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A new microsporidium, *Potaspora macrobrachium* n.sp. infecting the musculature of pond-reared oriental river prawn *Macrobrachium nipponense* (Decapoda: Palaemonidae)

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ABSTRACT

This paper described a novel microsporidian infection in the pond-reared oriental river prawn *Macrobrachium nipponense*. A conspicuous symptom of the infection was progressive white opacity associated with the musculature. Although neither bacteria nor viruses were detected in routine diagnostic tests, apparently degenerated microsporidian cells or spores were frequently observed in wet smears of the musculature from diseased prawns. Histological observations also revealed characteristics typical of microsporidian infection throughout the host. Transmission electron microscopy revealed multiple life stages of a microsporidian parasite within the cytoplasm of host muscle cells. In addition, partial small subunit ribosomal RNA (SSU rRNA) gene was obtained by a nested PCR using microsporidian specific primers. A consensus sequence was then deposited in GenBank (accession no. KU307278) and subjected to a general BLASTn search that yielded hits only for microsporidian sequence records. Phylogenetic analysis showed that the isolate was most similar to the fish microsporidian clade containing the genera *Kabatana*, *Microgemma*, *Potaspora*, *Spraguea*, and *Teramicra*. The highest sequence identity, 87%, was with *Potaspora* spp. Based on histological, ultrastructure and molecular phylogenetic data, we erected a new species, *Potaspora macrobrachium* for the novel microsporidium. The description of microsporidium in this important commercial host was fundamental for future consideration of factors affecting stock health and sustainability.

1. Introduction

The oriental river prawn, *Macrobrachium nipponense*, is an economically and nutritionally important crustacean. In China, the farming of *M. nipponense* has been in stable production for more than 10 years (Hongtuo et al., 2012; Xiu et al., 2015). The frequency of disease outbreaks for *M. nipponense* has been very low in the past. However, recently an epidemic affected the aquaculture industry for the prawns in the Nanjing City, Jiangsu Province of China. The diseased prawns were recognized by the progressive white opacity associated with the musculature. Such prawns were known as 'white tail' prawns. Generally, the survival rates of such diseased individuals in pond-reared system were poor. These infected prawns represented a significant loss to the aquaculture industry. To date, the cause of the disease has not been thoroughly investigated.

The Microsporidia are a diverse parasite phylum infecting host groups from all major taxa in all environments (Madyarova et al., 2015). Almost half of the known microsporidian genera infect aquatic hosts, however, microsporidians are probably ubiquitous and vastly underreported in aquatic systems (Stentiford et al., 2013). Several genera of microsporidia have been reported to infect crustacean hosts, including *Ameson*, *Agmasoma*, *Flabelliforma*, *Nosema*, *Nadelspora*, *Ordospora*, *Pleistophora*, *Thelohania*, *Tuzetia* and *Vavraia* (Moodie et al., 2003; Small et al., 2014; Sokolova et al., 2007; Stentiford et al., 2013). It was noteworthy that according to the most recent report, these parasites were a main mortality driver in the commercially important Chinese mitten crab, *Eriocheir sinensis* (Stentiford et al., 2011; Ding et al., 2015). The parasites have also been reported from prawns of several genera, notably *Penaeus*, *Pandalus* and *Crangon* (Ramasamy et al., 2000; Sprague and Couch, 1971). However, to date, there is no report about the microsporidian infection in the oriental river prawn *M.*

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nipponense. The majority of microsporidian species which have been described infecting crustaceans occur in feral populations; detailed information regarding the effect of microsporidia on the abundance of commercially reared populations is limited.

In the current study, histological, ultrastructural and phylogenetic evidence was used to describe a novel microsporidian pathogen in pond-reared oriental river prawns *M. nipponense*. The description of the pathogen infecting important commercial hosts is fundamental for future consideration of factors affecting stock health and sustainability.

2. Materials and methods

2.1. Sampling

In November 2015, a total of 110 prawns (with a carapace length of 18–35 mm) presented clinical signs out of approximately 800 prawns (apparent prevalence 13.75%), were collected in ten commercially reared ponds in Nanjing city of Jiangsu province, China. Prawns were examined visually for opacity in the tail musculature as an indication of the presence of microsporidian infection. These prawns were transported to the laboratory in sterile, aerated freshwater and maintained in aerated tanks containing filtered freshwater. The animals were then examined for the collection of exact carapace length (measured from the tip of the rostrum to the end of carapace), and were then fixed in 75% alcohol for use in PCR amplification, in 4% paraformaldehyde solution (pH 7.3) fixative for histopathological examination, and in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for electron microscopy observations. Each specimen was numbered and the different tissues were placed in vials. The remainder was frozen at −80 °C.

