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A new microsporidium, *Triwangia caridinae* gen. nov., sp. nov. parasitizing fresh water shrimp, *Caridina formosae* (Decapoda: Atyidae) in Taiwan

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ABSTRACT

A new microsporidium was isolated from the endemic, Taiwanese shrimp, *Caridina formosae* (Decapoda, Atyidae) from northern Taiwan. A conspicuous symptom of infection was presence of opaque white xenomas located in the proximity of the alimentary tract, the surface of the hepatopancreas, and the gills. A fully developed xenoma consisted of a hard, thick capsule filled with sporophorous vesicles containing multiple spores. Microsporidia developed synchronously within the same sporophorous vesicle, although the stage of parasite development differed among the vesicles. Fresh spores were pyriform, mononucleated and measured 6.53 × 4.38 μm. The polar filament was anisofilar with 9–11 coils. Phylogenetic analysis based on the small subunit ribosomal DNA sequence showed that the isolate is most similar to the fish microsporidian clade containing the genera *Kabatana*, *Microgemma*, *Potaspora*, *Spraguea*, and *Teramicra*. The highest sequence identity, 80%, was with *Spraguea* spp. Based on pathogenesis, life cycle and phylogenetic analysis, we erect a new genus and species, *Triwangia caridinae* for the novel microsporidium.

1. Introduction

Microsporidia are obligate intracellular eukaryotic parasites reported from nearly all invertebrate phyla. The majority of species are described from arthropod and fish hosts, particularly insects and crustaceans (Wittner and Weiss, 1999). Approximately 43 microsporidian genera from crustaceans have been described (Table 1) and 11 of these genera have been reported from shrimps including, *Agmasoma*, *Ameson*, *Enterocytozoon*, *Inodosporus*, *Myospora*; *Perezia*, *Pleistophora*, *Thelohania*, *Tuzetia*, *Vairimorpha* and *Vavraia* (Table 2).

At least 23 microsporidian species have been described from shrimps. Microsporidia have been reported from about 20 species of marine or estuarian shrimps and eight species of fresh water crayfish (Table 2). The microsporidia *Agmasoma penaei*, *Ameson* sp., *Enterocytozoon hepatopenaei*, *Perezia nelsoni*, *Pleistophora* spp., *Thelohania* spp., and *Tuzetia weidneri* collectively infect at least eight species of penaeid shrimp, a group that contains many species of economic importance including *Penaeus monodon* and *Litopenaeus setiferus* (Table 2). In addition, *Inodosporus spraguei* and *Indosporus octospora* were isolated from *Palaemon* spp. and *Palaemonetes* spp. (Azevedo et al., 2000; Overstreet and Weidner, 1974; Sprague and Couch, 1971), and *Pleistophora crangoni*, *Thelohania giardi* and *Vavraia mediterranica* were recovered from five species of crangonid shrimp (Azevedo, 2001; Breed and Olson, 1977; Krygier and Horton, 1975) (Table 2).

The tiny atyid shrimp, *Caridina formosae*, with an adult body length of 1.5–2.0 cm (Fig. 1), is an endemic species occurring in the streams of northern and western Taiwan (Shy et al., 2001). Shrimps complete their life cycle in the fresh water system and are often reared commercially as live food for aquaculture or are kept as aquarium pets (Hung et al., 1993; Shy et al., 2001). We first observed microsporidian infections in field collected shrimps and noted that symptoms of the disease were obviously different from those of known microsporidioses from marine or other freshwater shrimps. We studied the life cycle, morphology and ultrastructure of this new microsporidian species. We also analyzed the full small subunit ribosomal DNA sequence, compared it with those of other microsporidia in the NCBI public database and performed a phylogenetic analysis. Based on ultrastructural and molecular evidence, we propose that this microsporidium belongs to a new genus closely related to the genus *Spraguea*, a xenoma-forming fish microsporidium.

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Table 1
Microsporidia genera in crustaceans.

Genus	Crustacean host		Reference
Triwangia	Shrimp	*Caridina formosae*	This study
Agmasoma	Shrimp	*Farfantepenaeus duorarum*; *Fenneropenaeus* spp. (2); *Litopenaeus setiferus*; *Penaeus monodon*	Sprague and Couch (1971), Kelly (1979) and Pasharawipas and Flegel (1994)
Ameson	Crab	*Callinectes sapidus*	Zhu et al. (1993)
	Crayfish	*Austropotamobius pallipes*	Edgerton et al. (2002)
	Shrimp	*Penaeus monodon*	Anderson and Nash (1989)
Abelspora	Crab	*Carcinus maenas*	Azevedo (1987)
Agglomerata	Copepod	*Acanthocyclops vernalis*	Bronnvall and Larsson (2001)
	Cladoceran	*Sida crystallina*	Larsson and Yan (1988)
Amblyospora	Copepod[a]	*Acanthocyclops* spp.(2); *Cyclops strenuus*; *Diacyclops bicuspidatus*; *Mesocyclops annulatus*; *Paracyclops fimbriatus fimbriatus*	Micieli et al. (2000a,b) and Vossbrinck et al. (2004)
Baculea	Water flea	*Daphina pulex*	Loubès and Akbarieh (1978)
Berwaldia	Water flea	*Daphnia pulex*	Larsson (1981)
Binucleata	Water flea	*Daphnia magna Straus*	Refardt et al. (2008)
Cougourdella	Copepod	*Megacyclops viridis*	Larsson (1989)
Cucumispora	Amphipod	*Dikerogammarus villosus*	Ovcharenko et al. (2010)
Desmozoon	Copepod	*Lepeophtheirus salmonis*	Freeman and Sommerville (2009)
Dictyocoela	Amphipod	*Echinogammarus berilloni*; *Gammarus* spp. (3); *Orchestia* spp. (2); *Talorchestia deshayesei*	Hogg et al. (2002), Terry et al. (2004) and Krebes et al. (2010)
Hepatospora	Crab	*Eriocheir sinensis*	Stentiford et al. (2011) and Wang and Chen (2007)
Enterocytozoon	Shrimp	*Penaeus monodon*	Tourtip et al. (2009)
Enterosora	Crab	*Cancer pagurus*; *Eupagurus bernhardus*	Stentiford et al. (2007) and Stentiford and Bateman (2007)
Facilispora	Copepod	*Lepeophtheirus salmonis*	Jones et al. (2012)
Flabelliforma	Water flea	*Daphnia. magna*	Larsson et al. (1998)
Glugoides	Water flea	*Daphnia* spp. (2)	Larsson et al. (1996)
Gurleya	Water flea	*Atyephira* spp. (2); *Daphnia* spp. (2); *Macrocyclops albidus*; *Moina rectirostris*	Doflein (1898), Jirovec (1942), Sprague and Couch (1971), Green (1974), Voronin (1996), Friedrich et al. (1996)
Gurleyides	Water flea	*Ceriodaphnia reticulata*	Voronin (1986)
Holobispora	Copepod	*Thermocyclops oithonides*	Issi (1986)
Hyalinocysta	Copepod[a]	*Orthocyclops modestus*	Andreadis and Vossbrinck (2002)
Inodosporus	Shrimp	*Palaemon* spp. (2); *Palaemonetes* spp. (2)	Codreanu (1966), Sprague and Couch (1971), Overstreet and Weidner (1974) and Azevedo et al. (2000)
Lanatospora	Copepod	*Macrocyclops albidus*	Voronin (1986)
Larssonia	Water flea	*Daphnia* spp. (2)	Vidtman and Sokolova (1994), Bengtsson and Ebert (1998)
Marssoniella	Copepod	*Cyclops* spp. (2)	Vossbrinck et al. (2004) and Vávra et al. (2005)
Microsporidium	Water flea	*Daphnia pulex*	Refardt et al. (2002)
Mrazekia	Copepod	*Macrocyclops albidus*	Issi et al. (2010)
	Isopod	*Asellus aquaticus*	Leger and Hesse (1916)
Myospora	Lobster	*Metanephrops challengeri*	Stentiford et al. (2010)
Nadelspora	Crab	*Cancer* spp. (2)	Olson et al. (1994), Childers et al. (1996)
Nelliemelba	Copepod	*Boeckella triarticulata*	Milner and Mayer (1982)
Norlevinea	Water flea	*Daphnia longispina*	Vávra (1984)
Nosema	Amphipod	*Gammarus* spp. (2)	Terry et al. (1999) and Haine et al. (2004)
	Crab	*Carcinus maenas*; *Callinectes sapidus*; *Pachygrapsus marmoratus*	Leger and Duboscq (1909) and Sprague and Couch (1971)
	Ostracod	*Stenocypris major*	Diarra and Toguebaye (1996)
Ordospora	Water flea	*Daphnia magna*	Larsson et al. (1997)
Ormieresia	Crab	*Carcinus mediteraneus*	Vivarès et al. (1977)
Paranucleospora	Copepod	*Lepeophtheirus salmonis*	Nylund et al. (2010)
Perezia	Crab	*Carcinus maenas*	Sprague and Couch (1971)
	Shrimp	*Farfantepenaeus aztecus*; *Litopenaeus setiferus*	Sprague (1950), Sprague and Vernick (1969) and Canning et al. (2002)
Pleistophora	Amphipod	*Gammarus duebeni celticus*	Terry et al. (2003)
	Crab	*Callinectes sapidus*	Sprague and Couch (1971)
	Crayfish	*Cambarellus puer*	Sprague (1966) and Sprague and Couch (1971)
	Shrimp	*Atyephira sp.*; *Branchinella thailandensis*; *Crangon* spp. (4) *Farfantepenaeus* spp. (2); *Litopenaeus setiferus*; *Palaemonetes pugio*	Kudo (1924), Baxter and Rigdon (1970), Sprague and Couch (1971), Streets and Sprague (1974), Krygier and Horton (1975), Breed and Olson (1977), Kelly (1979) and Purivirojkul and Khidprasert (2009)
Thelohania	Crab	*Carcinus maenas*; *Eupagurus bernhardus*; *Petrolisthes armatus*	Sprague and Couch (1971)
	Crayfish	*Astacus* spp. (3); *Cambarellus* spp. (2); *Cherax destructor*	Henneguy (1892), Sprague (1950), Sogandares-Bernal (1962), Sprague and Couch (1971) and Moodie et al. (2003a,b)
	Shrimp	*Crangon crangon*; *Farfantepenaeus* spp. (3); *Palaemonetes variens*; *Pandalus jordani*; *Penaeus semisulcatus*	Sprague and Couch (1971), Vernick et al. (1977), Johnston et al. (1978) and Kelly (1979)
Tuzetia	Copepod	*Boeckella triarticulata*; *Cyclops albidus*	Kudo (1921) and Milner and Mayer (1982)
	Shrimp	*Farfantepenaeus aztecus*; *Litopenaeus setiferus*	Canning et al. (2002)
Vairimorpha	Crayfish	*Cherax destructor*	Moodie et al. (2003c)
Vavraia	Shrimp	*Crangon crangon*	Azevedo (2001)

