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ORIGINAL PAPER

Morphological and molecular biological characterization of *Pleistophora aegyptiaca* sp. nov. infecting the Red Sea fish *Saurida tumbil*

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Abstract One hundred three out of 225 (45.8%) of the Red Sea fish *Saurida tumbil* were infected with microsporidian parasites. The infection was recorded as tumor-like masses (whitish macroscopic cysts) or xenomas often up to 2 cm in diameter and embedded in the peritoneal cavity. Generally, the infection was increased during winter 63.8% (86 out of 135) and fall to 18.9% (17 out of 90) in summer. Light microscopic study revealed that xenomas were encapsulated by a fibrous layer encircling numerous sporophorous vesicles filled with mature spores measuring 1.7±0.6 (1.5–2.7 μm)×1.5±0.3 μm (1.2–1.8 μm) in size. Ultrastructural microscopic study showed the presence of smooth membranes of the sarcoplasmic reticulum forming a thick, amorphous coat surrounding various developmental stages of the parasite. The various recognizable stages of the parasite were uninuclear, binucleated, and multinucleated meronts followed by detachment of the plasmalemma of the sporont from the sporophorous vesicle producing sporoblasts. Mature spores consist of a spore coat and spore contents. The spore contents consist of the uninucleated sporoplasm and a posterior vacuole located at the posterior end. The polar tube consists of a straight shaft and a coiled region (26–32 coils) arranged in many rows along the inside periphery of the spore. The polaroplast consisted of an anterior region of closely and loosely packed membranes. Molecular analysis based on the small subunit rDNA gene was performed to determine the phylogenetic position of the present species. The percentage identity between this species and a range of other microsporidia predominantly from aquatic hosts demonstrated a high degree of similarity (>92%) with eight *Pleistophora* species. Comparison of the nucleotide sequences and divergence showed that the sequence of the present microsporidium was most similar to that of *Pleistophora anguillarum* (99.8% identity) differing in 13 nucleotide positions. So, the present species was recorded and phylogenetically positioned as a new species of *Pleistophora*.

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Introduction

Microsporidia are obligate intracellular parasites, a major cause of disease in fish, and may have an economically important impact on fish stocks (Dykova 1995; Lom and Nilsen 2003). Microsporidia have recently been added to the National Institutes of Health list of priority pathogens, as well as the Environmental Protection Agency list of waterborne microbial contaminants of concern (USEPA 2008). More than 140 genera and almost 1,200 species have been recognized (Basch 1971; Bayne et al. 1975; Weiss and Vossbrinck 1999; Andreadis and Vossbrinck 2002; Joh et al. 2007). Owing to the large number of genera and species, the phylum is characterized by a great diversity of morphology and life cycle strategies. However, the characters that unify all members of the phylum and qualify an organism as a microsporidium are that they exist only as spores outside the host cell and the invasion of a host involves eversion of the polar tube. This hollow tube pierces the host cell, inoculating the infective sporoplasm directly into its cytoplasm.

Many of the earlier studies on microsporidia based on morphology, ultrastructure, life cycle, and host–parasite

relationships have resulted in the unnecessary creation of a large number of new microsporidian species. The difficulties encountered in proper identification of them based on infection and light and electron microscopic examinations are well illustrated by Mercer and Wigley (1987)), who could not distinguish several *Nosema* species they have found in a stem borer, *Sceliodes cordalis*, from 12 other *Nosema* species known to infect Lepidoptera. However, they found the PCR method to be quite sensitive and useful in the diagnosis and differentiation of microsporidians from specimens infected with more than one microsporidian species. Classification based on ultrastructural differences has been replaced by phylogenetic analysis based on DNA marker profiles (Leipe et al. 1993; Baker et al. 1995; Hartskeerl et al. 1995; Mathis et al. 1997; Hung et al. 1998). Phylogenetic reconstruction based on comparison of small subunit ribosomal RNA (SSU-rRNA) sequences have successfully been used to detect and classify various microorganisms (Cavalier-Smith and Chao 1996; Dugourd et al. 1996) including microsporidia (Kawakami et al. 1992; Vossbrink et al. 1993; Baker et al. 1994, 1995; Hung et al. 1998; Raynaud et al. 1998; Hatakeyama et al. 2000; Rao et al. 2004, 2005). Small subunit rDNA sequence data have revealed that some of the developmental features and ultrastructural characters used to designate microsporidian taxa are the result of convergent evolution. As a result, a number of studies with Microsporidia are now including both ultrastructural and comparative rDNA sequence characters (Maddox et al. 1999; Fries et al. 1999; Andreadis and Vossbrinck 2002; Sokolova et al. 2003; Vavra et al. 2006), and eventually, with the help of phylogenetic analysis, the pattern of ultrastructural changes over evolutionary time will be determined.

The aim of the present study is to report on the natural occurrence and prevalence of microsporidian infections in the marine fish *Saurida tumbil* by means of light and transmission electron microscopy as well as to clarify the actual systematic and phylogenetic position of the parasite by molecular analysis of its SSU-rDNA sequence.

Materials and methods

Light and transmission electron microscopy

A total of 225 freshly caught living specimens of the fish *Saurida tumbil* were collected during the period of October 2009 to November 2010 from boat landing sites of Suez and Hurghada at the Gulf of Suez and Red Sea, respectively. Fishes were examined for microsporidian infections. Infection was determined by the presence of xenomas located along the wall of the abdominal cavity being recognizable by the naked eye. Descriptions and measurements of xenomas and fresh spores were done according to the guidelines of Lom et al. (1989)). After crushing the xenoma, the generated spores were examined under the light microscope. For transmission electron microscopy, the xenomas and surrounding tissues were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C for 20–24 h, rinsed overnight in the same buffer at 4°C, and postfixed in 2% OsO_4 in the same buffer at 4°C for 4 h. After dehydration in an ascending ethanol series (70%, 80%, 90%, 95%, and 100%, staying 2 h in each stage) and in propylene oxide (two changes for 3 h each), the infected tissues were embedded in Epon (staying 10–12 h in each change). Semithin sections were stained with toluidine blue. Meanwhile, measurements were based on 30 spores, and the range of the data is presented here. Ultrathin sections were examined with a Zeiss 902A transmission electron microscope.