2.2. Histological analysis

For histology, tissues of tail muscle, hepatopancreas and cardiac muscle were dissected, fixed in 4% paraformaldehyde solution for 24 h and then transferred to 70% ethanol. After dehydration in a graded ethanol series to absolute ethanol, samples were embedded in paraffin. Five 4 μm-thick sections were obtained from each piece in different orientations and planes using a Leica RM2235 manual rotary microtome. Sections were then stained with hematoxylin and eosin (H&E) (Bell and Lightner, 1988; Ding et al., 2013) and examined using a light microscope (Nikon, Eclipse 80i). An unstained wet smear of infected musculature was also examined under the phase contrast microscope for signs of microsporidian infection.

2.3. Electron microscopy observations

In preparation for ultrathin sectioning, the fixative solution (2.5% glutaraldehyde) was removed and the tissues were post-fixed in 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer at pH 7.4 for 2 h. The tissues were dehydrated through a graded series of acetone and embedded in Epon 812. Ultrathin sections were double stained with uranyl acetate and lead citrate, and observed with a HITACHI H-600 TEM (Bojko et al., 2015).

2.4. DNA extraction and PCR

Diseased prawns were also analyzed by a nested PCR. DNA extractions of tail muscle were prepared with the Tissues Genomic DNA Isolation Kit (Tiangen, China). The extraction processes were carried out as recommended by the manufacturer. Templates were tested further for concentration and purity with a spectrophotometer (Eppendorf, Germany).

Fig. 1. External signs of the diseased prawns *M. nipponense*. The transparent appearance of uninfected prawn (a) contrasts that of infected prawns (b) due to the opacity in underlying skeletal musculature. Infected shrimp are lethargic and less able to elicit a tail-flick response. Arrows show the white opacity of the musculature.

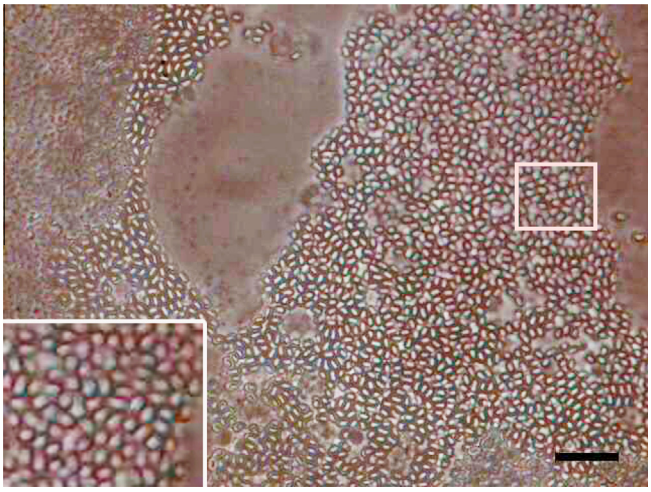

Fig. 2. An unstained wet smear of muscle tissue from the diseased prawns showed numerous degenerated microsporidian cells or spores. The insert (bottom) denoted area of magnified image in the white box (top). Scale = 100 μm.

The small subunit ribosomal RNA (SSU rRNA) gene sequences from the parasites were obtained by sequencing the PCR products resulting from a nested PCR. The primers V1F (5′-CACCAGGTT GATTCTGCCTGAC-3′) (Weissli, 1994) and 1492r (5′-GGTTACCTTGT TACGACTT-3′) (Weiss and Vossbrinck, 1998) were used for first round PCR, while the primers V1F and 964r (5′-CGCGTTGAGT CAAATTAA-GCCGCACA-3′) (Stentiford et al., 2011; Terry et al., 2003) were used for the second round. The PCR reactions were carried out in a final volume of 30 μl containing 5 μl template DNA, 3 μl 10×STR buffer (including dNTP, Mg^{2+}, Promega, USA), 0.75 units *Taq* DNA polymerase, 2 μl primers mix (2 μM) and 19.8 μl sterile water. In the first-round nested PCR, 3 ng of target DNA were used. For the second-round, 1 μl of a 1:100 dilution of the first-round PCR product was used as target DNA. Amplifications were performed starting with a 4 min denaturation step at 95 °C, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 47 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min.

