Parasitol Res (2012) 111:1587–1597
DOI 10.1007/s00436-012-3012-9

ORIGINAL PAPER

Ultrastructure and molecular phylogenetics of a new isolate of *Pleistophora pagri* sp. nov. (Microsporidia, Pleistophoridae) from *Pagrus pagrus* in Egypt

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Received: 31 May 2012 / Accepted: 4 June 2012 / Published online: 7 July 2012

Abstract The spore morphology and molecular systematic of a new microsporidian which was isolated from the common sea bream *Pagrus pagrus* (F: Sparidae Linnaeus, 1758) from the Red Sea, Egypt have been studied. Fifty-six out of 300 (18.7 %) of this fish were infected with microsporidian parasites. The infection was appeared as whitish, ellipsoid, round, or elongated nodules embedded in the epithelial lining of the peritoneum and also in the intestinal epithelium. Light microscopic study revealed that nodules were encapsulated by a fibrous layer encircling numerous mature spores measuring 1.7±0.6 (1.5–2.7 μm)×1.5±0.3 μm (1.2–1.8 μm) in size. Ultrastructure of spores was characteristic for the genus *Pleistophora*: dimorphic, uninucleate spores (each spore possesses three to five polar filament coils) and a posterior vacuole. Also, the early recognizable stages of the parasite within nodules include uninucleated, binucleated, and multinucleated meronts followed by detachment of the plasmalemma of the sporont producing sporoblasts which mature to spores that consist of a spore coat and spore contents. Also, we analyzed the small subunit ribosomal gene (SSUrDNA) using PCR and sequencing specimens from the marine populations of *P. pagrus* fish from the Red Sea. From blast searches, sequence analysis, and phylogenetic analysis, we did not find corresponding GenBank entries to our species. Comparison of the nucleotide sequences showed that the sequence of our microsporidium was most similar to five *Pleistophora* species with degrees of identity (>91.5 %). It was most similar (97.8 % identity) to that of *Pleistophora hyphessobryconis* (account no. GU126672) differing in 19 nucleotide positions and with lower divergence value, *Pleistophora ovariae* (96.2 % identity, account no. AJ252955), *Pleistophora hippoglossoideos* (91.9 % identity, account no. AJ252953), *Pleistophora mulleri* (91.9 % identity, account no. EF119339), and *Pleistophora typicalis* (91.9 % identity, account no. AJ252956). So, they likely represent new species named *Pleistophora pagri* sp. n. with accession number JF797622 and a GC content of 53 %.

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Introduction

Microsporidia infect a broad range of vertebrates and invertebrates including insects, fishes, and mammals (Canning 1976; Wittner and Weiss 1999; Wasson and Peper 2000; Weiss 2001; Cali and Takovorian 2003; Casal et al. 2009). They are responsible for infectious diseases in humans and considerable problems in industries such as fisheries and sericulture. There are at least 144 available genera of Microsporidia (Larsson 1999), 18 of them occurring in teleost fishes from the different geographic areas and habitat (Azevedo and Matos 2003; Lom and Nilsen 2003; Baquero et al. 2005; Casal et al. 2008). Fish Microsporidia are embedded directly in the cytoplasm of the host cell which they actually destroy or they induce enormous hypertrophy of the cell (Lom and Nilsen 2003; Lom and Dykova 2005). Microsporidia are eukaryotes with a distinct nucleus and nuclear envelope, but they do not have centrioles or mitochondria and are considered unique among the eukaryotes in that their small subunit ribosomal RNA (SSU-rRNA) genes are smaller than those of typical eukaryotes (Vossbrinck et al. 1993). The genus *Pleistophora* (Gurley 1893) that belong to the protozoan phylum Microspora (Sprague and

Vavra 1977; Wittner and Weiss 1999; Weber et al. 2000) are obligate intracellular protists that were traditionally considered to be a unique eukaryotic that are transmitted by means of minute unicellular infective spores producing xenomas in many invertebrate animals and ectothermic vertebrates, especially bony fishes (Canning and Lom 1986; Vossbrinck et al. 1987; Lom and Dykova 1992; Sprague et al. 1992; Leiro et al. 1996; Lom 2002; Nageswara et al. 2004; Fielding et al. 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 (Kawakami et al. 1992; Hartskeerl et al. 1995; Cavalier–Smith & Chao 1996; Hung et al. 1998). Therefore, taxa which were thought to be very different taxonomically based on their developmental 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 (Fries et al. 1999; Hatakeyama et al. 2000; Andreadis and Vossbrinck 2002; Sokolova et al. 2003; Rao et al. 2004, 2005; Vavra et al. 2006), and eventually with the help of phylogenetic analysis, the pattern of ultrastructural changes over evolutionary time will be determined.

This paper reports the occurrence of microsporidian infections as well as the pathological features and transmission electron microscopy (TEM) of the isolated *Pleistophora pagri* found in infected sea bream fish *Pagrus pagrus*. Also, we examine its small subunit rDNA sequence and determine its phylogenetic placement among other multisporous Microsporidia.