DNA extraction, PCR amplification

For DNA extraction from the ethanol preserved samples, plasmodia were isolated from epithelial cells of the gut and peritoneal cavity of *Saurida tumbil*, then ruptured by homogenization procedure, and spore suspensions were pipetted and centrifuged for 10 min at 5,000×*g*. The supernatant was discarded, and the spore pellet was dissolved in 50 μl STE buffer (10 mM Tris–HCl (pH 8), 100 mm NaCl, 0.25 mm EDTA, 0.5% SDS, and 0.4 mg ml^{-1} proteinase K); then, genomic DNA was extracted from spores using QIAamp DNA MiniKit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The DNA was stored at −20°C until use. DNA concentration and purity were determined spectrophotometrically by measuring absorbance at wavelengths of 260 and 280 nm. The small subunit ribosomal DNA gene cluster (16s SSU-rDNA) were targeted for amplification using PCR. Combinations of primers that can be used and were employed in PCR and sequencing reactions are listed in Table 1. For PCR amplification, the 16s rDNA was amplified using 18f and 1492r in a 25-μl reaction mixture comprising 1 μl of extracted genomic DNA, 5 μl of 1 mM deoxyribonucleotide triphosphates (MBI Fermentase), 0.25 μl of each primer (50 pmol μ^{-1}), 2.5 μl of 10× Taq polymerase buffer (MBI Fermentase), 2 μl of 25 mM $MgCl_2$, 1 μl Taq DNA polymerase (2U) (MBI Fermentase), and 13 μl of distilled water. The PCR cycle consisted of an initial denaturation step of 94°C for 4 min, followed by 35 cycles of 94°C for 50 s, 56°C for 50 s, 72°C for 80 s, and was finished with terminal extension at 72°C for 7 min, then rested at 4°C. The PCR products were electrophoresed in 1.0% agarose gel in Tris–acetate–EDTA-buffered gel stained with 1% ethidium bromide and visualized with a

Table 1 Sequencing primers for 16s SSU-rDNA of some microsporidian species (Vossbrinck and Andreadis 2007)

Primers	Sequence	Tm (°C)	GC Content
18 f	5′-CAC CAG GTT GAT TCT GCC-3′	50.4	55.6%
1492 r	5′-GGT TAC CTT GTT ACG ACT T-3′	42.8	42.1%
SS350 f	5′-CCA AGG AYG GCA GCA GGC GCG AAA-3′	73.1	64.6%
350 r	5′-TTT CGC GCC TGC TGC CRT CCT TG-3′	70.9	63.0%
SS530 f	5′-GTG CCA GCM GCC GCG G-3′	64.2	84.4%
SS530 r	5′-CCG CGG KGC TGG CAC-3′	59.5	83.3%
1061 f	5′-GGT GGT GCA TGG CCG-3′	54.0	73.3%
1047 r	5′-AAC GGC CAT GCA CCA C-3′	52.5	62.5%

Y=T/C, R=G/A, M=C/A, K=T/G

UV transilluminator. PCR products were purified using standard techniques (Qiaquick PCR Purification Kit, Qiagen company, CA) and run against a mass standard ladder (100 bp) on an agarose gel to estimate the concentration of DNA. Purified PCR products were sequenced in both directions with the remaining primers listed in Table 1. Sequencing templates were prepared using a plasmid preparation kit of Machery-Nagel and sequenced on automated sequencer used for cycle sequencing of 16s fragments in a 48 capillary ABI 310 automatic DNA sequencer (Applied Biosystems) using the BIO Dye Terminator v 3.1 Ready Sequencing Kit (Applied Biosystems) using universal forward and reverse primers. To evaluate the relationship of the present studied species, a homology search was performed using BLAST program on GenBank (Altschul et al. 1997). Partial to complete 16s SSU-rDNA sequences and NCBI accession number for 17 microsporidian species (Table 2) were available from GenBank database; some microsporidian parasites presented in this investigation were aligned separately with these data, and the sequences were truncated for homology.

The alignment was made using CLUSTRAL_X (Thompson et al. 1997). The alignment was manually corrected using the alignment editor of the software BioEdit 4.8.9 (Hall 1999) to eliminate minor inconsistencies between different taxa and with reference to known elements of secondary structure (Van de Peer and De Wachter 1997). The resulting sequences fragments were assembled into a single contiguous sequence using the multiple-alignment algorithm in Megalign (DNASTAR, Windows version 3.12e). Ambiguous regions, incomplete sequences, invariant sites, and gaps were removed, and remaining 521 variable sites were used for phylogenetic analysis. *Beauveria bassiana* was chosen as an outgroup (Lom et al. 2001; Moodie et al. 2003).