Note: Figure in brackets refers to number of species belong to the genus.
[a] Intermediate host.

Table 2
Microsporidia species in shrimps.

Microsporidian species	Host species (habitat)	Symptoms	Tissue infected	Reference
Triwangia				
Triwangia caridinae	*Caridina formosae* (F)	Xenomas	Alimentary canal Hepatopancreas Gills	This study
Agmasoma				
Agmasoma penaei	*Fenneropenaeus merguiensis* (M)	Whitened tissue	Hepatopancreas Gonads	Pasharawipas and Flegel (1994)
	Fenneropenaeus indicus (M)	Whitened tissue	Ovary	Sprague and Couch (1971)
	Farfantepenaeus duorarum (E)	Whitened tissue	Musculature Digestive tract Ovary	Kelly (1979)
	Litopenaeus setiferus (E)	Whitened tissue	Gonads	Sprague and Couch (1971)
	Penaeus monodon (M)	Whitened tissue	Hepatopancreas Gonads	Pasharawipas and Flegel (1994)
Ameson				
Ameson sp.	*Austropotamobius pallipes* (F)	Whitened tissue	Musculature	Edgerton et al. (2002)
Ameson sp.	*Penaeus monodon* (M)	Whitened tissue	Hepatopancreas	Anderson and Nash (1989)
Enterocytozoon				
Enterocytozoon hepatopenaei	*Penaeus monodon* (M)	Not described	Hepatopancreas	Tourtip et al. (2009)
Inodosporus				
Inodosporus spraguei	*Palaemonetes pugio* (E)	Whitened tissue	Musculature	Overstreet and Weidner (1974)
Inodosporus octospora	*Palaemon elegans* (M)	Whitened tissue	Musculature	Codreanu (1966)
	Palaemon serratus (M)	Whitened tissue	Musculature	Azevedo et al. (2000)
	Palaemonetes rectirostris (E)	Whitened tissue	Musculature	Sprague and Couch (1971)
Myospora				
Myospora metanephrops	*Metanephrops challenger* (M)	Xenomas	Musculature	Stentiford et al. (2010)
Perezia				
Perezia nelsoni	*Farfantepenaeus aztecus* (E)	Whitened tissue	Musculature	Sprague (1950) and Canning et al. (2002)
	Litopenaeus setiferus (E)	Whitened tissue	Musculature	Sprague and Vernick (1969) and Canning et al. (2002)
Pleistophora				
Pleistophora crangoni	*Crangon franciscorum* (E)	Whitened tissue	Musculature	Krygier and Horton (1975)
	Crangon nigricauda (E)	Whitened tissue	Musculature	Krygier and Horton (1975)
	Crangon nigromaculata (E)	Whitened tissue	Musculature	Breed and Olson (1977)
	Crangon stylirostris (E)	Whitened tissue	Musculature	Breed and Olson (1977)
Pleistophora lintoni	*Palaemonetes pugio* (E)	Whitened tissue	Musculature	Streets and Sprague (1974)
Pleistophora miyairii	*Atyephira sp.*(F)	Whitened tissue	Digestive tract	Sprague and Couch (1971) and Kudo (1924)
Pleistophora penaei	*Farfantepenaeus aztecus* (E)	Whitened tissue	Musculature	Baxter and Rigdon (1970)
	Litopenaeus setiferus (E)	Whitened tissue	Musculature Heaptopancreas	Baxter and Rigdon (1970)
Pleistophora sogandaresi	*Cambarellus puer* (F)	Whitened tissue	Musculature	Sprague (1966) and Sprague and Couch (1971)
Pleistophora sp.	*Farfantepenaeus duorarum* (E)	Whitened tissue and flaccid	Musculature	Kelly (1979)
Thelohania				
Thelohania butleri	*Pandalus jordani* (M)	Not described	Musculature	Vernick et al. (1977) and Johnston et al. (1978)
Thelohania cambari	*Cambarellus bartonii* (F)	Whitened tissue	Musculature	Sprague (1950)
Thelohania contejeani	*Astacus astacus* (F)	Whitened tissue	Musculature	Sprague and Couch (1971)
	Astacus fluviatilis (F)		Musculature	Henneguy (1892)
	Astacus palipes (F)	Whitened tissue	Musculature	Sprague and Couch (1971)
Thelohania duorara.	*Farfantepenaeus duorarum* (E)	Whitened tissue	Musculature Digestive tract Hemocyte-forming organ	Kelly (1979)
	Farfantepenaeus brasiliensis (E)	Whitened tissue	Musculature	Sprague and Couch (1971)
	Farfantepenaeus aztecus (E)	Whitened tissue	Musculature	Sprague and Couch (1971)
Thelohania giardi	*Crangon crangon* (E)	Whitened tissue	Musculature	Sprague and Couch (1971)
Thelohania macrocystis	*Palaemonetes varians* (E)	Whitened tissue	Musculature	Sprague and Couch (1971)
Thelohania montirivulorum	*Cherax destructor* (F)	Not described	Musculature	Moodie et al. (2003a)
Thelohania parastaci	*Cherax destructor* (F)	Whitened tissue	Musculature	Moodie et al. (2003b)
Thelohania soganderesi	*Cambarellus shufeldti* (F)	Whitened tissue	Musculature	Sogandares-Bernal (1962)
Thelohania sp.	*Penaeus semisulcatus* (M)	Whitened tissue	Musculature Gonads	Sprague and Couch (1971)
Tuzetia				
Tuzetia weidneri	*Litopenaeus setiferus* (E)	Whitened tissue	Musculature	Canning et al. (2002)
	Farfantepenaeus aztecus (E)	Whitened tissue	Musculature	Canning et al. (2002)