Materials and methods

Samples of 300 individual sea bream fish, *P. pagrus*, were collected throughout the whole year of 2010 from the coasts at the Gulf of Suez and Hurghada City at the Red Sea. Fish were immediately transported in water tanks to the laboratory at the Zoology Department, Faculty of Science, Cairo University. The captured fish were kept alive in aquaria filled with the same water source to prevent the loss of mobile and temporary ectoparasites. Microsporidian infections were firstly elucidated by the presence of visible masses embedded in the muscles of the abdominal cavity and along the intestinal wall of the infected fish. These were individually spread on glass microscope slides and pressed under cover slips to the thickness of one cell. Fresh spores were observed under a microscope, and the diameter of the spores was measured as mean± SD (range) according to the guidelines of Lom et al. (1989). Photomicrographs were taken using Zeiss Axiovert 135 microscope with Cannon digital Camera.

For TEM, small pieces of the isolated microsporidian cysts were fixed for 48 h in 2.5 % glutaraldehyde in a cacodylate buffer at 4 °C for 3 h and then post-fixed in 2 % osmium tetroxide in 0.1 M phosphate buffer, pH 7.2 at 4 °C for 2 h. After dehydration in a graded ethanol series, the infected tissues were embedded in Spurr's resin. The semi-thin sections (1 μm thick) were cut on a Leica ultracut (UCT) ultramicrotome and stained with toluidine blue. The ultrathin sections were cut on a Leica UCT ultramicrotome and stained with uranyl acetate and lead citrate. Electron micrographs were made with a Jeol JEM 1220 electron microscope operated at 80 kV.

Genomic DNA was extracted from the sporoplasms discharged from spores using the glass bead method described by Undeen and Cockburn (1989). The microsporidians isolated were suspended in an aqueous solution containing 100 mM NaCl, 200 mM sucrose, 10 mM EDTA, and 30 mM Tris–HCl buffer (pH 8.0), to osmotically protect the nuclei from disruption. To this, equal volumes of spore suspension (5×10^{10}) and glass beads (0.425–0.600 μm, 30–40 US sieve size; Sigma, St. Louis, MO, USA) were added in a 1.5-ml Eppendorf tube. The tubes were vigorously shaken for 1–5 min at a maximum speed on the vortex. When >85 % of the spores had ruptured, as indicated by their dark and empty appearance under a phase contrast microscope, the homogenate was centrifuged at 3,000 rpm for about 2 min, and the supernatant solution was transferred to a fresh Eppendorf tube using a Pasteur pipette. Proteinase K (100 μg/ml; Bangalore Genei, Bangalore, India), 1/4th volume of 2.5 % sodium dodecyl sulfate (SDS), 250 mM EDTA, and 500 mM Tris–HCl (pH 9.2) were added, and the mixture was incubated for 1 h at 55 °C to release DNA from the nuclei. The SDS and proteins were precipitated by adding 1/5th volume of 1 M potassium acetate, incubated for 1 h at 4 °C, and centrifuged at 5,000 rpm for 20 min. The DNA was precipitated by adding double the volume of cold absolute ethanol. RNA was degraded by digestion with100 μg/ml RNase (Bangalore Genei, Bangalore, India) for 1 h at 37 °C. DNA concentration and quality was determined both by spectrophotometry at 260 and 280 nm and on 0.8 % agarose gel, using a known quantity of λDNA (10 μg/ml) as a standard before use in subsequent PCRs. A working solution of DNA (10 μg/ml) was prepared in sterile double-distilled water.

For PCR amplification, the microsporidian-specific primers (Table 1), 18f (5′-CAC CAG GTT GAT TCT GCC-3′) and 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′) in 20 μl of reaction mixture containing approximately 30 ng template DNA, 0.2 μm of a single primer, 200 μm each dNTP's (Fermentas Life Sciences, Vilnius, Lithuania), and 1 U of Taq DNA polymerase in 1× PCR buffer containing 75 mM Tris–HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01 % Tween 20, and 2 mM $MgCl_2$ both provided by the

Table 1 Primer sequences for the 16 SSU-rDNA of the investigated microsporidian parasites under study (Vossbrinck and Andreadis 2007)

Primers	Sequence	T_m (°C)	G+C content
18f	5′-CAC CAG GTT GAT TCT GCC-3′	50.4	55.6 %
1492r	5′-GGT TAC CTT GTT ACG ACT T-3′	42.8	42.1 %
SS350f	5′-CCA AGG AYG GCA GCA GGC GCG AAA-3′	73.1	64.6 %
350r	5′-TTT CGC GCC TGC TGC CRT CCT TG-3′	70.9	63.0 %
SS530f	5′-GTG CCA GCM GCC GCG G-3′	64.2	84.4 %
SS530r	5′-CCG CGG KGC TGG CAC-3′	59.5	83.3 %
1061f	5'-GGT GGT GCA TGG CCG-3'	54.0	73.3 %
1047r	5′-AAC GGC CAT GCA CCA C-3′	52.5	62.5 %