Table 2 Microsporidian species used in the phylogenetic analysis of the present *P. aegyptiaca* sp. nov

Organism	Host/Host group	Source	Accession no.	Sequence length (bp)	Percent identity (%)	Divergence
Pleistophora typicalis	*Myoxocephalus scorpius* (F)	GenBank	AF044387	1,864	93.0	7.4
Pleistophora sp. (3)	*Aedes sierrensis* (I)	GenBank	AF044390	1,879	93.5	6.9
Pleistophora ehrenbaumi	*Anarhichas lupus* (F)	GenBank	AF044392	1,379	92.6	7.9
Pleistophora finisterrensis	*Micromesistius poutassou* (F)	GenBank	AF044393	1,372	90.8	9.8
Glugea stephani	*Pleuronectes americanus* (F)	GenBank	AF056015	1,165	90.7	9.9
Glugea anomala	*Gasterosteus aculeatus* (F)	GenBank	AF056016	1,165	90.7	9.9
Loma acerinae	*Gymnocephalus cernua* (F)	GenBank	AJ252951	1,352	89.9	10.9
Pleistophora sp. (TB)	*Myoxocephalus scorpius* (M)	GenBank	AJ252957	1,373	93.5	6.9
Loma psittaca	*Colomesus psittacus* (F)	GenBank	FJ843104	1,260	90.1	10.6
Dictyocoela muelleri	*Gammarus duebeni*(C)	GenBank	AJ438956	1,498	81.6	21.5
Pleistophora mulleri	*Gammarus duebeni* (C)	GenBank	EF119339	3,698	93.7	6.6
Glugea hertwigi	*Osmerus mordax* (F)	GenBank	GQ203287	1,856	91.2	9.4
Glugea atherinae	*Atherina presbyter* (F)	GenBank	GAU15987	1,335	91.2	9.4
Glugea plecoglossi	*Plecoglussus altivelis* (F)	GenBank	AJ295326	1,774	91.0	9.6
Pleistophora anguillarum	*Anguilla japonica* (C)	GenBank	U47052	1,183	99.8	0.2
Pleistophora ovariae	*Notemigonus crysoleucas* (F)	GenBank	AJ252955	1,397	94.9	5.3
Pleistophora hyphessobryconis	*Danio rerio* (F)	GenBank	GU126672	1,361	97.3	2.8

F fish, *C* crustacean, *M* mammal, *I* insect

Results

Light microscopic observations

Thirty five out of 225 of the examined fish were infected with microsporidian parasites at a percentage of 15.5%. Whitish macroscopic cysts embedded in the peritoneal cavity were observed to infect many organs of the body including muscles, connective tissue of ovaries, and the intestinal epithelium (Fig. 1(1, 2)). The infection was developed as tumor-like masses of often up to 2 cm in diameter inducing an enormous hypertrophy. Concerning the seasonal prevalence of the microsporidian parasites, it was noted that they generally increased during winter to 24.4% (31/125) and fall to 4% (4/100) in summer. Sporophorous vesicles containing a large number of mature spores were usually observed after rupture of the xenoma (Fig. 1(3)). The spores were elongated ovoid or ellipsoidal in shape with a posterior vacuole reaching the midpoint of the spore (Fig. 1(4,5)) and were identified to belong to the phylum Microsporidia. Unfixed spores measured 1.7±0.6 (1.5–2.7 μm)×1.5± 0.3 μm (1.2–1.8 μm) in size. The coiled polar filament becomes spontaneously released or under pressure (Fig. 1(5)). Histopathological observations showed that parasitic foci or xenomas seen in semithin sections were encapsulated by a wall or fibrous layer produced by the host encircling numerous sporophorous vesicles filled with mature spores (Fig. 1(7, 8)).

Transmission electron microscopic observations

All developmental stages of the recorded *Pleistophora* sp. were found within the single xenoma isolated from the infected fish. Starting from the host cytoplasm and going toward the parasite, we found that the smooth membranes of the sarcoplasmic reticulum forming a thick-walled coat (Fig. 2(9)). This amorphous coat surrounds the various developmental stages of the parasite (Fig. 2(9)). An additional feature characteristic for the present species is the presence of sporophorous vesicles surrounding a number of spores (Fig. 2(10)). The number of spores in the sporophorous vesicle varied due to the different planes of sectioning. The sporophorous vesicle was bounded by the sporophorous wall which is a system of two membranes made of cisternae from the endoplasmic reticulum (Fig. 2(10)). The first recognizable stage of parasite observed was the uninuclear meront with a nucleus (Fig.2(11)), which divides to produce binucleated meronts with two nuclei (Fig. 2(12)). Additional multinucleated meronts with three to eight irregularly shaped nuclei arose by plasmotomy (Fig. 2(13)). The next step was the detachment of the plasmalemma of the sporont from the sporophorous vesicle (SPV) wall. This space became filled with a fine granular substance containing strands of the endoplasmic reticulum. Separate sporoblasts with moderately dense cytoplasm resulted from sporogonial plasmodium division (Fig. 2(14)). As development proceeded, advanced stages of sporoblasts contained already most of the typical structures of spores including the electron pale exospore (Fig. 2(15)). It became separated from the sporoblast to build a dense exospore around the mature spore. An evidence for spore morphogenesis is the presence of the polar tube primordial or paramural bodies which are the precursors of the polar tube and endospore formation (Fig. 2(16)). The spores consist of a spore coat and spore contents. The spore contents consist of the extrusion apparatus, the sporoplasm with the single nucleus, and a posterior vacuole located at the posterior end (Fig. 3(17)). The content of the posterior vacuole varies; in some spores, it contains amorphous materials, but in others, it is empty. The polar tube consists of a straight shaft and a coiled region (Fig. 3(20, 21)). At the anterior end of the spore, the anchoring disk was found in a central position (Figs. 3(20, 21)). The polaroplast consisted of an anterior region of closely packed membranes and a posterior region comprising a series of loosely packed membranes (Fig. 3(22)). The number of coils of the polar tube is about 26–32 arranged in many rows along the inside periphery of the spore (Fig. 3(23)). In cross sections, the polar filament appears to be divided into three regions (Fig. 3(23)). It has an outer electron-dense layer with ridges on its inner surface projecting into an electron-transparent layer, with the innermost region of the polar filament filled with an amorphous material of varying electron densities. The outermost layer, the exospore, is electron dense and has some ridges on its outer surface, while the endospore is much thinner than the exospore (Fig. 3(24)).