(*continued on next page*)

Table 2 (*continued*)

Microsporidian species	Host species (habitat)	Symptoms	Tissue infected	Reference
Vairimorpha				
Vairimorpha cheracis	*Cherax destructor* (F)	Whitened tissue	Musculature	Moodie et al. (2003c)
Vavraia				
Vavraia mediterranica	*Crangon crangon* (M)	Whitened tissue	Musculature	Azevedo (2001)

E: Estuary; M: Marine; F: Fresh water.

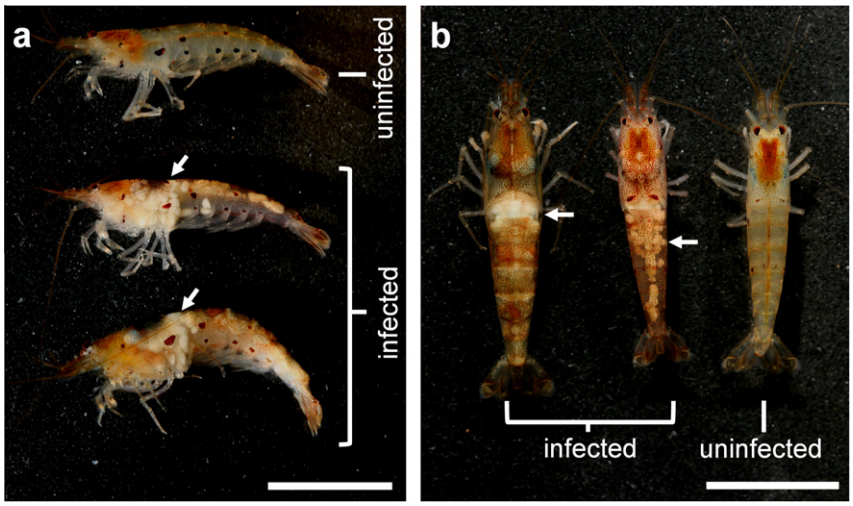

Fig. 1. Lateral views (a) and dorsal views (b) of uninfected and infected atyid shrimps, *Caridina formosae*. White xenomas (arrow) located at hemocoel, gills and dorsal part of abdomen along alimentary tract. Scale bar = 1 cm.

2. Materials and methods

2.1. Source of specimens

The atyid shrimp, *C. formosae*, were collected from Yuantan Stream, Huangtan Village, Wanli District, New Taipei City in northern Taiwan (121°38′50.22″E, 25°12′1.14″N). Heavily infected shrimps with obvious white xenomas in the dorsal abdomen were easily observed and were captured with dip nets.

2.2. Light microscope observations of fresh and stained spores

The xenomas were dissected from the infected shrimp and immersed in PBS, then ruptured to release their contents (spores and sporophorous vesicles). The material was smeared and stained with 5% Giemsa solution (Merck). Semi-thin sections processed for electron microscopy (see below) were stained with 1% toluidine blue. The slides were observed under phase-contrast microscopy (Olympus IX71) and photographed using a CCD camera (Olympus IX71). The fresh and stained spores and the sporophorous vesicles were measured by Amira 3.1.1 program for MacOSX.

2.3. Ultrastructural observations

The xenomas were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, pH = 7.2, at 4 °C for 1 week and post-fixed in 1% OsO_4 in the same buffer at 4 °C for 2 h. The fixed samples were dehydrated in ethanol series (50–100%) (Wang et al., 2009). For scanning electron microscopy, dehydrated samples were dried at the critical point in a critical point drier (Hitachi HCP-2), and coated with gold, then observed and photographed with a JSM-5600 (JEOL) scanning electron microscope. For transmission microscopy, dehydrated samples were embedded in Spurr's resin (Spurr, 1969). The ultra-thin sections were cut on a Reichert OMU 3 ultramicrotome and stained with 2% aqueous uranyl acetate followed by lead citrate. The electron micrographs were taken with a Hitachi H7100 transmission electron microscope at an accelerating voltage of 80 kV (Wang et al., 2009).

2.4. Nucleic acid extraction

DNA was extracted from infected tissues using a EDNA HISPEX™ kit (Saturn Biotech); the procedure was modified from the manufacturer's instructions. In brief, approximately 1 mm^3 of infected tissues was homogenized in TE buffer (0.1 M Tris, 0.01 M EDTA, pH 9.0) with a pestle. The macerated solution was centrifuged and the supernatant was discarded. Solution 1A (64 μl) and Solution 1B (16 μl) were added to the pellet, mixed gently, and then incubated at 95 °C for 90 min. Solution 2 (20 μl) was added to the sample, mixed gently, and stored at −20 °C for PCR amplification.

2.5. PCR amplification and sequencing of SSU rDNA

The SSU rDNA fragment of the microsporidium was amplified using primer set 18f (5′-CAC CAG GTT GAT TCT GCC-3′): 1537r (5′-TTA TGA TCC TGC TAA TGG TT-3′) (Vossbrinck et al., 1993). Each 50-μl PCR mix contained 5 μl 10× reaction buffer (Bioman), 4 μl 2.5 mM dNTPs, 0.5 μl 100 mM of each primer, 1 μl 1.25 U HiFi *Taq* polymerase (RBC) and 1 μl template DNA. PCR amplifications were performed as follows on an AG9600 Themal Station (Biotronics Corp.): thermal cycler was preheated at 95 °C for 5 min, 35 cycles at 94 °C for 30 s, 50 °C for 1 min, and 72 °C for 2 min, followed by a 10 min final extension at 72 °C and storage at 20 °C. The PCR product was cloned into T&A cloning vector (RBC Bioscience) and commercially sequenced (Genomics Biosci. & Tech. Company).

2.6. Phylogenetic analysis

SSU rDNA sequences of 36 microsporidian isolates, including this isolate (Table 3), were selected for phylogenetic analysis. The microsporidium *Nosema bombycis* was chosen as outgroup. Multiple sequences were aligned using Clustal X, Version 1.81 (Thompson et al., 1997) and then manually edited with the GeneDoc (Nicholas et al., 1997). Maximum-likelihood (ML) and maximum parsimony (MP) analyses were conducted using *MEGA* version 4 (Tamura et al., 2007). A maximum-likelihood (ML) tree was generated using Kimura 2 parameter of substitution model (Kimura, 1980) using the close neighbor interchange (CNI) heuristic method, and the initial tree was generated automatically. Maximum parsimony (MP) analyses were generated using a CNI heuristic search with search level of 2 and random initial trees addition of 2000 replicates (Casal et al., 2008). Bootstrap analyses (100 times) for ML and MP were performed to evaluate the robustness of the phylogenies.

Pairwise sequence comparison performed for the following species exhibited highest similarity in the BLAST search: Spraguea gastrophysus *Spraguea gastrophysus*, *Spraguea* sp. (1) and (2), *Spraguea lophii* (1), and (2), *Microgemma* sp., *Potaspora morhaphis*, *Tetramicra brevifilum*, and *Kabatana* sp. JS-2012 (Table 3) from the most recent GenBank database (NCBI network) using BLAST (Altschul et al., 1990). Pairwise identity was calculated by GenDoc using the Blossum 35 matrix algorithm (Nicholas et al., 1997).