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

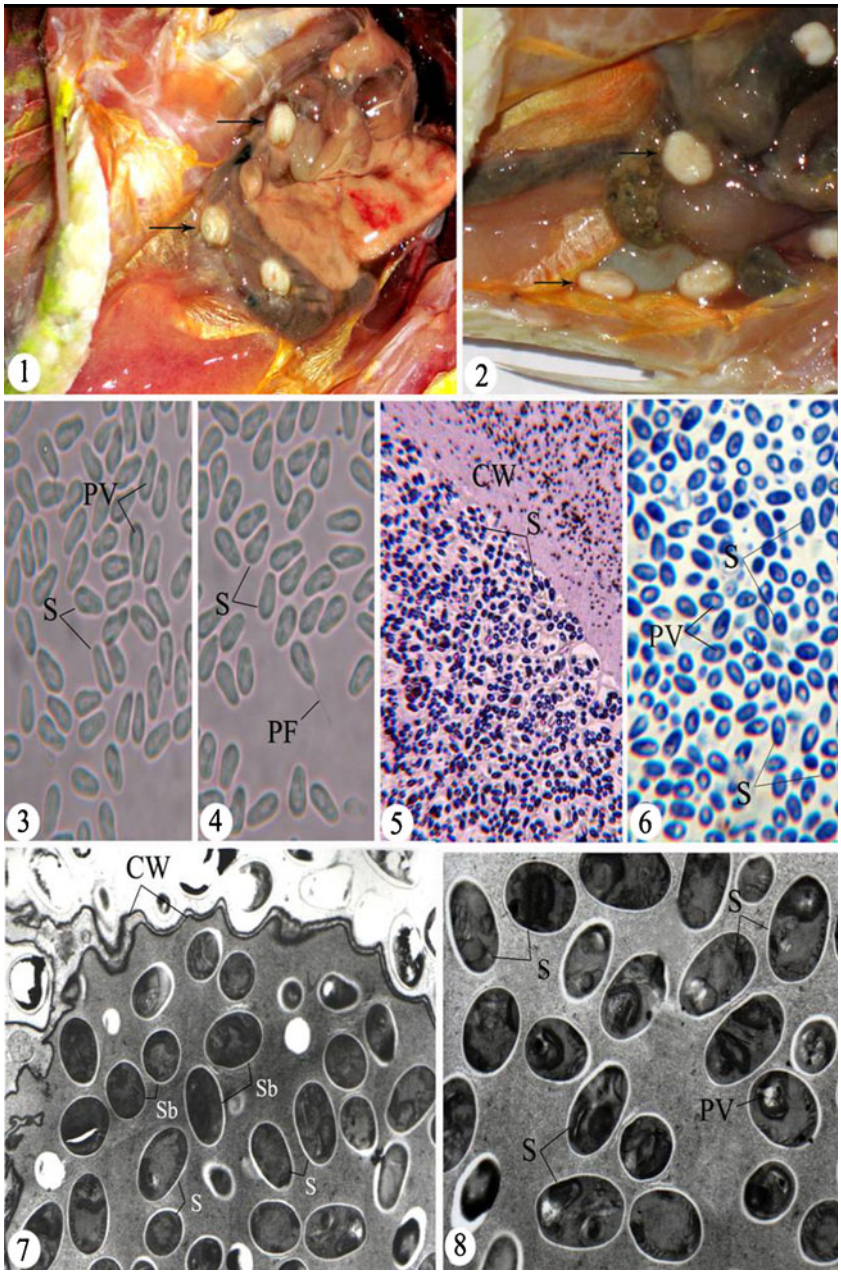
manufacturer of the enzyme (Fermentas Life Sciences, Vilnius, Lithuania). Amplification reactions were carried out for 35 cycles after an initial denaturation for 4 min at 94 °C. Each PCR cycle comprised three steps: denaturation at 94 °C for 50s, annealing at 56 °C for 50s, and extension at 72 °C for 80 s with a final extension of 7 min at 72 °C then rested at 4 °C. A negative control without DNA was run in parallel in the same thermocycler. The amplified PCR products were size-fractionated by electrophoresis on 1.5 % agarose gel (Gibco-BRL, Grand Island, NY, USA) in 1× Tris–borate–EDTA buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0), and gels were stained with ethidium bromide (0.5 μg/ml) for 30 min (Sambrook et al. 1989). A standard molecular weight marker (mass ruler DNA ladder, MBI Fermentas) was used in each electrophoretic run, and the UV-transilluminated gels were photographed by using Gel Documentation System (Syngene Corporation, Cambridge, UK). The PCR product was then purified on a Qiaquick PCR purification kit (Qiagen Company, Valencia, CA, USA) and prepared for sequencing. Sequencing was done in ABI 310 automatic DNA sequencer (Applied Biosystems) with the following microsporidian primers: 18f, CACCAGGTTG ATTCTGCC; SS350f, CCAAGGA(T/C) GGCAGCAGG CGCGAAA; 350r, TTTCGCGCCTGCTG CC(G/A)TC CTTG; SS530f, GTGCCAGC(C/A)GC CGCGG; SS530r, CCGCGG(T/G)GCTGGCAC; 1047r, AACGGCCATG CACCAC; 1061f, GGTGGTGCAT GGCCG; and 1492r, GGTTACCTTGTTACGACTT. Sequencing templates were prepared using a plasmid preparation kit of Machery–Nagel using the BIO Dye Terminator v 3.1 Ready Sequencing Kit (Applied Biosystems) using universal forward and reverse primers. SSU-rDNA sequences and accession numbers for 16 different microsporidian species were obtained from the NCBI GenBank database and were aligned using the Clustal X program (Thompson et al. 1997) and corrected using the alignment editor BioEdit 4.8.9 (Hall 1999). The resulting sequence fragments were assembled into a single contiguous sequence using the multiple-alignment algorithm in Megalign (DNASTAR, Windows version 3.12e). *Beauveria bassiana* was included as outgroup. Aligned sequences were analyzed by maximum parsimony using PAUP version 3.1b (Swofford 1998). Bootstrap analysis was accomplished using 1,000 neighbor joining replicates.