Molecular analysis

In order to investigate the microsporidian isolated from the marine fish *Saurida tumbil*, molecular analysis based on the small subunit rDNA gene was performed. The PCR product was cloned and sequenced. The 650-kb sequence was submitted to a GenBank BLAST search to find the most similar sequences. Calculation of the percentage identity (number of base differences/total number of bases) between this novel sequence and a range of other microsporidia predominantly from aquatic hosts demonstrated a high degree of similarity (>92%) with eight *Pleistophora* species (Table 1). The six highest BLAST scores were aligned with CLUSTAL-X; these are *Pleistophora ovariae* (accession no. AJ252955), *Pleistophora mulleri* (accession no. EF119339), *Pleistophora hyphessobryconis* (accession no. GU126672), *Pleistophora anguillarum* (accession no. U47052), *Pleistophora typicalis* (accession no.

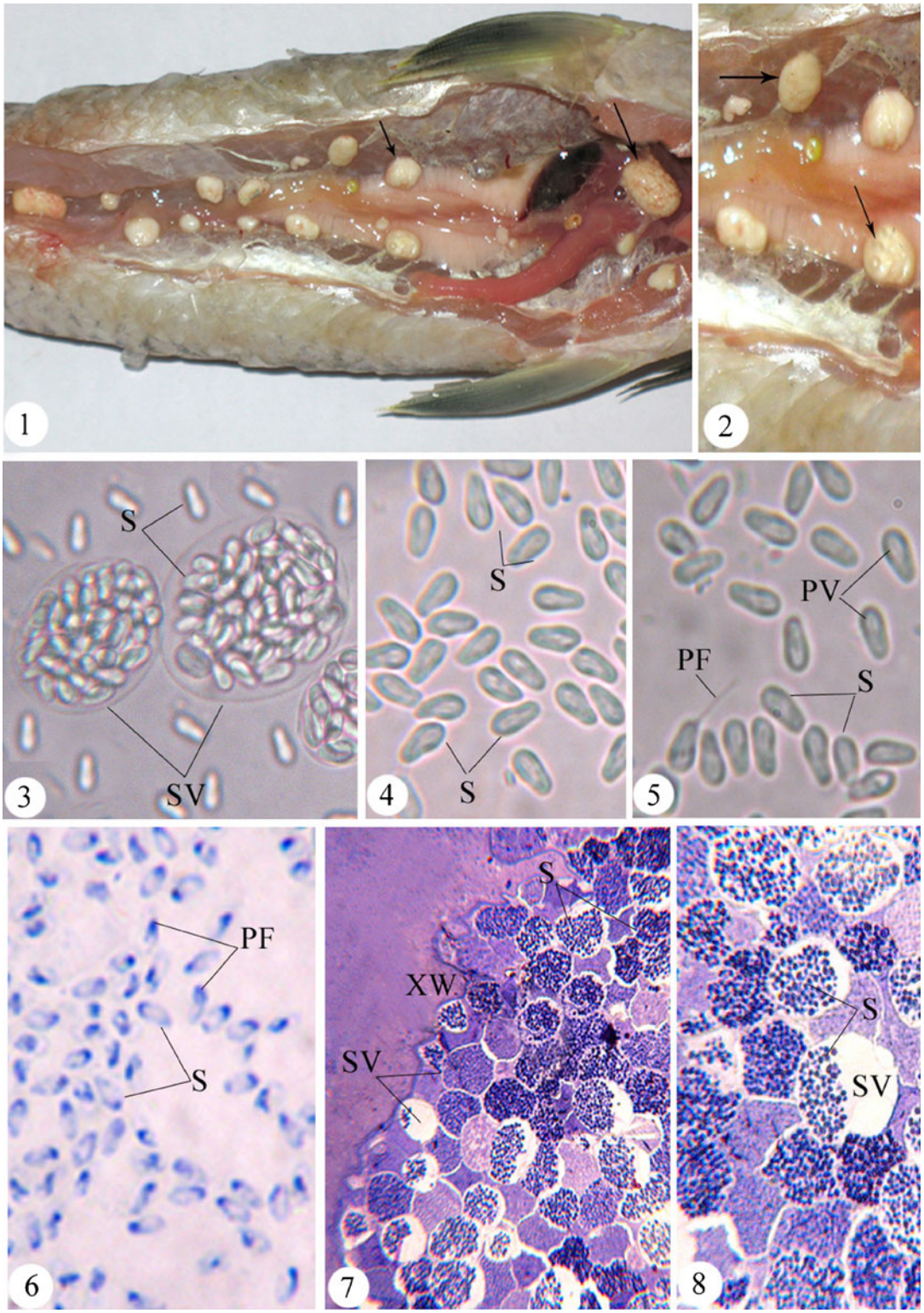

Fig. 1 *1*, *2* Photographs of the infected fish showing the large whitish xenomas (*arrows*) of *P. aegyptiaca* in their body cavity. *3–5* Photomicrographs of the fresh spores (*S*) of *P. aegyptiaca* after rupture of xenomas. The spores may be present within sporophorous vesicles (*SV*), or they can be released. *6* Photomicrographs of fixed spores, stained with Giemsa and showing the spore body and darkly stained blue polar filament coil region. *7–8* Photomicrographs of semithin sections through parasite xenomas surrounded externally with xenoma wall (*XW*) and filled with spores (*S*) within sporophorous vesicles (*SV*)