3. Results

3.1. Gross pathology

Numbers of white or sometimes yellow, opaque xenomas were observed dorsally in infected *C. formosae* shrimps, particularly in the abdominal area beneath the dorsal median carina. In heavily infected shrimp, the carapace appeared swollen and xenomas were also found on gills and surface of hepatopancreas (Fig. 1a and b). Infected individuals swam slowly and walked with a lurch, and died soon after collection. The number of xenomas ranged from 27 to 54 per individual ($n = 3$) (Fig. 1a and b). The spores were enclosed in a sporophorous vesicle (SPOV) of approximately 37 μm in diameter; each vesicle contained approximately 40 spores (Fig. 2a). Mature spores were pyriform in shape (Fig. 2b).

3.2. Xenoma morphology

Xenomas were irregular in shape with a relatively smooth surface (Fig. 3a). Each xenoma consisted of a mass of SPOVs enclosed in a capsule (Figs. 3b and 4a). TEM revealed that each capsule was composed of three layers: an external acellular layer (0.3–05 μm thick), an intermediate acellular layer (0.7–0.9 μm), and an internal cellular layer (1.2–1.5 μm). The first two layers consisted of fibers oriented perpendicularly to each other. Fibers composing the intermediate layer were loosely arranged and formed meshwork-like

Table 3
Microsporidian SSUrDNA sequences used in phylogenetic analysis.

Microsporidian species[c]	Host species	Accession number
Dictyocoela berillonum (1)	*Echinogammarus marinus* (A)	JQ673481
Dictyocoela berillonum (2)	*Echinogammarus berilloni* (A)	AJ438957
Dictyocoela cavimanum (1)	*Talitrus* sp. (A)	AJ438959
Dictyocoela cavimanum (2)	*Orchestia cavimana* (A)	AJ438960
Dictyocoela deshayesum	*Talorchestia deshayesei* (A)	AJ438961
Dictyocoela duebenum (1)	*Gammarus duebeni duebeni* (A)	FN434091
Dictyocoela duebenum (2)	*Echinogammarus marinus* (A)	JQ673482
Dictyocoela duebenum (3)	*Gammarus duebeni* (A)	AF397404
Dictyocoela duebenum (4)	*Echinogammarus marinus* (A)	JQ673483
Dictyocoela gammarellum	*Orchestia gammarellus* (A)	AJ438958
Dictyocoela muelleri (1)	*Gammarus duebeni celticus* (A)	AJ438955
Dictyocoela muelleri (2)	*Gammarus duebeni duebeni* (A)	FN434090
Dictyocoela muelleri (3)	*Gammarus roeseli* (A)	AJ438956
Dictyocoela sp. GPM1	*Gammarus pseudolimnaeus* (A)	HM991451
Dictyocoela sp. GL	*Gammarus lac* (A)	GU196256
Glugea hertwigi	*Osmerus mordax* (F)	GQ203287
Glugea plecoglossi	*Plecoglossus altivelis* (F)	AB623035
Kabatana sp. JS-2012[c]	*Clevelandia ios* (F)	JQ062989
Kabatana sp. JI-2008	*Gobiusculus flavescens* (F)	EU682928
Loma acerinae	*Gymnocephalus cernuus* (F)	AJ252951
Microgemma sp.[c]	*Taurulus bubalis* (F)	AJ252952
Microsporidium prosopium	*Prosopium williamsoni* (F)	AF151529
Myosporidium merluccius	*Merluccius sp.* (F)	AY530532
Nosema bombycis[a]	*Bombyx mori* (I)	D85504
Pleistophora sp. 3	*Taurulus bubalis* (F)	AF044390
Pleistophora sp. (PA)	*Farfantepenaeus aztecus* (S)	AJ252958
Potaspora morhaphis[c]	*Potamorrhaphis guianensis* (F)	EU534408
Spraguea gastrophysus[c]	*Lophius gastrophysus* (F)	GQ868443
Spraguea lophii (1)[c]	*Lophius piscatorius* (F)	AF104086
Spraguea lophii (2)[c]	*Lophius piscatorius* (F)	AF033197
Spraguea sp. (1)[c]	*Lophius litulon* (F)	AY465876
Spraguea sp. (2)[c]	*Lophius piscatorius* (F)	JF927624
Spraguea sp. Sdu-2008	*Seriola dumerili* (F)	AB623034
Spraguea sp. MB2010	*Seriola quinqueradiata* (F)	JQ820238
Spraguea sp. MB2011	*Seriola quinqueradiata* (F)	JQ820239
Tetramicra brevifilum[c]	*Scophthalmus maximus* (F)	AF364303
Trachipleistophora hominis	*Homo sapiens* (M)	AJ002605
Triwangia caridinae[cb]	*Caridina formosae* (S)	JQ268567

A = amphipod; F = fish; I = insect; M = mammal; S = shrimp.
[a] Outgroup.
[b] This study.
[c] Selected for sequences comparison.

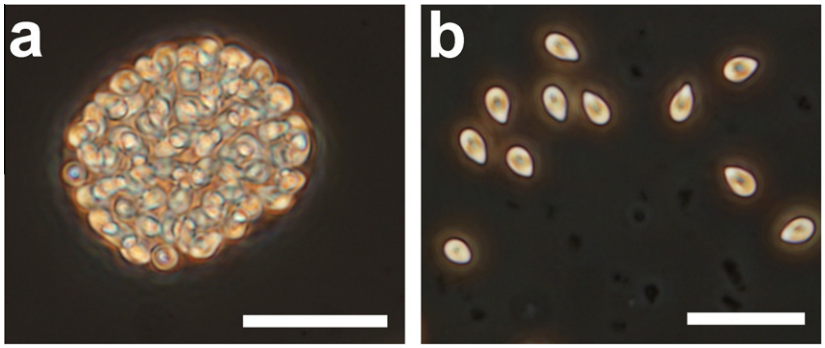

Fig. 2. Fresh preparations of a sporophorous vesicle filled with spores (a) and released spores (b); spores are pyriform in shape. Scale bar = 20 μm.

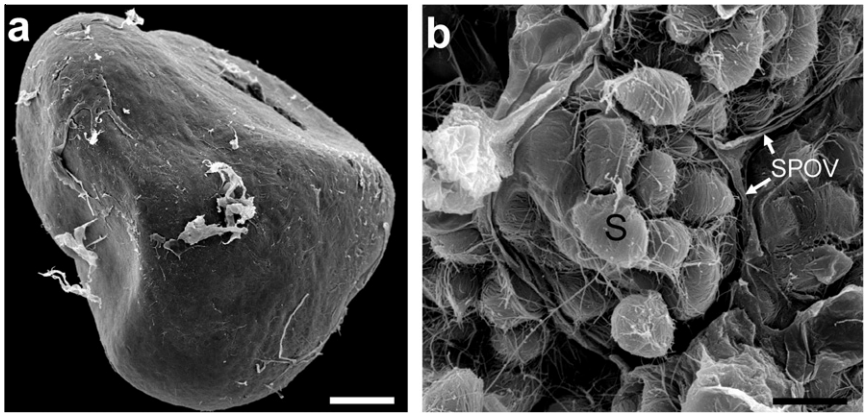

Fig. 3. Scanning electronmicrographs of a xenoma (a) xenoma, scale bar = 100 μm; (b) sporophorous vesicles (SPOVs) within a xenoma. Scale bar = 5 μm. S = spores.