Results

Light microscopic observations

Fifty-six out of 300 of the examined fish were infected with microsporidian parasites at a percentage of 18.7 %. Whitish macroscopic cysts embedded in the peritoneal cavity were observed to infect many organs of the body including muscles, connective tissues, and the intestinal epithelium (Figs. 1 and 2). The infection was developed as tumor-like masses often up to 2 cm in diameter inducing an enormous hypertrophy to the infected organs. It was noted that infection with this microsporidian is generally increased during winter to 10 % and fall to 6 % in summer. A large number of mature spores were usually observed after rupture of cysts (Fig. 3). The spores were elongated ovoid or ellipsoidal in shape with a posterior vacuole. Fresh, 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. 4). Histopathologically, parasitic foci seen in semithin sections were encapsulated by a wall or fibrous layer produced by the host encircling numerous immature and mature spores (Figs. 5 and 6). Ultrastructurally, the isolated parasite cysts were bordered by a double-layered cyst wall enclosing different developmental stages of the parasite (Fig. 7), the early stages were found to be present under the cyst wall while the mature stages of spores were found at the center of these cysts (Fig. 8).

The life cycle of the microsporidian pathogen described herein included four stages: proliferation (merogony), sporogony, sporoblast, spores, and liberation. The first stage, the merogony (proliferative phase), is initiated by piercing the host cell with the polar tube of the spore and extruding infective sporoplasm into the cytoplasm of the host cell.



Figs. 1–8 Photographs of dissected *P. pagrus* fish infected with the microsporidian parasite *P. pagri* sp. n. The infection occurs in the form of whitish cysts (*arrows*) appeared embedded in different organs in the peritoneal cavity of infected fish. Photomicrographs of the microsporidian parasite showing *3*, *4* fresh spores (*S*) which release after rupture of cysts. Each spore consists of a posterior vacuole (*PV*) located posteriorly. Observe the extruded polar filament from spores (*PF*) in *4* (×2,400). *5* Semi-thin section of the parasite cyst surrounded by cyst wall (*CW*) which is seen to encapsulate the parasite and spores (*S*) in various degrees of development (×1,000). *6* High magnification of the semi-thin section showing the microsporidian spores (*S*). Note that each mature spore contains a posterior vacuole (*PV*) (×2,200). Transmission electron micrographs of *7* cyst periphery showing the double-layered cyst wall (*CW*) enclosing spores (*S*) and sporoblasts (*Sb*) (×3,300). *8* Mature spores within the center of parasite cyst (×7,000)

The sporoplasm of generative cells (Fig. 9) develops and forms proliferative cells referred to as meronts (Figs. 10, 11, 12, and 13) which are roundish cells encircled by a typical unit membrane. The uninucleated meronts have a large nuclear region with a single nucleus (Fig. 10) followed by binucleated meronts (Fig. 11) and then form rounded plasmodial multinuclear cells (Fig. 12) that divide by plasmotomy (Fig. 13). This process essentially involves conversion of a meront into a sporont, the cell that produces the sporoblasts (Fig. 14). Sporoblasts are cells that mature and transform into spores without further division but involve a general, progressive increase in cytoplasmic density as more ribosomes and endoplasmic reticulum are formed. Sporogenesis began with the concurrent formation of the exospore and polar filament followed by cell elongation (Fig. 5). Mature spore contained a short isofilar polar filament composed of three to five coils which were arranged such that the first turn began near the midpoint of the spore and then angled to the posterior part of the spore (Figs. 16 and 17). A region of the polaroplasm can be distinguished (Figs. 14 and 15) called polaroplast which is a laminar membrane system region. A posterior vacuole can be found at the posterior pole of mature spore (Figs. 16 and 17). All these stages of the parasite are haplokaryotic and develop in a vacuole bounded by a single membrane in contact with host cell cytoplasm (Figs. 14, 15, 16 and 17). Spores are released from the host cells when they are mature and infect new host cells.