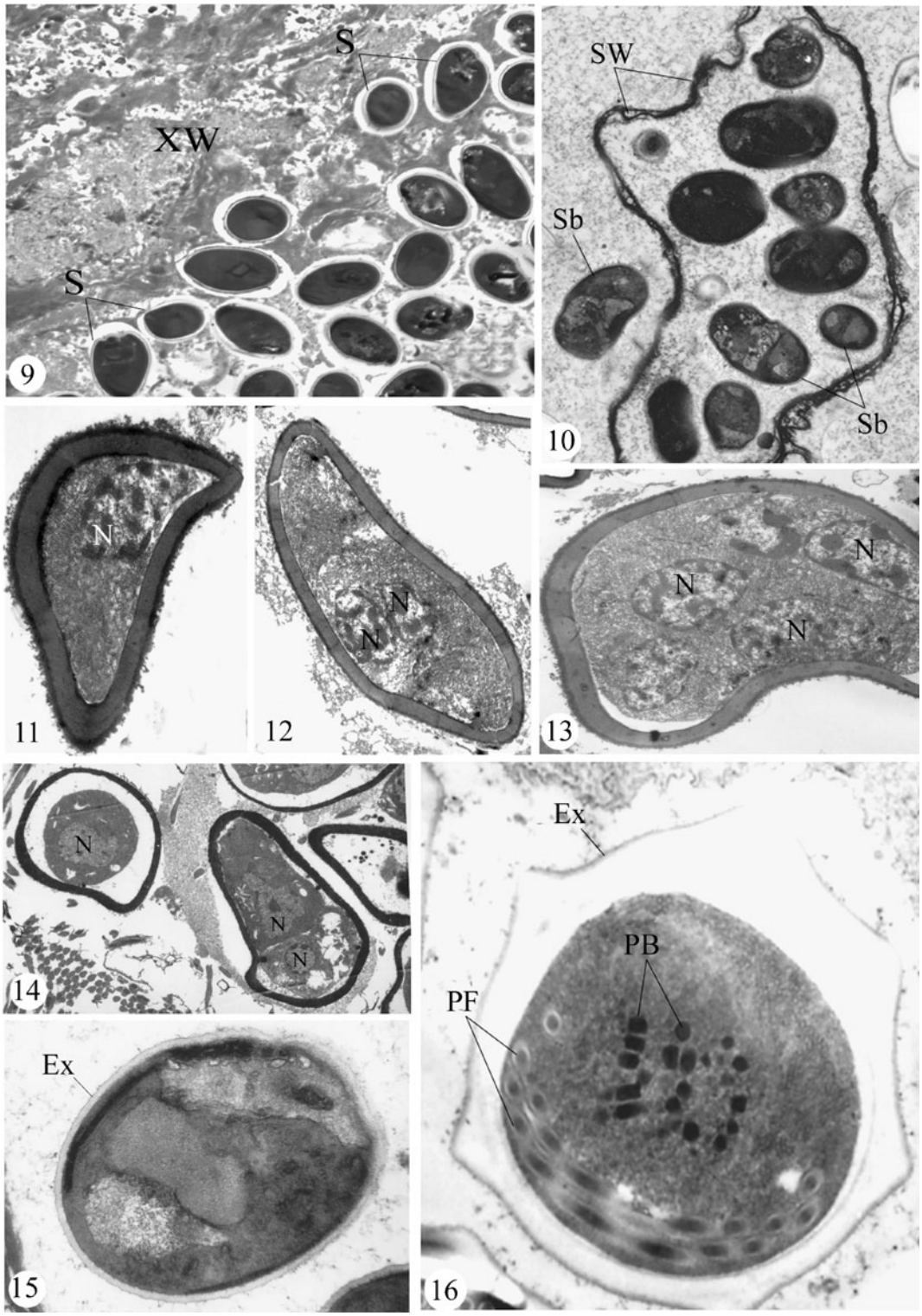

Fig. 2 Transmission electron micrographs showing the developmental stages of *P. aegyptiaca* within its xenoma. *9* Xenoma wall (*XW*) covering spores (*S*); the wall is composed of layers of the parasite cell coat and connective tissue as manifestation of the host response. *10* The double layers of the sporophorocyst walls (*SW*) enclosing a number of sporoblasts (*Sb*) and spores (*S*). *11* Uninucleated meront. *12* Binucleated meront. *13* Multinucleated meront (these meronts are surrounded by a dense wall). *14* Uninucleated sporonts surrounded with a dense wall and being ready for the start of spore maturation. *15* Enlarged sporoblast in an advanced stage of development showing the future exospore (*Ex*) appears pale. Note that the endospore and the filament coils are not yet formed. *16* Immature spore surrounded by exospore (*Ex*) and paramural bodies (*PB*) are still present to aid in filament coils formation