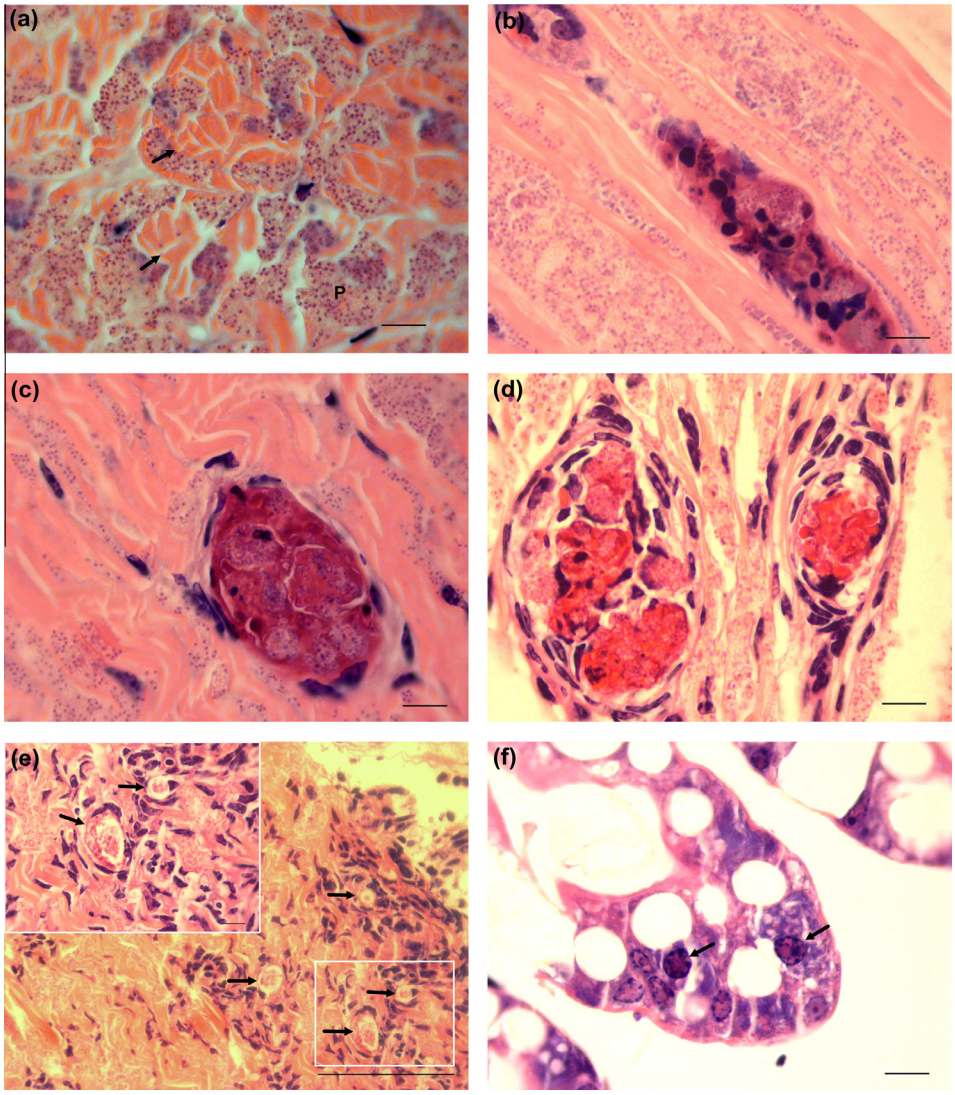

Fig. 3. Histopathology of the diseased oriental river prawn *M. nipponense*. (a) Muscle tissue of the diseased prawns. Note the 'islands' of unattached muscle blocks (arrows) surrounded by masses of parasitic cells (P). (b) Large infection replacing areas of the muscle block and hemocyte aggregation was often observed. (c) A large melanised granuloma was present with phagocytised microsporidian spores. (d) Two large focal granulomas in the cardiac muscle. (e) Inflammatory foci (arrows) were evident in the cardiac muscle. The insert (top) denoted the area of magnified image in white frame (bottom). (f) Numbers of hypertrophic and eosinophilic nuclei within the epithelial cells of hepatopancreas. All images H&E staining. Scale = 100 μm.

All the amplified products were detected using a 1.5% agarose gel followed by UV visualization after ethidium bromide staining.