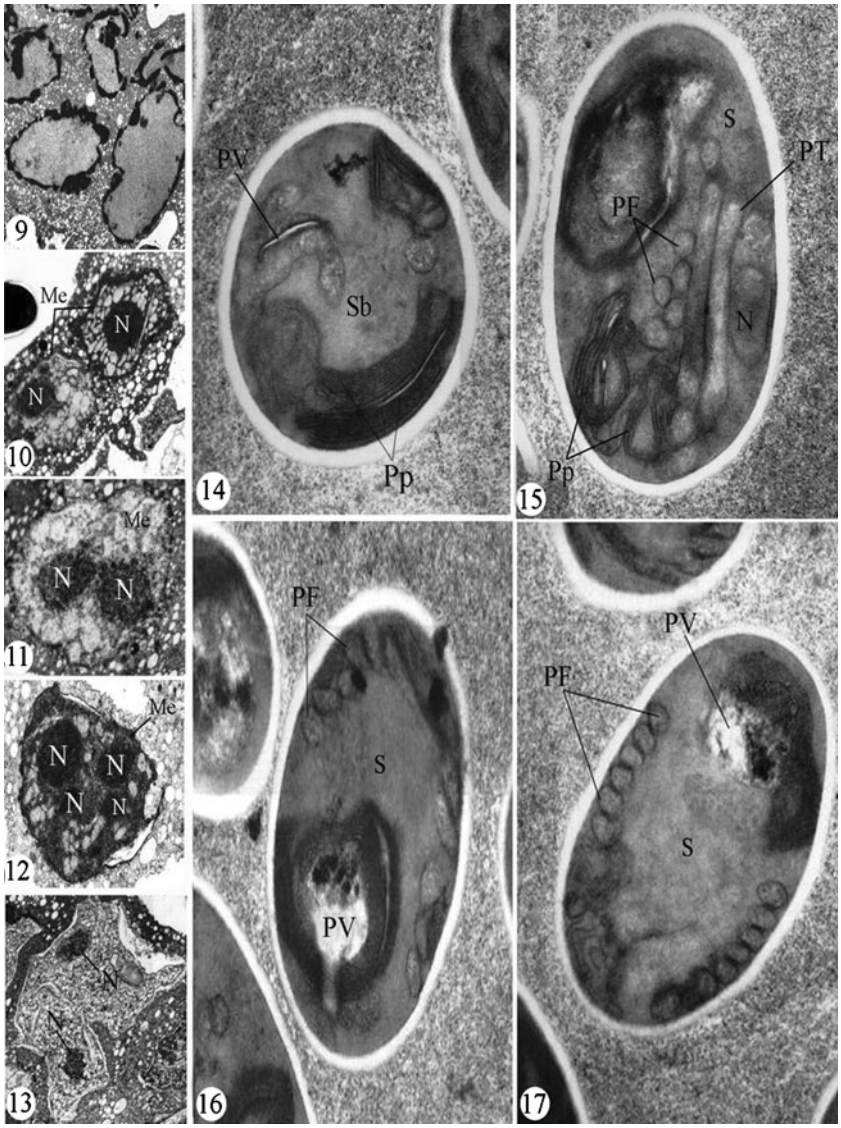


Fig. 9–17 Transmission electron micrographs showing the development of *P. pagri* sp. nov. within their cyst inside their fish host. *9* Early stage of development, the generative cells (×3,500). *10* Uninucleated meront (*Me*) with one nucleus (×3,300). *11* Binucleated meront (*Me*) with four nuclei (×3,300). *12* The multinucleated meront (*Me*) with four nuclei (×3,300). *13* Binucleated meront (*Me*) undergoing cytoplasmic cleavage (to form further uninucleated sporonts (×3,300). *14* Sporoblasts (*Sb*) with poorly developed posterior vacuole (*PV*) and with lamellar polaroplast (*Pp*) (×20,000). *15* A nearly mature uninucleated spore (*S*). The extrusion apparatus formed of a polar tube (*PT*) followed by polar filament coils (*PF*) (×22,000). *16*, *17* Mature uninucleated spore (*S*). A posterior vacuole (*PV*) is located posteriorly and may be empty or filled with dense components. The extrusion apparatus formed of polar filament coils (*PF*) followed from a polar tube (*PT*) and arranged on both sides of the posterior part of spore around the PV area and below the spore wall (×20,000)

Taxonomy summary

Type host Common sea bream *P. pagrus* (F: Sparidae Linnaeus 1758).

Type locality Gulf of Suez and Hurghada city of the Red Sea, Egypt.

Infection site Epithelial cells of gut and viscera in the peritoneal cavity of host fish.

Transmission Unknown. Liberation of spores through feces and persistent high prevalence suggest horizontal transmission.

Presporulation stages Rounded binucleate and tetranucleate cells. Proliferation by binary fission.