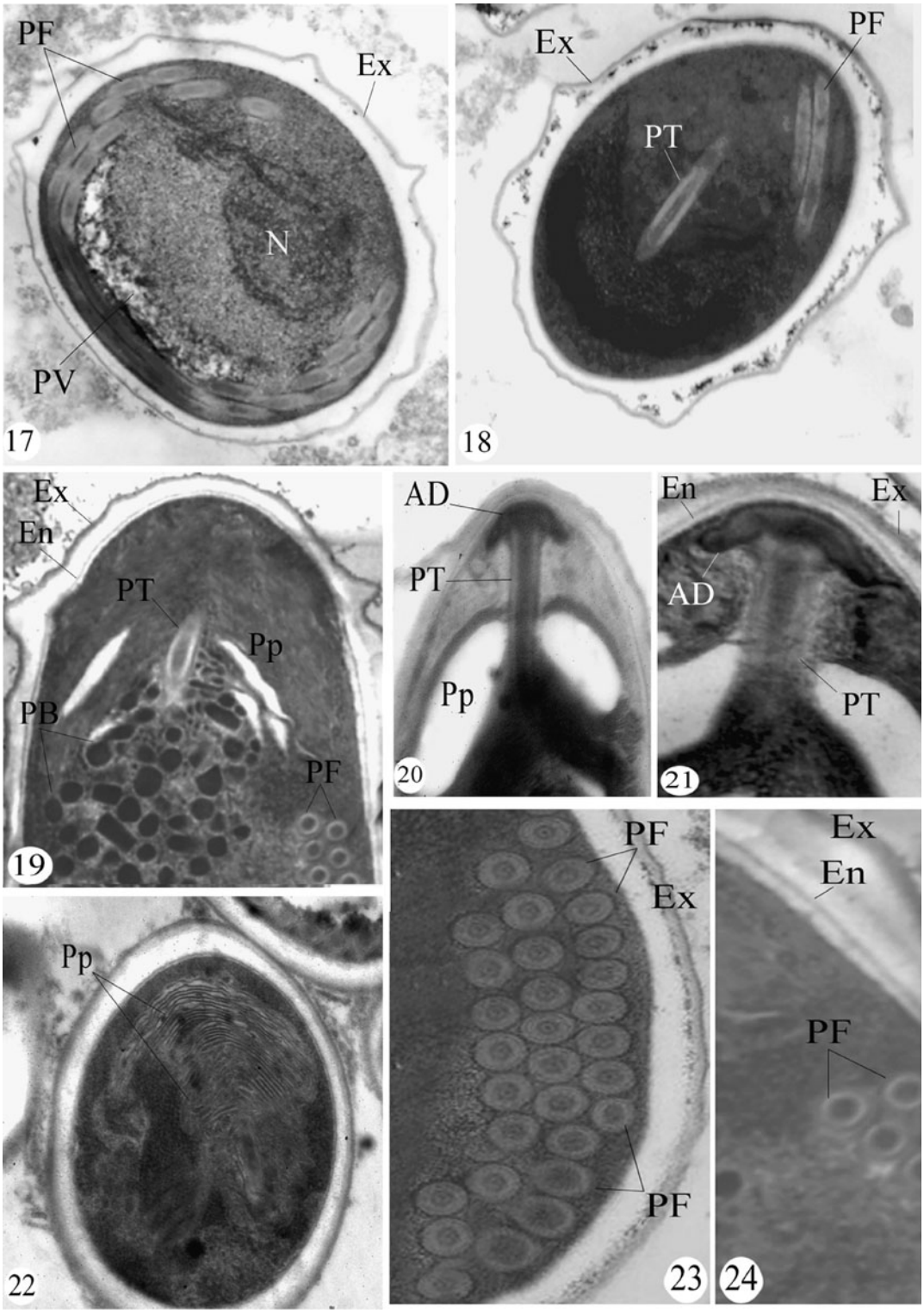

Fig. 3 Transmission electron micrographs showing the typical structural contents of mature spores. *17* Longitudinal section of a uninucleated spore showing the spore wall, the polar filament coils (*PF*), and the posterior vacuole (*PV*). *18* Oblique section through mature spore showing the polar tube (*PT*) and polar filaments (*PF*). *19* The apical structure of microsporidian spore of *P. aegyptiaca* showing the exospore (*Ex*), endospore (*En*), polaroplast (*Pp*), polar tube (*PT*) in cross section, and paramural bodies (*PB*). *20*, *21* The anchoring disk (*AD*) at the spore apex showing the lucent layer of the shaft of the future polar tube. *22* A nearly mature uninucleate spore with the polaroplast (*Pp*) that reveals two portions—the anterior one consists of tightly arranged membranes and the posterior one consisting of loosely arranged membranes. *23* Details of the polar filament coils of a spore (*PF*); observe the multilayered structure of the coil. *24* Details of the spore wall, thick-walled exospore (*Ex*) covering thin-walled endospore (*En*)

AF044387), and *Pleistophora* sp. (accession no. AF044390). All other microsporidia, including *Glugea* spp., *Loma*, and *Dictyocoela muelleri*, which are closely related to *Pleistophora*, showed homology between 81.6% and 90.7% (Table 3).The small subunit rDNA sequence of *B. bassiana* (accession no. AB 576868) was included as outgroup. Comparison of the nucleotide sequences and divergence showed that the sequence of the present microsporidium was most similar to that of *P. anguillarum* (99.8% identity, accession no. U47052) differing in 13 nucleotide positions and showed the lower divergence value followed by *P. hyphessobryconis* and *P. ovariae* which had a percent identity of 97.3%, 94.9% and differing in 33, 35 nucleotide positions respectively. *D. muelleri* (81.6%, 21.5), *Loma acerinae* (89.9%, 10.9), and *Loma psittaca* (90.1%, 10.6) showed the low percent of identity and divergence values. The resulting alignment consisted of 521 bases after trimming the 3′ end (79 ambiguously aligned positions were excluded). Before phylogenetic analysis, only those sites which could be unambiguously aligned amongst all microsporidians were used. Phylogenetic analyses using DNASTAR program and maximum likelihood and maximum parsimony methods placed our novel sequence within the *Pleistophora* clade containing the type species *Pleistophora aegyptiaca* and deposited in the GenBank with accession number JF514548 and a GC content of 54%. A consensus and most parsimonious tree (tree length 723.0) based on 1,000 bootstrap replicates for MP and on 100 replicates for ML using DNASTAR is given in Fig. 4.

