (ssrDNA) analysis is helpful for phylogenetic analysis of the microsporidia (Vossbrinck and Debrunner-Vossbrinck, 2005). We have tried to do this with the microsporidian universal primer but the sequence was too short to be used for taxonomic analysis. The longer sequences failed to obtained due to the long period of fixation with glutaraldehyde (we collected samples in 1999). And fresh samples were also unavailable because the farmers had changed aquatic animals for aquaculture. However the ultrastructural characteristics have provided worthwhile information because ultrastructural data is still a very important evidence for taxonomic classifications.

4.3. Parasitophorous-like vacuole

The microsporidia-like organism from Chinese mitten crab is characterized by the life cycle stages including merogony, sporogony and sporoblast spores that developed within a vacuole bounded by a single membrane (Figs. 1, 5–7). The vacuole was a parasitophorous-like vacuole according to ultrastructural observations, in which many small vesicles frequently lay in immediate proximity to the vacuole membrane in the host cell, indicating its host origin (Fig. 1). The proximity of host cell mitochondria to the vacuole membrane showed that they appear capable of meeting the energy requirements of an increasing parasitic load (Fig. 5).

It is generally believed that the parasitophorous vacuole (PV) membrane is derived from the host cell while the spor-

ophorous vesicle (SV) is a membrane produced by the parasite (Bigliardi and Sacchi, 2001; Keeling and Fast, 2002; Canning and Curry, 2004; Franzen, 2004). Although in many papers, the origin of the wall has not been clearly observed and stated (Lom and Nilsen, 2003). Canning et al. (1985) studied *Cystosporogenes operophterae* and found that spores were produced in groups within a fine, semi-persistent envelope but were unable to determine whether the envelope was a SV or a PV of host origin. In the case of *Endoreticulatus fidelis*, the vacuole was bound by two membranes and, by virtue of the ribosome studding on the outer of the two membranes, the vacuole was identified as being of host origin, derived from the endoplasmic reticulum (Brooks et al., 1988). Microsporidia of the genus *Encephalitozoon* develop inside a PV of unknown origin. Using colocalization studies, the PV was found to be absent from the endocytic pathway markers, early endosomal autoantigen, transferrin receptor, and lysosome-associated membrane protein and for the endoplasmic reticulum marker calnexin (Fasshauer et al., 2005). Further investigations of the origin and genesis of the vacuole in the epithelial cells of hepatopancreas of Chinese mitten crab are necessary in order to understand the properties of this microsporidian pathogen interface.

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