Transition to sporogony Stages become enclosed by host rough endoplasmic reticulum, show vacuolated cytoplasm.

Sporogony Stages are enclosed by host rough endoplasmic reticulum or coated with an electron-dense material of increasing thickness.

Molecular analysis

For the classification of the isolated microsporidium, a phylogenetic analysis based on the small subunit rDNA gene was performed. The PCR product was cloned and sequenced. The 249-bp sequence was submitted to a GenBank BLAST search to find the most similar sequences. Sequences with the 16 highest BLAST scores were aligned with Clustal W. The small subunit rDNA sequences of *B. bassiana* (account no. HQ697270) was included as an outgroup. Comparison of the nucleotide sequences showed that the sequence of our microsporidium was most similar to five *Pleistophora* species with degrees of identity (>91.5 %). It was most similar (97.8 % identity) to that of *Pleistophora*

Table 2 Microsporidian species used in the phylogenetic analysis of the present *P. pagri* sp. n. assigned as JF797622

Organism	Host/host group	Source	Accession no.	Percent of identity (%)	Divergence
Glugea anomala	*Gasterosteus aculeatus* (F)	GenBank	AF056016	83.6	18.6
Glugea atherinae	*Atherina presbyter* (F)	GenBank	U15987	84.5	17.4
Glugea hertwigi	*Osmerus mordax* (F)	GenBank	GQ203287	84.5	17.4
Glugea plecoglossi	*Plecoglussus altivelis* (F)	GenBank	AJ295326	85.0	16.7
Glugea stephani	*Pleuronectes americanus* (F)	GenBank	AF056015	83.6	18.6
Ichthyosporidium sp.	*Leiostomus xanthurus* (F)	M.D. Baker et al.	L39110	79.8	23.5
Loma acerinae	*Gymnocephalus cernua* (F)	M. Pekkarinen	AJ252951	81.4	21.6
Loma psittaca	*Colomesus psittacus* (F)	G. Casal et al.	FJ843104	80.0	23.6
Microsporidium sp.	*Calliptamus italicus* (I)	Issi and Keylova	AY140647	89.5	11.4
Pleistophora hippoglossoideos	*Hippoglossoides platessoides* (F)	F. Nilsen; K. McKenzie	AJ252953	91.9	8.6
Pleistophora hyphessobryconis	*Danio rerio* (F)	J.L. Sanders	GU126672	97.8	2.3
Pleistophora mulleri	*Gammarus duebeni* (C)	GenBank	EF119339	91.9	8.6
Pleistophora ovariae	*Notemigonus crysoleucas* (F)	E. Weidner	AJ252955	96.2	3.9
Pleistophora typicalis	*Myoxocephalus scorpius* (F)	R. Turner	AJ252956	91.9	8.6
Trachipleistophora hominis	*Homo sapiens* (Ma)	J.J. Becnel	AJ002605	91.2	9.4
Vavraia culicis	*Aedes albopictus* (HI)	J.J. Becnel	AJ252961	90.3	10.5

F fish, *C* Crustacean, *HI* hematophagous insect, *Ma* mammal, *I* insect

hyphessobryconis (account no. GU126672), differing in 19 nucleotide positions and with lower divergence value; *Pleistophora ovariae* (96.2 % identity, account no. AJ252955) differing in 17 nucleotide positions; *Pleistophora hippoglossoideos* (91.9 % identity, account no. AJ252953); *Pleistophora mulleri* (91.9 % identity, account no. EF119339); and *Pleistophora typicalis* (91.9 % identity, account no. AJ252956) differing in 26 nucleotide positions (Tables 2 and 3). All other Microsporidia, including *Glugea* spp., *Loma* sp., *Vavraia culicis*, *Microsporidium* sp., *Ichthyosporidium* sp., and *Trachipleistophora hominis*, showed homology between 79.8 and 91.2 % (Table 2). Phylogenetic analyses were carried out using maximum likelihood (ML), maximum parsimony (MP), and DNASTAR program. A consensus tree based on 1,000 bootstrap replicates for MP and on 100 replicates for ML is given in Fig. 18. In all analyses, our isolate clustered within *Pleistophora* clade, indicating a close relationship between the two organisms and deposited in the GenBank with accession number JF797622 and a GC content of 53 %.