Discussion

The present recorded microsporidian parasite was recorded as whitish xenomas embedded directly in the host tissues destroying them by induction of an enormous hypertrophy of the infected host cell. Similar observations have been recorded previously (Canning 1976; Maurand et al. 1988; Weissenberg 1976; Lom et al. 1993; Matos et al. 2005; Kent and Speare 2005; Lom and Dyková 2005; Lovy et al. 2007; Casal et al. 2008; Abdel-Ghaffar et al. 2009, 2011; Stephens 2009); these authors stated that in fish, a type of host–parasite relationship occurs when microsporidia induce the development of xenomas. Muscle tissue turns opaque and cloudy in affected fish, appearing granular and unappetizing for anglers (Lom et al. 2000a, b, c; Sutherland et al. 2000; Lom 2002; Sutherland 2002; Sutherland 2002; Sutherland et al. 2004). *Pleistophora* sp. is a parasite that occurs within the skeletal muscle cells of fish. Genus *Pleistophora* infections may also have significant pathogenic effects on their hosts. This species infects the tissues diffusely and might eventually be surrounded by host connective tissue. Of particular economic significance are those species infecting the muscle tissue because infected host cells produce grossly visible lesions that render the flesh unfit for human consumption. The data obtained from the dimensions of mature spores of the present *Pleistophora* sp. with those obtained by other authors revealed that the dimensions obtained here were in agreement with those of Kabata (1959) and only slightly different from those obtained by Bossanquet (1910). These differences may be due to the changes that occurred during the preparation. The key feature of this genus on electron microscopy images is the presence of sporophorous vesicles with a dense, rather solid, wall enclosing all the developmental stages of the parasite, i.e., meronts, sporonts, and spores. Either 8 or 16 spores grow inside vesicles found in the sporophorocysts. The polar filament within the spore is extended posteriorly from the anterior mass (polar tube) and then laterally to form a coil just beneath the membrane occupying about two thirds of the spore. In cross sections, the coils appeared as a pair of beaded extensions along the two inner sides of the spore wall. The same observation was reported by Sprague (1966)), Rodriguez-Tovar et al. (2003), and McGourty et al. (2007). Inside the SPV wall, the plasmodia divide stepwise into uninucleate sporoblasts, which finally mature into spores as recorded by Lorn and Dykova (1992)). Also, a mature spore is characterized by the presence of a small posterior vacuole in the spore body; similar results were recorded by Canning and Nicholas (1980) and Casal et al. (2008)). Paramural bodies which are associated with the inner side of the cell membrane are a characteristic and marked structure for the present parasite. The presence of these bodies, which help in the formation of sporoblast wall and the endospore, is one of the reasons to keep this species in the genus *Pleistophora*. The polaroplast is the first apparent vacuole reported by Lom and Corliss (1997). The center of the filament is filled with an electron-dense substance. A similar observation was reported by Kudo and Daniels (1963) from *Thelohania calefornia* and by Lom and Corliss (1997) from *P. hyphessobryconis*.

Molecular analysis

Many of the earlier studies on microsporidia based on morphology, ultrastructure, life cycle, and host–parasite relationships have resulted in the unnecessary creation of a large number of new microsporidian species. The difficulties encountered in proper identification of them based on infection and light and electron microscopic examinations are well illustrated by Mercer and Wigley (1987)), who could not distinguish several microsporidian species they have found in a stem borer, *S. cordalis*, from other species known to infect Lepidoptera. However, they found the PCR method to be quite sensitive and useful in the diagnosis and differentiation of microsporidians from specimens infected

Table 3 Alignment of 521-bp region of *P. aegyptiaca* 16s rDNA sequence with the most related *Pleistophora* species (Only variable sites are shown. Dots represent bases identical to those of the first sequences; dashes indicate gaps, and unknown bases are represented by 'N')

Species	GenBank acc. no.	417	419	422	423	424	427	463	473	509	522	527	543	545	546	547	548	549	500	501	555	567	568	571	575	578	586	588	599
P. aegyptiaca	JF514548	G	A	C	T	T	G	G	=	A	G	G	G	C	=	=	=	=	=	=	T	C	A	G	G	G	A	G	T
P. anguillarum	U47052	·	·	·	·	=	·	=	T	·	·	·	·	=	=	=	=	=	=	=	·	·	·	·	·	·	·	·	·
P. hyphessobryconis	GU126672	T	G	T	C	=	A	G	·	T	·	A	A	C	=	=	=	=	=	=	·	T	·	·	·	·	·	·	·
P. ovariae	AJ252955	·	A	C	A	=	·	·	·	·	·	·	·	·	=	=	=	=	=	=	·	·	·	·	·	·	·	A	·
P. mulleri	EF119339	G	·	·	·	=	·	·	·	A	A	G	·	·	C	T	T	T	T	A	A	C	G	·	C	A	G	·	G
P. sp. (3)	AF044390	·	·	·	·	=	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
P. typicalis	AF044387	·	·	·	·	=	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	=	·	·	·	·	·

Species	GenBank acc. no.	600	601	602	606	607	645	669	670	671	675	676	682	686	723	758	772	775	776	777	778	779	780	781	782	783	784	785	786
		T	T	T	G	C	G	G	C	G	G	C	G	A	C	T	T	G	T	=	=	=	=	=	=	G	C	G	C
		·	·	·	·	·	·	·	·	=	·	·	=	·	·	·	·	·	=	=	=	=	=	=	=	=	=	T	G
		·	·	·	·	·	C	·	·	G	·	·	G	·	·	·	·	·	A	T	T	=	A	G	A	G	T	·	C
		·	·	·	·	T	·	·	·	·	A	·	·	·	·	C	A	·	·	G	G	=	·	C	·	A	·	·	·
		C	C	G	C	A	·	·	·	·	T	G	·	T	T	T	G	A	G	T	T	C	T	T	·	G	A	C	T
		·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
		·	·	·	·	·	·	C	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·