2.5. Phylogenetic analysis

The resulting PCR products were purified by ethanol precipitation and sequenced. Sequenced data were then compared to known sequences using Basic Local Alignment Search Tool (BLASTn) (Altschul et al., 1990) to retrieve similar sequences. Multiple alignments were also performed using Clustal W (Thompson et al., 1997). An 827 bp partial SSU rDNA consensus sequence derived from V1F/964r amplicons from five prawn samples was deposited in genbank and placed into a neighbour-joining (NJ) tree containing 47 representative microsporidia that parasitize fish, crayfish, crab and other groups. Phylogenetic analyses were conducted using MEGA version 5 (Tamura et al., 2011). The NJ tree was constructed using a maximum composite likelihood model and the robustness of the tree was tested using 1000 bootstrap replicates. Bootstrap values were shown at each node.

3. Results

3.1. Gross pathology

Whitish discolouration[1] of the tail and abdominal musculature was a characteristic feature of the disease. Heavily infected prawns had a distinctly chalky white appearance, and were generally lethargic and less able to elicit a tail-flick response (Fig. 1). In addition to their reduced survivability during holding and transportation (>30%, farmers' description), the severe pathology ('white meat') was likely to cause considerable alteration in the yield and appearance of the individuals, which was also of potential commercial significance.

An unstained wet smear of muscle tissues from infected prawns showed numerous degenerated microsporidian cells or spores (Fig. 2). Neither bacteria nor viruses were detected in routine diagnostic tests.

[1] For interpretation of colour in Fig. 1, the reader is referred to the web version of this article.