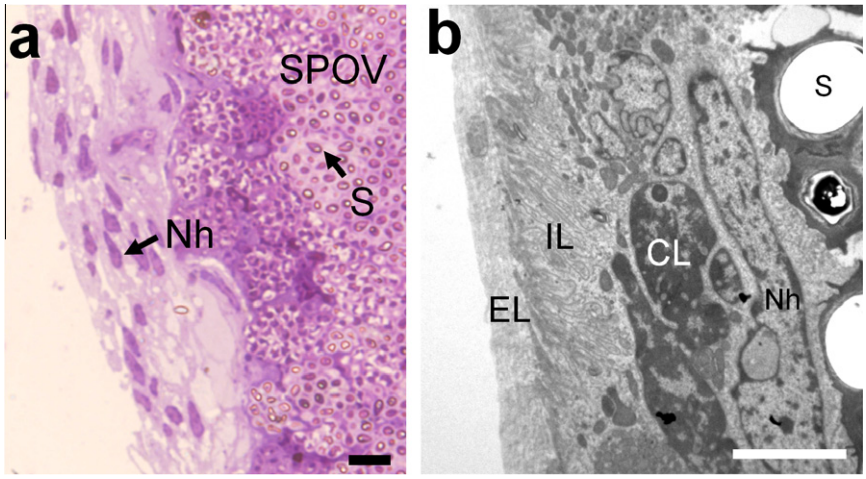

Fig. 4. Xenoma structure. (a) Semithin section through xenoma periphery stained with toluidine blue. The 60–100 μm capsule encloses SPOVs with mature spores (S). Scale bar = 20 μm; (b) ultrathin section of the xenoma capsule showing the external acellular layer (EL), intermediate (meshwork) acellular layer (IL) and internal (cellular) layer (CL). Scale bar = 1 μm. M = meront; Nh = nucleus of elongated flattened cells.

structures. The internal cellular layer was composed of elongated flattened cells with large nuclei, abundant mitochondria and well developed ER (Fig. 4b). Individual SPOVs contained parasites at approximately the same developmental stage, but stages varied among vesicles. SPOVs with early developmental stages of this new microsporidium were located at outmost region of the xenoma near or adjacent to the host cell nucleus, and SPOVs with mature spores tended to be located near the center of xenoma. Fibrillar material was found between spores within a vesicle (Fig. 3b). Microsporidian development appeared to be more synchronized within SPOVS with more advanced stages (Fig. 5a).

3.3. Merogony

Meronts were roundish diplokaryotic cells surrounded by a plasma membrane (Fig. 6a and b) that increased in size and underwent

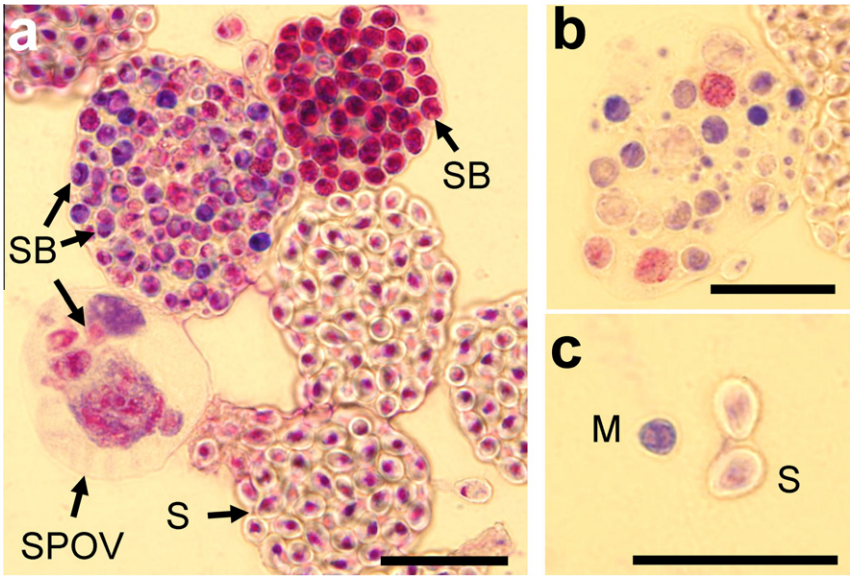

Fig. 5. Giemsa-stained sporophorous vesicles (SPOV). (a) SPOVs from a xenoma containing parasites in different development stages; (b) sporoblasts (SB) developing asynchronously in a SPOV; (c) diplokaryotic meront (M) and two mature spores. Scale bar = 20 μm.

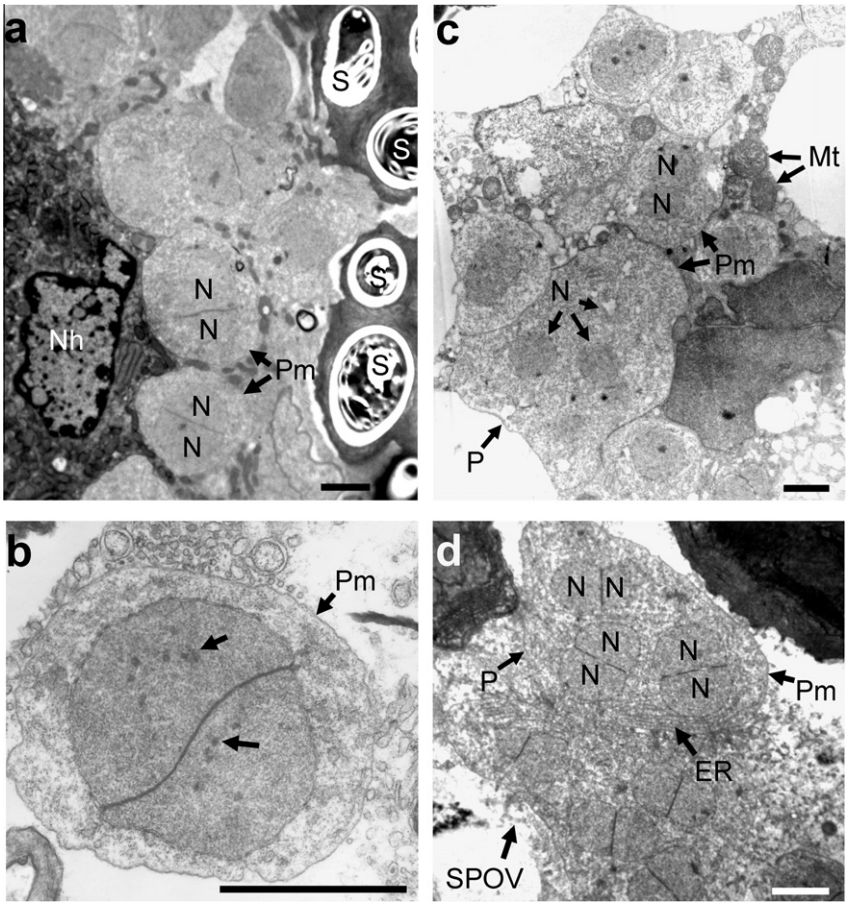

Fig. 6. Merogony. (a) The periphery of the xenoma containing proliferating parasites; (b) diplokaryotic meront; (c) multinuclear plasmodium (P); (d) multinucleate plasmodium at the latest merogonial stage. Diplokarya are surrounded by endoplasmic reticulum (ER). Scale bar = 2 μm. Mt = mitochondria; N = nucleus of parasite; Nh = nucleus of elongated flattened cells; Pm = plasma membrane; S = spore.

multiple nuclear division to produce multinucleate diplokaryotic plasmodia (Fig. 6c and d). Host mitochondria were often observed adjacent to the plasmodia (Fig. 6c).

3.4. Sporogony

3.4.1. Sporonts and sporogonial plasmodia

No diplokaryotic stages were observed during sporogony. The earliest sporogonial stage was a plasmodium with numerous nuclei, most of which were in the process of division (Fig. 7a). More than 20 nuclei were observed in a section through one plasmodium (Fig. 7b). Individual sporoblasts eventually segregated inside sporophorous vesicles (Fig. 7c).