Discussion

The parasite that causes the microsporidiosis found in this study was classified as phylum Microspora Sprague 1977, class Microsporea Delphy 1963, order Microsporidia Balbiani 1892, family Pleistophoridae Doflein 1901, and genera Pleistophora Gurley 1893. The phylum Microspora is comprised of unicellular organisms living as intracellular parasites in a variety of invertebrates and in all five classes of vertebrate hosts (Lom and Dykova 2006) consisting of approximately 144 genera and over 1,000 species (Mathins 1997; Wittner and Weiss 1999; Mathins et al. 2000). These microsporidia organisms contain a nucleus with a nuclear envelope, an intracytoplasmic membrane system, and they undergo chromosome separation on mitotic spindles, but do not have centrioles or mitochondria (Weiss 2001). The genera *Glugea*, *Heterosporis*, *Ichthyosporidium*, *Inodosporus*, *Kabatana*, *Loma*, *Microfilum*, *Microgemma*, *Microsporidium*, *Neonosemoides*, *Nucleospora*, *Pleistophora*, and *Thelohania* have been found in aquatic environments (Lom et al. 1999; Azevedo et al. 2000; Yokoyama et al. 2002), but only the genera *Heterosporis*, *Kabatana*, *Microsporidium*, *Pleistophora*, and *Thelohania* have been found to infect muscle tissue (Kelly 1979; Flegel et al. 1992; Lom and Dykova 2006). There are many microsporidians causing diseases in fish aquaculture. They may seriously endanger whole stocks of feral fish and thus reduce the productivity of fisheries (Maurand et al. 1988; Lom and Dykova 1992; Pekkarinen 1996). In natural habitats, notably marine, microsporidia may have the largest impact on young fry and yearling fish and may constitute one of the factors limiting the growth of stocks (Lom and Dykova 1992). Microsporidia are embedded directly in the cytoplasm of the host cell, and they subsequently destroy the cell (Lom and Nilsen 2003). Microsporidia are currently classified on the basis of their ultrastructural features, including size and morphology of the spores, number of coils of the polar tube, developmental life cycle, and host–parasite relationship (Sprague et al. 1992). The number and

Table 3 Alignment of 249-bp region of *P. pagri* sp. n. 16S rDNA sequence with the most related *Pleistophora* species

Species	GenBank account no.	407	408	409	410	411	412	414	415	416	417	419	422	423	426	428	471	484	488	508	521	526	542	545
P. ovariae	AJ252955	T	G	C	A	G	G	A	A	A	T	A	C	A	A	T	G	C	G	T	G	A	A	T
P. hyphessobryconis	GU126672	C	A	.	.	.	.	.	.	.	.	G	T	G	.	.	G	C	G	.	.	.	.	.
P. pagri	JF797622	A	G	G	T	T	T	T	C	C	G	A	C	T	G	=	.	.	.	A	.	G	G	.
P. typicalis	AJ252956	T	.	C	A	G	G	A	A	A	.	.	.	A	A	T	.	.	.	.	A	.	A	C
P. hippoglossoideos	AJ252953	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. mulleri	EF119339	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Species	547	550	551	552	553	554	555	556	557	558	559	570	571	578	581	589	591	602	603	604	605	609	610	656	659
P. ovariae	C	=	=	=	=	=	=	=	=	=	=	T	A	G	G	A	A	T	T	T	T	G	T	=	C
P. hyphessobryconis	.	=	=	=	=	=	=	=	=	=	=	.	.	.	.	.	.	G	.	.	.	.	C	=	.
P. pagri	.	=	=	=	=	=	=	=	=	=	=	C	.	.	.	.	.	.	.	.	.	.	.	G	G
P. typicalis	T	A	T	T	C	G	T	T	C	A	T	.	G	C	A	G	A	G	C	C	G	C	A	=	C
P. hippoglossoideos	.	.	.	.	.	=	=	=	=	.	.	.	.	.	.	.	.	.	.	.	.	.	.	=	.
P. mulleri	.	.	.	.	.	=	=	=	=	.	.	.	.	.	.	.	.	.	.	.	.	.	.	=	.

Only variable sites are shown. Dots represent bases identical to those of the first sequences; dashes indicate gaps

arrangement of coils found within the spore will vary depending on the genus and species of microsporidia (Weiss 2001). Whitish xenomas (cysts) were embedded in all body organs, including the muscles, liver, intestine, and stomach. Similar results were previously recorded in other microsporidians (Weissenberg 1976; Matos et al. 2003; Kent and Speare 2005; Lom and Dykova 1992; Bcker and Speare 2004; Lovy et al. 2004; 2007; Casal et al. 2008). The key feature of this genus on ultrastructural level 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). Uninucleated, binucleated, and multinucleated meronts divided by plasmotomy and had a thick amorphous wall which agreed with (Ferguson et al. 2007; Casal et al. 2008; Abdel-Ghaffar et al. 2009). Small posterior vacuole is seen in the present species. Similar observations were recorded by Canning and Nicholas (1980) in *P. typicalis*, where also a vacuole appears at both ends of the spore. The polaroplast is the first apparent vacuole. Similar structures were reported by Lom and Corliss (1997). 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 which appeared. In cross sections as a pair of beaded extensions along the two inner sides of the spore wall occupying about two thirds of the spore, the same observation was reported by (Sprague 1966; Rodriguez-Tovar et al. 2003; McGourty et al. 2007). Mature spores were found to be ovoid in shape surrounded by two membranes, thick exospore and thin endospore. In the polar filament within the spore coil just beneath the membrane along the two inner sides of the spore wall, similar records were observed by (Abdel-Ghaffar et al. 2009; Morsy 2010).