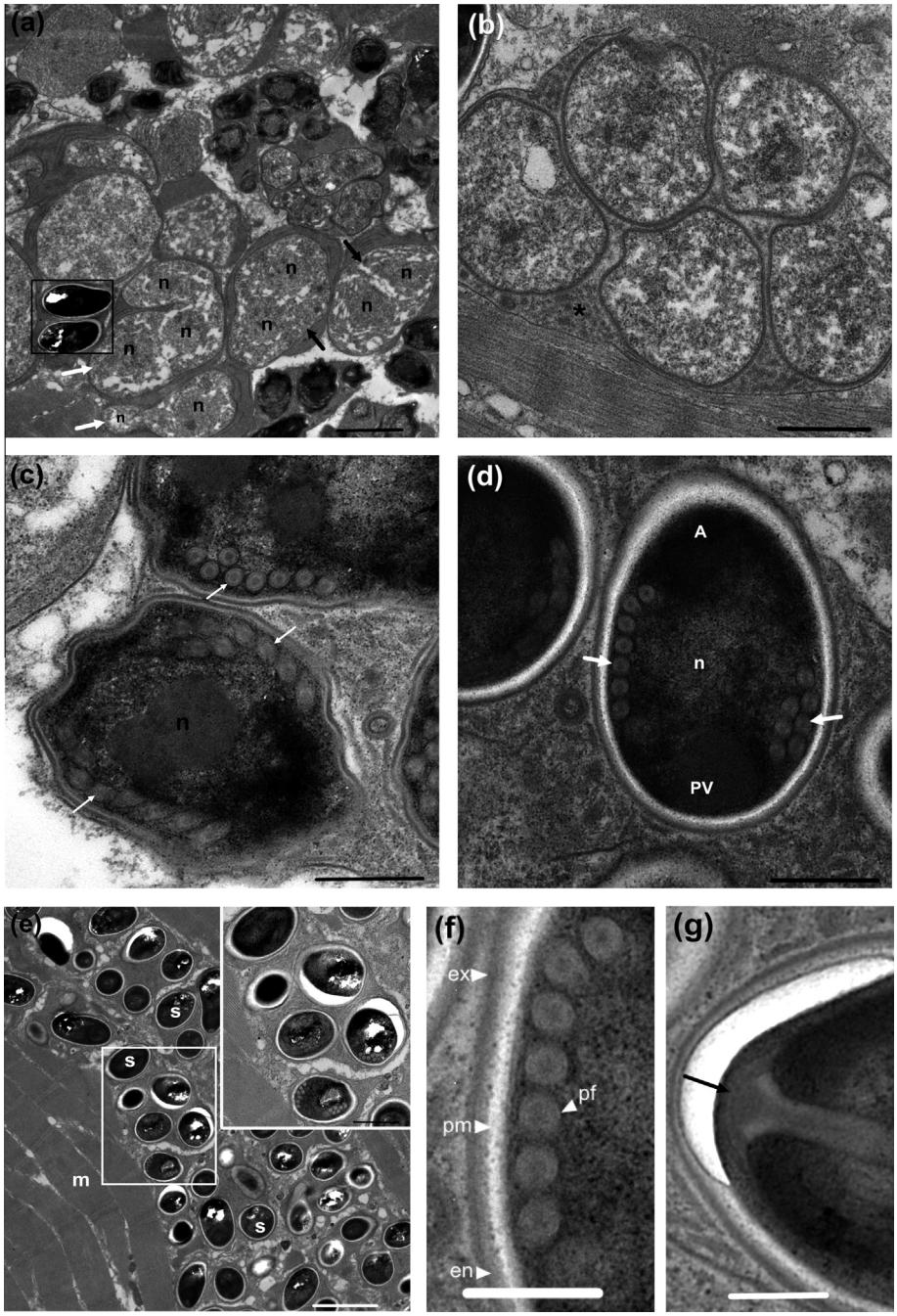

Fig. 4. Ultrastructure of spore maturation in the muscle tissue of the infected prawns *M. nipponense*. (a) Multinucleate sporogonial plasmodia within a sporophorous vesicle. Clear interfacial envelopes surrounding each Plasmodium which contained amorphous material in the episporontal space were observed (arrows). Mature spores (frame). Scale 2 μm. (b) A divided sporogonial Plasmodium (showing 5 sporonts) each of which would transform into a sporoblast was observed. The envelope of the sporophorous vesicle and episporontal secretions were clearly seen (asterisk). Scale 1 μm. (c) The sporoblast, present with nuclei (n) and developing polar filament (white arrows). Scale 500 nm. (d) Mature uninucleate spore with darkened cytoplasm. Polar vacuole (PV), nuclei (n), polar filaments (white arrows) and anchoring disk (A). Scale 500 nm. (e) A group of mature spores (s) among myofibres (m). Insert: Magnification of the frame area. Scale 2 μm (scale of insert 1 μm). (f) Detail of trilaminar spore wall comprising exospore (ex), endospore (en), and plasma membrane (pm). Polar filament (pf). Scale 250 nm. (g) High magnification image of the anchoring disk (black arrow). Scale 250 nm. All transmission electron microscopy.

3.2. Histopathology

In the infected prawns, muscle tissue was almost completely replaced by large numbers of parasites, with only 'small islands' of identifiable muscle fibres remaining (Fig. 3a). Spores were known to develop initially between individual muscle fibres; eventually replacing these tissues. Hemocyte aggregation was also often observed (Fig. 3b). Intense multi-focal inflammatory granulomas containing parasitic spores were commonly seen in heavily infected prawns (Fig. 3c). In the cardiac muscle, similar encapsulation responses were also observed (Fig. 3d) and inflammatory foci with phagocytised microsporidian spores were evident (Fig. 3e). Multiple hypertrophic and eosinophilic nuclei within the epithelial cells of the hepatopancreas were also observed (Fig. 3f).

3.3. Ultrastructure

Transmission electron microscopy (TEM) revealed multiple life stages of a microsporidian parasite within the cytoplasm of host muscle cells. Sporulation stages (sporogony plus spore morphogenesis) could be observed and given the advanced state of the infection meronts would be rare. Multinucleate sporogonial plasmodia within a sporophorous vesicle was also shown. There was a clear interfacial envelope surrounding each Plasmodium which contained an amorphous material in the episporontal space (Fig. 4a). A divided sporogonial Plasmodium (showing 5 sporonts) each of which would transform into a sporoblast was observed. The envelope of the sporophorous vesicle and episporontal secretions were clearly seen (Fig. 4b). Organelles including the anchoring disk, polar filament and condensed polaroplast began to form during development of the sporoblast (Fig. 4c) and eventually developed into mature spores (Fig. 4d). Groups of mature spores were often observed among myofibres (Fig. 4e). The polar filament was comprised of concentric rings of varying electron density, which was anisofilar with 7–8 coils. The spore wall was trilaminar with a thin electron lucent exospore and a thickened, electron lucent endospore overlaying the plasma membrane (Fig. 4f). Oval shaped mature spores possessed an umbrella-shaped anchoring disk covering the anterior region of the bi-laminar polaroplast (Fig. 4g).

3.4. Molecular phylogeny

The great discrepancies between the evolutionary relationships among Microsporidia based on morphology and developmental cycles has been clarified to some extent through the comparative analysis of the SSU rRNA gene sequences (Vossbrinck and Debrunner-Vossbrinck, 2005). So in this study, molecular phylogeny of the microsporidian parasite infecting *M. nipponense* was based upon a partial SSU rRNA gene retrieved from histopathology confirmed infected host material.