3.4.2. Sporoblast

Sporoblasts gradually matured into spores (Fig. 8a–c). The most prominent feature of sporoblasts was the presence of electron lucid and electron dense granules. The former eventually transformed

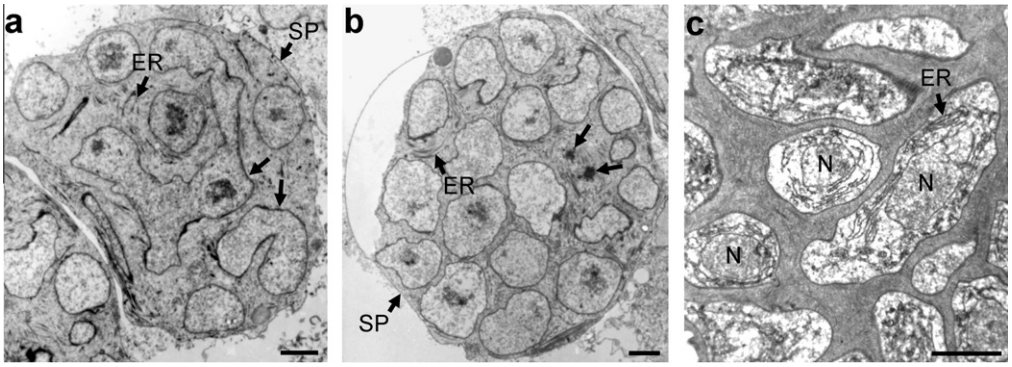

Fig. 7. Sporogony. (a) A sporogonial plasmodium (SP) with numerous irregularly shaped nuclei presumably undergoing divisions; (b) multinucleate sporogonial plasmodium within SPOV membrane. Arrow indicates the nucleus in the process of division. (c) Sporonts with single nuclei surrounded by endoplasmic reticulum (ER), segregate inside SPOV. Scale bar = 2 μm. N = nucleus of parasites.

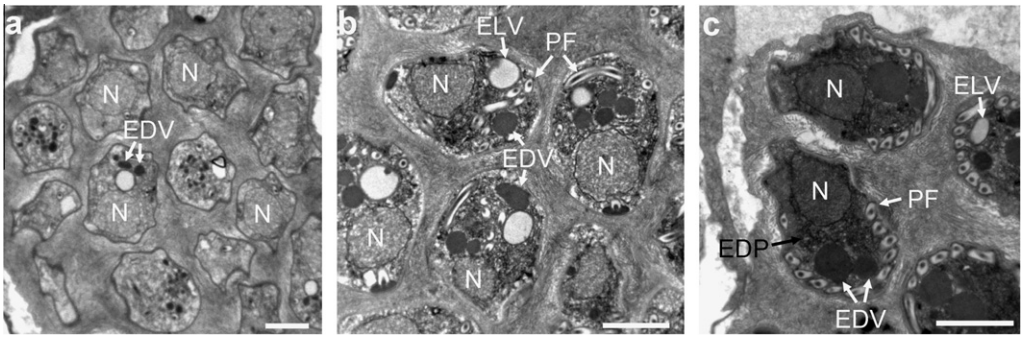

Fig. 8. Sporoblast. (a) Early sporoblasts showing nucleus (N) and some electron-dense vesicles (EDV); (b) advanced sporoblastic stage: the sporoblasts contain one nucleus, coiled polar filament (PF), an electron-lucent vacuole (ELV), and electron-dense vesicles; (c) late sporoblastic stage: the sporoblasts contain one nucleus (N), two to three electron-dense vesicles at the posterior end (arrows), polar filament with six to seven coils. Spore wall is thickened and electron-dense. Scale bar = 2 μm. EDP = electron-dense particles.

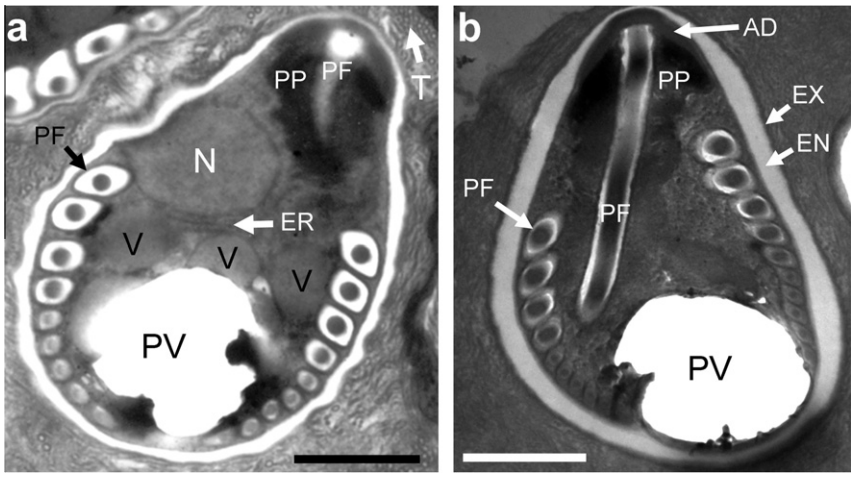

Fig. 9. Immature and mature spores. (a) Immature spore is surrounded by tubular structures (T). Spore constituents are electron dense polaroplast (PP), polar filament (PF), large posterior vacuole (PV), one nucleus (N), three vesicles (V) and endoplasmic reticulum (ER); (b) mature spore is pyriform and has a lamellar polaroplast (PP), mushroom-like anchoring disc (AD), polar filament (PF) and large posterior vacuole (PV). Scale bar = 1 μm. EN = endospore wall; EX = exospore wall.

into membranes surrounding the anchoring disc and polar tube coils. Electron dense granules were numerous and dispersed in early sporoblasts, and were probably precursors of polar filament proteins associated with the Golgi complex (Fig. 8a). At later stages only one or two large dense granules could be observed posteriorly (Fig. 8c). Presumably these granules gave rise to the posterior vacuole in mature spores. The number of regularly arranged polar filament coils increased during sporoblast maturation.

3.5. Spores

Immature spores were enclosed in sporophorous vesicles and were surrounded by tubular structures (T) (Fig. 9a). The mature spores were pyriform in shape with a smooth surface (Fig. 9b). The average size of fresh mature spores was 6.53 ± 0.34 × 4.28 ± 0.27 μm (n = 51) and of fixed spores was 4.96 ± 0.04 × 3.10 ± 0.2 μm (n = 3). The spore wall was comprised of a thin (31 ± 3 nm, n = 7) exospore and a thick (274 ± 19 nm, n = 7) electron-lucent endospore (Fig. 9). The spores were uninucleate and the nucleus was surrounded by endoplasmic reticulum (Fig. 9a). The polaroplast was composed of tightly packed lamellae and appeared electron dense in the mature spores. The anisofilar polar filament was arranged in 10–12 coils (4–5 + 6–7). The diameter of the largest proximal coils was 260 nm; distal coils ranged in diameter from 131 to 156 nm. The anchoring disc was mushroom-like. The polar sac encircled the apical portion of the polaroplast. The posterior vacuole was large but poorly preserved in sections of mature spores (Fig. 9a and b).