Comparison between the present *Pleistophora* species and those recorded previously in Egypt, we found that our species differs from those species being with a small dimensions of spore and five to eight coils of polar filament coils, which is a small number than those recorded by (Abdel-Ghaffar et al. 2008, 2011) for *Pleistophora* sp. and *Pleistophora aegyptiaca* recorded from *Epinephelus chlorostigma* and *Saurida tumbil* fish, respectively. So on the basis of our morphological observations, we believe that this microsporidium is described as a first time by as *P. pagri*. Earlier classification of microsporidia based on the features of life cycle, spore size, shape, and ultrastructure of spores including the number of coils of the polar tube and the host–parasite relationship resulted in the unnecessary creation of new *Nosema* species, which resulted in confusion in the systematic classification and placement of microsporidian species. Sato et al. (1982) based on spore ultrastructure identified that *Nosema* M12 was not really *Nosema* but a species of *Vairimorpha*. The difficulties encountered in proper identification of microsporidians using ultrastructural studies

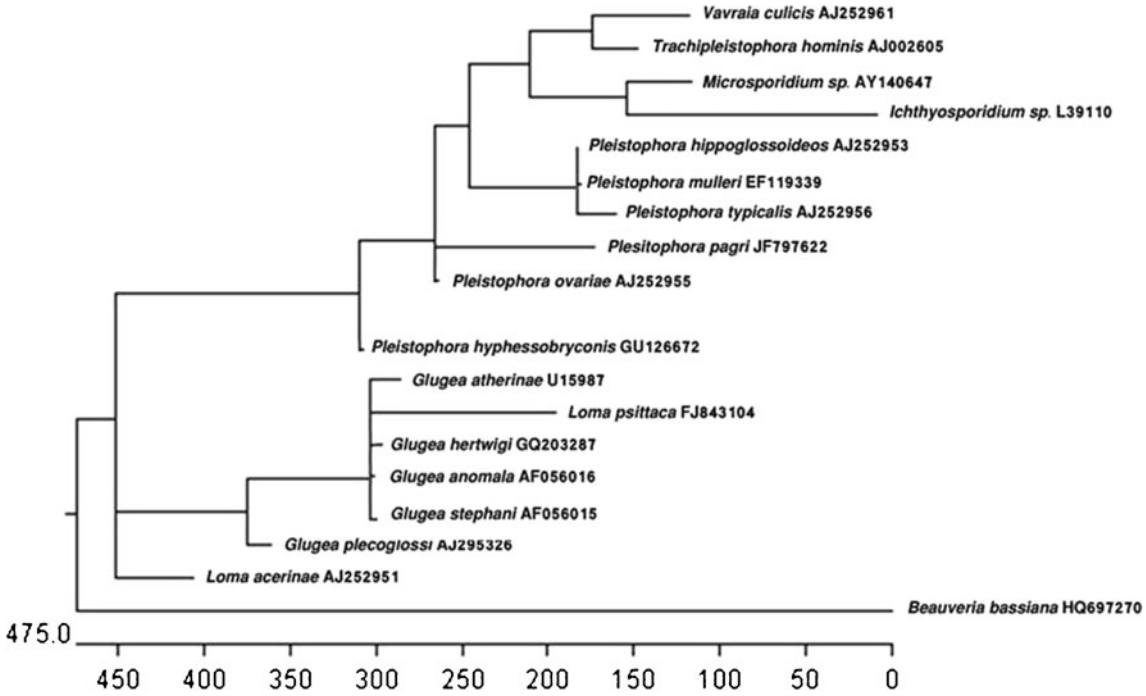


Fig. 18 Phylogenetic relationship based on 16 small subunit (SSU) rDNA showing the relationship between the present *P. pagri* sp. n. to other 17 microsporidian species obtained from GenBank. The tree was oriented by using the 16S rRNA sequence of *B. bassiana* that was used as outgroup (tree length=475)

are well documented by various researchers (Raynaud et al. 1998; Muller et al. 1999). Molecular markers that were developed during the past two decades have considerably reduced the problems associated with distinguishing the microsporidians. PCR-based techniques such as RAPD and SSUrRNA sequence analysis provide DNA markers and sequences, which are found to be more reliable than others in distinguishing the organisms (Hatakeyama et al. 1997; Muller et al. 2000; Tsai et al. 2003; Rao et al. 2005; Rao et al. 2007; Refardt et al. 2008).

Assignment of the present parasite to the genus *Pleistophora* is also strongly supported by the molecular data. Our molecular investigations revealed a close taxonomic relationship between *P. pagri* and *P. hyphessobryconis* (97.8 %) showing that they are clearly separate species differing in 19 nucleotide positions in its 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) 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 91 % similarity to our sequence, while parasites from other clades showed only 79.8–91.2 % as similarity has been observed in other recent publications (Hartskeerl et al. 1995; Hung et al. 1998; Terry et al. 1999; Matthews et al. 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). 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).

Acknowledgment This work is supported by Faculty of Science, Cairo University, Egypt.

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