Two regions: An 1127 nucleotide and 868 nucleotide section of the SSU rRNA gene, were obtained for the unidentified microsporidian parasite from diseased *M. nipponense* using the nested PCR (V1F and 1492r; V1F and 964r) (Fig. 5a and b). A consensus sequence was concluded from five prawn samples and an 827 bp portion of the amplicon sequence (GC content 50.54%), which was then deposited in GenBank (accession no. KU307278) was subjected to a general BLASTn search that yielded hits only for microsporidian sequence records. The top hits from the BLASTn search included *Potaspora aequidens* isolated from the freshwater fish *Aequidens plagiozonatus* (GenBank KP404613) at 87% identity (with 99% coverage) and *Potaspora morhaphis* isolated from the teleostean fish, *Potamorhaphis guianensis* (GenBank EU534408) at 87% identity (with 100% coverage).

Phylogenetic trees displayed four distinct clades, which were assigned as groups 1, 2, 3 and 4 (Fig. 6) (Stentiford et al., 2011; Wang et al., 2013). Group 1 is represented by fish microsporidia including genera *Spraguea*, *Microgemma*, *Tetramicra*, *Kabatana* and *Potaspora*. Group 2 is composed of the genus *Dictyocoela*, pathogens of fresh water amphipods. Group 3 is comprised of microsporidia infecting several vertebrate and invertebrate hosts including humans, fish and shrimps. Group 4 is composed of both the family Enterocytozoonidae (genera *Enterospora*, *Enterocytozoon*, *Nucleospora*) and a new microsporidia, *Hepatospora eriocheir* parasitizing the Chinese mitten crabs (*E. sinensis*). The microsporidia recovered from the prawns *M. nipponense* shared most sequence similarity with Group 1 genera. The highest sequence identity was with *Potaspora* sp. which was consistent with the BLASTn analysis. This new lineage was supported by bootstrap values of 98%.

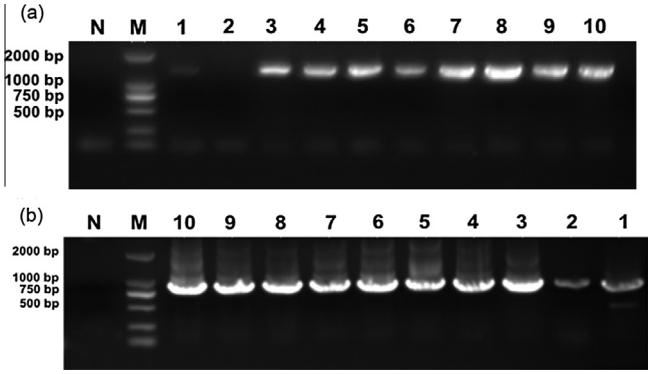

Fig. 5. Agarose gels of a nested PCR for microsporidia detection in 10 diseased prawns *M. nipponense* sampled from Nanjing city of Jiangsu Province, China. Two regions: a 1127 nucleotide (a) and 868 nucleotide (b) section of the SSU rRNA gene, were obtained for the unidentified microsporidian parasite. The two sequences were subjected separately to a general BLASTn search that all yielded hits only for microsporidian sequence records. The top hits included *Potaspora aequidens* isolated from the freshwater fish *Aequidens plagiozonatus* (GenBank KP404613) at 87% identity (with 99% coverage) and *Potaspora morhaphis* isolated from the teleostean fish, *Potamorhaphis guianensis* (GenBank EU534408) at 87% identity (with 100% coverage).

Thus, the disease was most probably caused by the infection of a hitherto unknown microsporidian parasite that has a genetic affinity to the genus *Potaspora*. The available morphological, ultrastructural, and molecular data supported that this microsporidium was a new species and was classified as *Potaspora macrobrachium*.

4. Taxonomic summary

Name: P. macrobrachium n.sp. (Microsporidia, Tetramicridae)

Species description: Majority of observed developmental stages of the parasite are unikaryotic. There is a clear interfacial envelope surrounding each Plasmodium which contains an amorphous material in the episporontal space. The interfacial membrane separates life stages from the host cell cytoplasm. Oval shaped mature spores uninucleate with mean length 1.578 μm (±0.0438 μm SE) and width 0.966 μm (±0.0193 μm SE) (n = 35). Spores possess 7–8 polar filament coils in a single or double rank, umbrella-shaped anchoring disk covering the anterior region of bi-laminar polaroplast and electron dense posterior vacuole. Spore wall trilaminar with a thin electron lucent exospore and a thickened, electron lucent endospore overlaying the plasma membrane.

Specific diagnosis: Presence of a microsporidian parasite with descriptive features of the above in cytoplasm of the host muscle cells. Diagnosis of morphological features by histology and TEM. Nucleic acid-based diagnosis via PCR amplification, accompanied by analysis of the SSU rRNA gene sequence and comparison to Genbank.