3.6. Molecular phylogenetic analysis

The SSU rDNA fragment of this isolate consisted of 1361 nucleotides (GenBank Accession No. JQ268567) and the GC content was 46.58%. Maximum-likelihood (ML) and maximum parsimony (MP) trees were similar in topology; only the MP tree is shown (Fig. 10). Phylogenetic trees displayed three distinct clades, which we assigned as groups I, II and III (Fig. 10). Group I is composed of the genus *Dictyocoela*, pathogens of fresh water amphipods. Group II

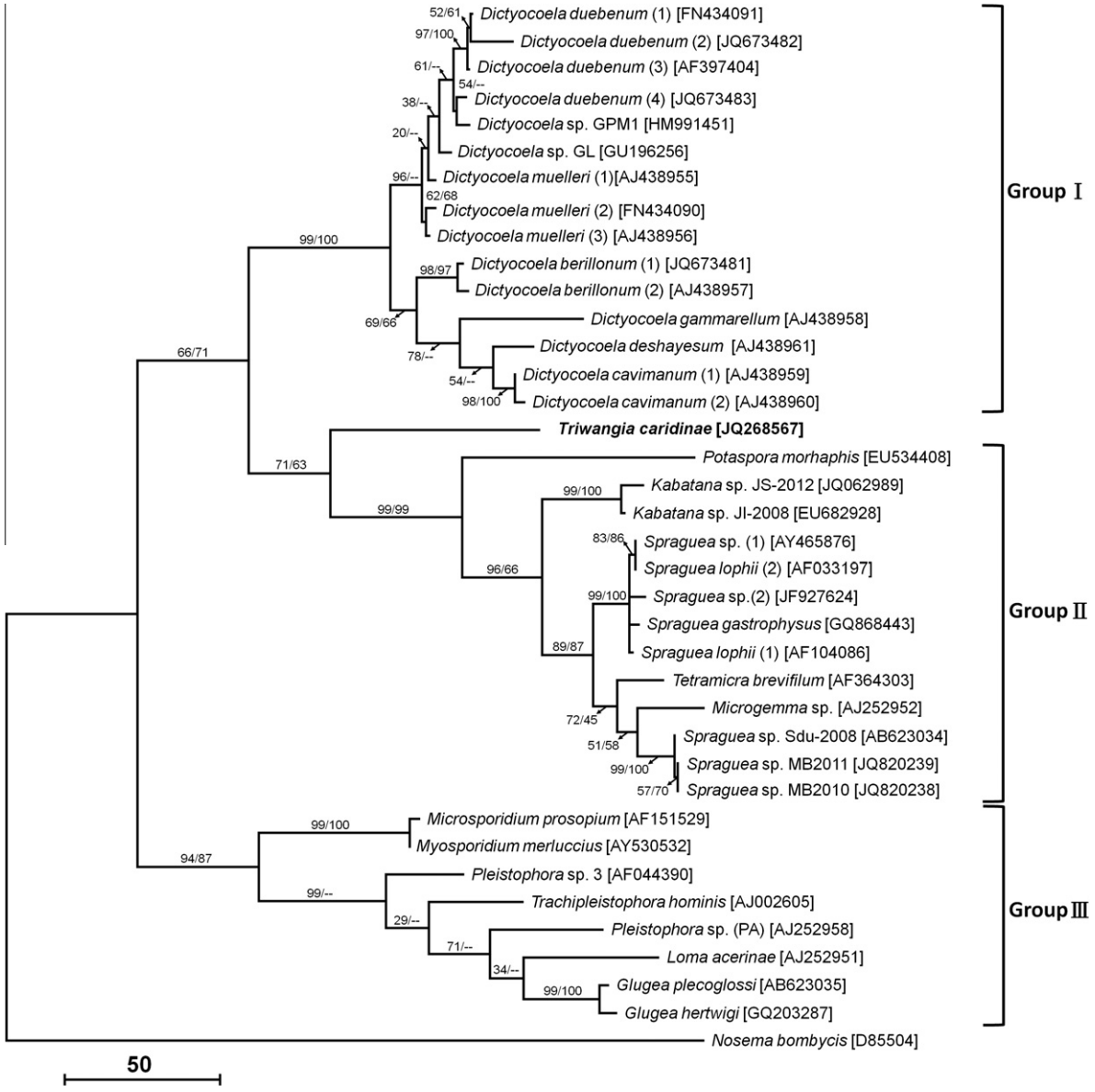

Fig. 10. Phylogenetic tree constructed by maximum parsimony (MP) revealed that *T. caridinae* is most closely related to the clade containing fish microsporidia (Group II). *N. bombycis* was used as outgroup. Bootstrap values for MP and ML trees are shown at each node.

Table 4
SSUrDNA identity of the Group II microsporidia in the phylogenetic tree.

	Spraguea sp. (1) (%)	*Spraguea lophii* (1) (%)	*Spraguea lophii* (2) (%)	*Spraguea* sp. (2) (%)	*Tetramicra brevifilum* (%)	*Microgemma* sp. (%)	*Kabatana* sp. JS-2012 (%)	*Potaspora morhaphis* (%)	*Triwangia caridinae* (%)
Spraguea gastrophysus	99	98	98	98	92	93	88	83	81
Spraguea sp. (1)		98	99	98	93	93	89	83	81
Spraguea lophii (1)			98	98	92	92	88	83	81
Spraguea lophii (2)				97	92	92	88	83	80
Spraguea sp. (2)					92	92	88	83	80
Tetramicra brevifilum						92	87	82	79
Microgemma sp.							87	83	79
Kabatana sp. JS-2012								82	78
Potaspora morhaphis									76

is represented by fish microsporidia including genera *Spraguea*, *Micorgemma*, *Tetramicra*, *Kabatana* and *Potaspora*. Group III is comprised of microsporidia infecting several vertebrate and invertebrate hosts including humans, fish and shrimps. The microsporidium recovered from atyid shrimps shared closest homology to Group II genera.

Pairwise sequence comparison of the novel microsporidium with representatives of Group II genera, revealed 76–81% sequence identities, the highest (81%) with *Sprague* spp. and the lowest (76%), with *P. morhaphis* (Table 4).

4. Discussion

4.1. Peculiarities of pathogenesis and xenoma formation

Most shrimp microsporidia infect muscle tissues and cause prominent whitish lesions, a disease known as "cotton" or "milk" syndrome of shrimps. Representatives of the genera *Ameson*, *Agmasoma*, *Pleistophora*, *Encephalitozoon* and *Thelohania* also have been reported from the hepatopancreas, digestive tract, reproductive organs, and other tissues of several species of pennaeid shrimps (Table 2). In addition, two species, *A. penaei* and *Thelohania* sp., parasitize the reproductive glands (gonad or ovary) of six shrimp species, including two *Penaeus* spp., two *Fenneropenaeus* spp., *Farfantepenaeus duorarum* and *L. setiferus* (Table 2). *Thelohania*-like spp. infect most tissues and organs of the host, including heart, connective tissues, hepatopancreas, hemocyte forming organs and other tissues (Kelly, 1979; Langdon, 1991; Edgerton et al., 2002), while *E. hepatopenaei* is specific to the hepatopancreas of black tiger shrimp (Tourtip et al., 2009).

The *C. formosae* microsporidium does not infect muscle tissues and the musculature of the host remains transparent. The most conspicuous symptom of infection is the occurrence of white, opaque xenomas located in the proximity of the alimentary canal, the surface of the hepatopancreas, and the gills.

Three xenoma-forming microsporidian species from crustacean hosts have been described, including *Mrazekia argoisi* (Debaisieux, 1931) from the waterlouse, *Asellus aquaticus*; *Abelspora portucalensis* (Azevedo, 1987) from the common littoral crab, *Carcinus maenas*; and *Myospora metanephrops* (Stentiford et al., 2010) from marine lobsters, *Metanephrops challengeri*. *M. argoisi* infection induces xenomas and hypertrophy of host fat cells and their nuclei that surround the stomach of *A. aquaticus* (Debaisieux, 1931). White xenomas of *A. portucalensis* are irregularly dispersed in the hepatopancreas of the common littoral crab and are more frequently observed at periphery of the organ. The xenomas are composed of numerous cysts consisting of hypertrophic host cells (Azevedo, 1987). The xenomas produced by *M. metanephrops* in the muscular system of heavily infected marine lobsters cause muscle lesions and transformation (Stentiford et al., 2010). In contrast to these species, xenomas produced by the *C. formosae* microsporidium were typically observed along the alimentary tract, particularly in the abdominal area beneath the dorsal median carina. Xenomas were found in the hepatopancreas and gills of only heavily infected shrimps. The most important distinction between the *C. formosae* microsporidium and the three xenoma-forming crustacean microsporidian species is that xenomas of *C. formosae* microsporidium are enclosed in hard, thick capsules. Compared to other xenoma-forming microsporidia reported from invertebrate hosts and fish (Lom and Dyková, 2005; Shaw and Kent, 1999), xenomas of the novel microsporidium were structurally most similar to the ones produced by *Glugea atherinae* and *G. anomala* in sand smelt and three-spine stickleback respectively (Lom and Nilsen, 2003). The novel microsporidium completes its life cycle in the infected atyid shrimp and different developmental stages can be found in different SPOVs within a single xenoma.