Species	GenBank acc. no.	789	790	797	806	832	838	839	840	841	899	900	909	911	912	913	917	923	924	925	930	932	934	939	940	941	942	943	945
		G	A	G	G	C	C	G	A	G	T	G	C	N	T	T	C	G	A	A	A	C	C	T	C	C	G	A	G
		·	·	·	·	=	·	·	·	·	·	·	·	G	G	A	A	C	C	C	·	·	·	G	T	G	T	T	T
		·	·	A	C	C	·	·	·	·	C	A	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
		T	T	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·
		G	C	·	G	·	·	·	·	·	T	G	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·
		·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	=	G	·	·	·	C	T	·
		·	·	·	·	·	=	=	=	=	·	·	·	·	·	·	·	·	·	·	A	C	C	·	·	·	T	G	·

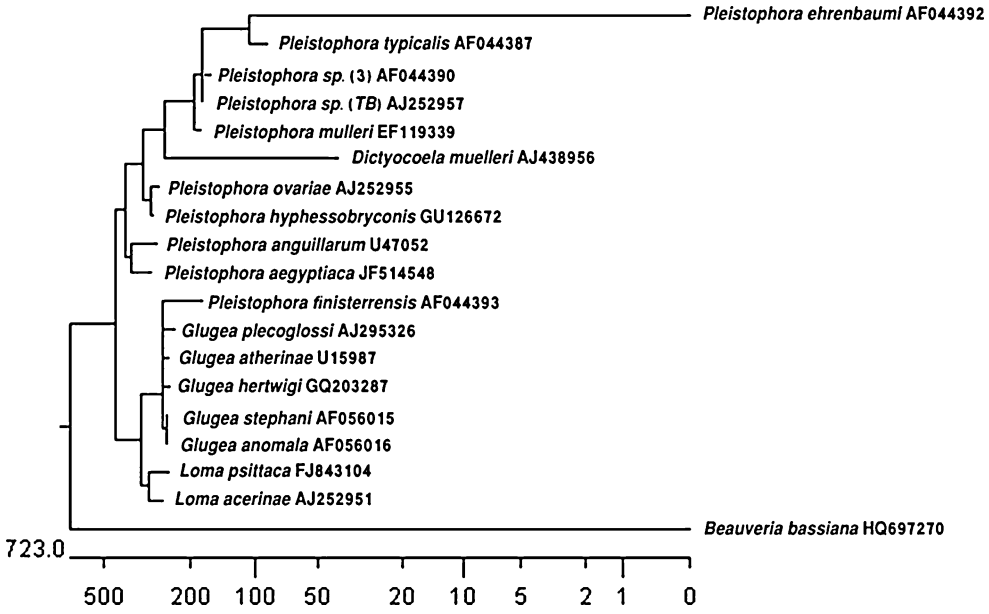

Fig. 4 Phylogenetic tree representing the relationship of *P. aegyptiaca* to other microsporidian species obtained from GenBank. (Tree length=723.0)

with more than one microsporidian species. Phylogenetic reconstruction based on comparison of SSU-rRNA sequences has successfully been used to detect and classify various organisms (Cavalier-Smith and Chao 1996; Dugourd et al. 1996) including microsporidia (Kawakami et al. 1992; Vossbrink et al. 1993; Baker et al. 1994, 1995; Hung et al. 1998; Raynaud et al. 1998; Hatakeyama et al. 2000; Rao et al. 2004, 2005). Small subunit rDNA sequence data have revealed that some of the developmental features and ultrastructural characteristics used to designate microsporidian taxa are the result of convergent evolution. Therefore, taxa which were thought to be very different taxonomically based on their development and ultrastructural characteristics are, based on rDNA analysis, very similar. At the same time, differences in development, morphology, and ultrastructure can suggest possible taxonomic lines. As a result, a number of studies with Microsporidia are now including both ultrastructural and comparative rDNA sequence characters (Maddox et al. 1999; Fries et al. 1999; Andreadis and Vossbrinck 2002; Sokolova et al. 2003; Vavra et al. 2006). Ultrastructural morphology in addition to phylogenetic analysis using SSU-rRNA genes has been shown to be very useful in microsporidian taxonomy and phylogeny (Maddox et al. 1999; Andreadis and Vossbrinck 2002; Canning et al. 2002; Sokolova et al. 2003). Assignment of the present parasite to the genus *Pleistophora* is also strongly supported by the molecular data. The general structure of the phylogram obtained in this study is consistent with the previous analyses. However, the addition of the new sequences from this study identifies the ancestral marine origin of this species, and it strongly aids the understanding of the cladistic arrangement within the more recent clades due to the addition of new species belonging to poorly represented genera or so far unavailable on the molecular database. The sequence shows 99.8% similarity to that of the type species *P. anguillarum*, with only 13 base changes in SSU-rDNA sequence. Previous molecular phylogenetic studies have demonstrated a high degree of sequence similarity between a subset of *Pleistophora* species (Cheney et al. 2000; Nilsen et al. 1998), and these are here designated into the *Pleistophora* forming clades in the phylogenetic trees that are distinct from other clades. We also observed that all *Pleistophora* showed at least 92% similarity to our sequence, whilst parasites from other clades showed only 81.6–90.6% similarity as has been observed in other recent publications (Matthews et al. 2001; Terry et al. 1999; Leipe et al. 1993; Baker et al. 1995; Hartskeerl et al. 1995; Mathis et al. 1997; Hung et al. 1998); our results clearly demonstrate the importance of using classical ultrastructural information in combination with molecular data when describing novel parasites (Baker et al. 1995; Weiss 2001). Recognition of the polyphyletic nature of the genus *Pleistophora* has resulted in reclassification of many species into other genera (Canning and Hazard 1982; Cheney et al. 2000; Nilsen et al. 1998).

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