Site of infection: Infection located in skeletal musculature and cardiac muscle.

Prevalence: Peak infection occurs during the winter (November and December) and the spring (March and April).

Type host: oriental river prawn *M. nipponense* (Decapoda: Palaemonidae)

Type locality: Nanjing city of Jiangsu province, China (32°4′46.10″ N, 118°24′47.46″ E).

Etyology: The specific epithet "macrobrachium" refers to the genus name of the host species.

Type material: Histological sections and TEM resin blocks from China have been deposited in Jiangsu Key Laboratory for Biofunctional Molecule, Jiangsu Second Normal University. *P. macro-*

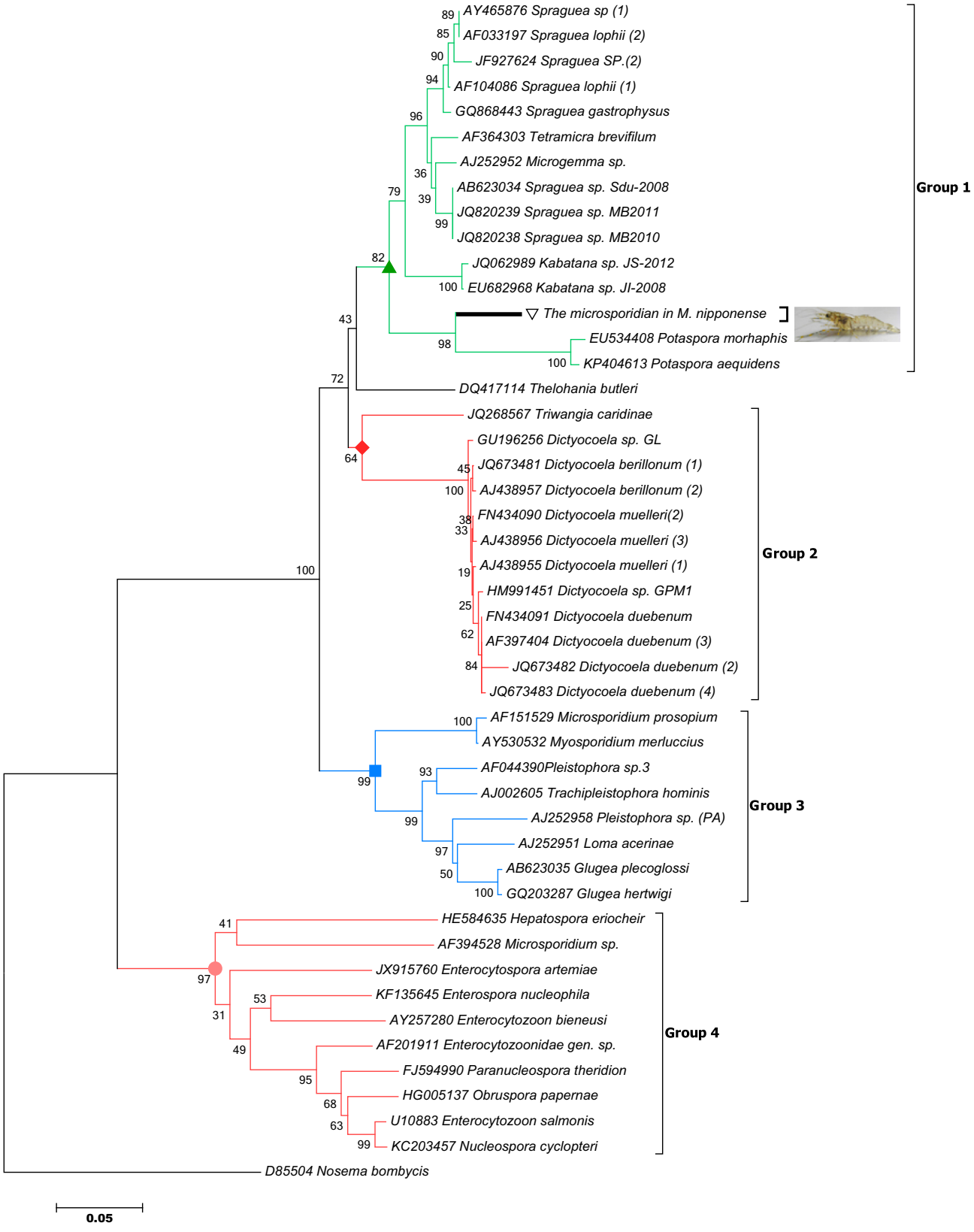

Fig. 6. Phylogenetic tree constructed by neighbour-joining (NJ) revealed that the microsporidia species whose sequences were obtained in the present study was most closely related to the clade containing fish microsporidia (Group 1). *Nosema bombycis*, a microsporidian parasite prevailed in sericulture, was used as outgroup. Analysis was done on 1000 bootstrapped data sets. Bootstrap values were shown at each node. The scale bar represented substitutions per nucleotide site.

brachium SSU rRNA gene sequences from samples collected in China have been deposited in GenBank (accession no. KU307278).