4.2. Life cycle of the Caridina formosae microsporidium and its comparison with other crustaceans and fish microsporidia

The development of *C. formosae* microsporidium is similar to species of *Inodosporus* and *Pleistophora*, which develop within SPOVs, while the species of *Agmasoma*, *Thelohania*, *Tuzetia* and *Varimorpha* develop in direct contact with the host cell cytoplasm during early stages and become isolated from the host cell cytoplasm by the parasite-produced membranes at the sporogonic phase. Species of genera *Ameson*, *Enterocytozoon*, *Myospora* and *Perezia* develop in direct contact with the host cell cytoplasm (Table 5) (Sprague et al., 1992; Shaw and Kent, 1999).

Unlike the described microsporidium, *Tuzetia infirma* from copepods is monokaryotic and has smaller spores (3.8×2.7 μm) with isofilar polar filaments. Representatives of genera *Thelohania*, *Vairimorpha* and *Agmasoma* produce octospores and also can be easily distinguished from the *C. formosae* microspopridium by other developmental and structural characters (Table 5). *Inodosporus* spp. and *Pleistophora* spp. are uninucleate pathogens from marine shrimps and develop in SPOVs (Table 5) Representatives of genera *Inodosporus* and *Pleistophora* produce octospores and also can be distinguished from the *C. formosae* microsporidium by other developmental and structural characters (Table 5). *Vavraia culicis* (type species of *Vavraia*) completes its life cycle within a merontogenetic sporophorous vacuole (MSV) and meronts are monokaryotic.

The four microsporidian genera that develop in direct contact with host-cell cytoplasm (Table 5) differ from *C. formosae* microsporidium by structural characters. The xenoma-forming microsporidium from marine lobsters, *M. metanephros*, is diplokaryotic throughout its lifecycle (Stentiford et al., 2010) (Table 5).

4.3. Genetic relationships of C. formosae microspopridium based on SSUrDNA sequence analysis

Although the xenoma structure of the described microsporidium appeared to be similar to the *Glugea* spp. (Group III), sequence analyses revealed closer homology to representatives of the

Table 5
Comparison of life cycle in the genera of shrimp microsporidia.

Genera	Xenoma	Interfacial relationship	Meront	Sporont	Sporoblast	Mature spore	Reference
Triwangia	Yes	Indirect contact by parasite-produced isolation with all stages (SPOV)	Diplokaryotic cell - multinucleate diplokaryotic plasmodia	Monokaryotic cell	Monokaryotic sporoblast	Uninucleate	This study
Agmasoma	No	Indirect contact by parasite-produced isolation with sporogonic phase	–	–	Monokaryotic sporoblast (Octosporoblastic)	Uninucleate	Sprague et al. (1992)
Ameson	No	Direct contact	Diplokaryotic cell	Diplokaryotic cell	Diplokaryotic sporoblast (Octosporoblastic)	Uninucleate	Sprague et al. (1992)
Enterocytozoon	No	Direct contact	Multi-nuclei	–	–	Uninucleate	Sprague et al. (1992) and Tourtip et al. (2009))
Inodosporus	No	Indirect contact by parasite-produced isolation with all stages (SPOV)	Diplokaryotic cell	Monokaryotic cell	Monokaryotic sporoblast (Octosporoblastic)	Uninucleate	Sprague et al. (1992) and Azevedo et al. (2000)
Myospora	Yes	Direct contact	Monokaryotic cell Diplokaryotic cell (Octo-nucleate meront)	Diplokaryotic cell (Two diplokaryotic cells)	Diplokaryotic sporoblast (Two diplokaryotic sporoblasts)	Binucleate	Stentiford et al. (2010)
Perezia	No	Direct contact	Diplokaryotic cell	–	–	Uninucleate	Sprague et al. (1992)
Pleistophora	No	Indirect contact by parasite-produced isolation with all stages (SPOV)	Monokaryotic cell	Monokaryotic cell	Monokaryotic sporoblast	Uninucleate	Sprague et al. (1992)
Thelohania	No	Indirect contact by parasite-produced isolation with sporogonic phase	Diplokaryotic cell	Diplokaryotic cell Monokaryotic cell	Diplokaryotic sporont Monokaryotic sporont (Octosporoblastic)	Binucleate Uninucleate	Sprague et al. (1992) and Moodie et al. (2003a,b)
Tuzetia	No	Indirect contact by parasite-produced isolation with sporogonic phase	Uninucleate schizonts	Monokaryotic cell	Monokaryotic sporoblast	Uninucleate	Sprague et al. (1992)
Vairimorpha	No	Indirect contact by parasite-produced isolation with sporogonic phase	Diplokaryotic cell	Diplokaryotic cell	Monokaryotic sporoblast (Octosporoblastic)	Binucleate Uninucleate	Sprague et al. (1992)
Vavraia	No	Indirect contact by parasite-produced isolation with all stages (MSV)	Monokaryotic cell – multinucleate diplokaryotic plasmodia	Monokaryotic cell – multinucleate diplokaryotic plasmodia	Monokaryotic sporoblast	Uninucleate	Sprague et al. (1992) and Azevedo (2001)

Spraguea–Kabatana–Microgemma clade, Group II (Table 4 and Fig. 10). Given relatively low statistical support for the *Triwangia*-Group II cluster (71% and 63% bootstrap support for MP and ML respectively), we believe that relationships among the groups of fish microsporidia and the new species need to be elucidated in further studies. Based on life cycle characters, peculiar pathogenesis, host specificity and SSU sequence analysis we propose to assign the described above microsporidium to a new genus and species and propose the name *Triwangia caridinae*.

5. Taxonomic summary

Triwangia caridinae n. g., n. sp. Y. Nai, H. Hsu and C. Lo.

Type host. The fresh water atyid shrimp *Carindia formosae* (Crustacean: Decapoda) (Hung et al., 1993).

Transmission. Unknown.

Site of infection. Alimentary tract, gills and hepatopancreas.

Xenoma composition. Consisting of a mass of SPOVs containing mature spores, sporoblasts and sporogonial plasmodia, enclosed in a capsule composed of an external acellular layer (0.3–0.5 μm thick), an intermediate acellular layer (0.7–0.9 μm), and an internal cellular layer (1.2–1.5 μm).

Interface. Sporophorus vesicles are generated from the initial diplokarytic merogony, then spores continue to develop inside the vesicles. Developmental stages are not in direct contact with host cytoplasm.

Merogony. Merogony results in diplokaryotic meronts or a plasmodium with diplokaryotic nuclei following binary division or multiple division.

Sporogony. Sporogony involves the formation of toruliform sporonts to produce mononucleate sporonts.

Spore. Mature spores measure 6.53 × 4.28 μm. Fixed spores are 4.95 × 3.10 μm. Sporophorous vesicles contain up to 41 monokaryotic spores. Polarplast is lamellar and the posterior vacuole appears to be constructed of four to five compartments. The anchoring disc is a mushroom-like structure at the apical end of the mature spore. Nine to ten polar filament coils with first four coils of diameter approximately 260 nm and of smaller size distally, approximately 140 nm. The spore wall consists of a 31-nm electron-dense exospore and 270 nm electron-lucent endospore. Tubular-like structures within the sporophorous vesicles are 55 nm in diameter.

Type location. Yuantan Stream (121°38′50.22″E, 25°12′1.14″N), Huangtan Village, Wanli District, New Taipei City, Taiwan, ROC.

Molecular data. GenBank Accession No. JQ268567 for SSU-rDNA.

Etyology. The genus *Triwangia* is named with reference to the specimens first collected and identified by Tai-Chuan Wang, Chih-Yuan Wang and Chung-Hsiung Wang, and the species name follows the host genus name, *caridina*.

Acknowledgments

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