5. Discussion

Histological and ultrastructural pathology, nested PCR detection and molecular phylogeny all confirmed the epidemic in commercially reared prawns *M. nipponense* was accompanied by a hitherto unknown microsporidian parasite, which is most likely the causative agent for the disease. This was the first description of a microsporidian parasite from the prawn *M. nipponense*. Microsporidians are known to infect a variety of other crustaceans, but this prawn has not previously been reported as hosts. This is significant because *M. nipponense* has few reported parasites whereas other crustaceans harbour a plethora of parasitic agents (Stentiford et al., 2013).

Phylogenetic analysis showed that the SSU rDNA of the microsporidium from *M. nipponense* was most identical with *Potaspora* spp. (87% sequence identity). However, the species was dissimilar with the initially described *Potaspora* spp. (*P. aequidens and P. moraphis*) in terms of spore size (1.578×0.966 vs. 3.4×1.9 and 2.8×1.5 μm), the number of coils in the PF (7–8 vs. 9–10 and 8–9) and host habitat (prawns vs. fish) (Casal et al., 2008; Videira et al., 2015). So based on histological, ultrastructure and phylogenetic data, we erected a new species, *P. macrobrachium* for the novel microsporidium.

In this study, the significance of the parasite as a mortality driver in *M. nipponense* stocks was assessed. Generally, the survival rates of such diseased individuals in pond-reared system were poor. Sometimes, infected prawns were rarely observed in landed catches. It may possibly be a result of the death of infected prawns, or to the greater vulnerability of infected prawns to predation, hence, greatly reducing the numbers of infected individuals available for sampling. Considering that the microsporidia found in prawns were highly pathogenic to the host, the discovery of this microsporidian pathogen may present a great threat as a novel disease agent in the crustacean aquaculture in China.

The presence of this parasite may be easily recognized in the advanced phase of the disease, since the abdominal musculature of infected individuals assumed an opaque white colouration. The infected prawns appeared lethargic and less able to elicit a tail-flick response, mainly as a result of the disintegration of muscle fibres. The body muscle fibres which were initially surrounded by a few microsporidian spores were gradually lost as the myofibrils are crystallized, probably by the process of depolymerization, until eventually these are destroyed and replaced by the invading spores (Ramasamy et al., 2000).

Granuloma-like foci were observed within the remaining blocks of tail muscle and also within the cardiac muscle. Such foci have been described as aggregations of flattened hyaline cells encapsulating foreign material and which lead to the deposition of melanin either on the object or within the haemocyte matrix (Stentiford et al., 2002). In the case of parasite infection, the parasite is destroyed as the inner layers of these foci became necrotic (Smith and Söderhäll, 1986). Such encapsulating lesions have been thought to indicate a previous microbial or parasitic infection.

The route of transmission of the parasite has not yet been resolved. Movement of the infected prawns facilitated the movement of their pathogens (Bojko et al., 2015), so tracking the spread of this invasion was an important endeavor. It was suggested that during translocations, done for restocking purposes, great care should be taken to select parasite-free prawn populations. In addition, prawn should be kept in quarantine ponds, under periodical control, before their introduction in large-scale aquaculture. Vertical transmission occurs in the life cycle of a number of species of Microsporidia. Transovarial transmission via infected eggs was common among microsporidia of invertebrates and has been suspected for some fish microsporidia suggesting that vertical transmission or contamination of progeny may also partially be responsible for the spread of the parasite.

It was observed that the disease showed a seasonal epizootiology, with peak infection occurring during the winter (November and December) and the spring (March and April), and with a latent infection or absence during the summer and early autumn. Prevalence of infections by parasitic dinoflagellates of the genus *Hematodinium* in some crab species also suggested highly seasonal disease outbreaks, with peak infection occurring over a relatively narrow time period, followed by a longer period of undetectable or low level prevalences (Stentiford et al., 2002). The virulence of the pathogens could increase owing to environmental stresses such as pollution, increase in prawn density, paucity of food etc. So adequate measures should also be taken in the susceptible months to avoid parasitic infections.

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