

Ultrastructure and phylogenetic characterization of the microsporidian parasite *Heterosporis lessepsianus* n. sp. (Microsporidia: Glugeidae) infecting the lizardfish *Saurida lessepsianus* (Pisces: Synodontidae) inhabiting the Red Sea



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ABSTRACT

Heterosporosis is an increasingly important microsporidian disease distributed worldwide, impacting wild and farmed-raised fish in both fresh and marine water environments. Twenty three out of 130 (17.69%) of the lizardfish *Saurida lessepsianus* were found to be naturally infected with microsporidian parasites. The rate of parasitic infection was increased during winter reaching 29.23% (19/65) and fall to 6.15% (4/65) in summer. The infection was recorded as numerous macroscopic whitish cysts reached 3.8–6.5 mm in diameter embedded in the abdominal cavity, skeletal muscles and mesenteric tissues of the infected fish inducing an enormous hypertrophy of infected tissues. Light microscopic examination revealed that parasitic foci were encapsulated by a host-derived fibrous membrane containing different developmental stages of the parasite. Spores were oval to pyriform in shape. Transmission electron microscopic study showed the presence of smooth membranes of the sarcoplasmic reticulum forming a thick, amorphous coat surrounding the various developmental stages of the examined parasite (meronts, sporont, sporoblasts, and spores). Mature spores were electron dense and uninucleate. The anchoring disk was found in a central position at the anterior end of the spore and a large vacuole was located at the posterior end. There was a definite number (7–8) of the polar filament turns. Molecular analysis based on the 16 small subunit (SSU) rDNA gene was performed to determine the phylogenetic position of the present parasite species. A 615 bp region of the 16SSU rDNA gene of the studied parasite was sequenced and deposited in GenBank under the accession number MF769371. Multiple sequence alignment demonstrated a high degree of similarity (> 82%) with other twenty microsporidian species isolated from different aquatic hosts. The most closely related sequence was provided by the GenBank entry JF745533 for *Heterosporis saurida* isolated from the marine fish *Saurida undosquamis* with the highest percentage of identity (98%) and lowest divergence value (0.9). The ultrastructural characteristics and phylogenetic analysis support the recognition of a new species, herein named *Heterosporis lessepsianus* sp. n.

1. Introduction

Phylum Microsporidia Balbiani [1] is a large and diverse group of spore-forming unicellular eukaryotes, only live as obligate intracellular parasites that infect broad range of invertebrate and vertebrate hosts [2–4]. This group of parasites were historically regarded as protozoa

and now considered highly specialized and reduced organisms with fungal affinities [5]. Regarding the recent taxonomic position of the phylum Microsporidia was established alongside the Aphelida and Cryptomycota within the superphylum Opisthosporidia [6]. Phylum Microsporidia includes more than 1.300 species belonging to over 180 genera infecting many hosts ranging from invertebrates to humans

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[7,8]. Among these genera, only few are xenoma-forming parasites of Microsporidia [9]. It is quite clear that microsporidian parasites are known as a cause of a dangerous disease in invertebrates such as silk-worms and bees, as well as fish and crustaceans [4]. Outbreaks of infection may result in significant economic losses due to significant muscle damage, growth deformities, reduced productivity and unsightly flesh, most notably in farmed fish and crustaceans [10].

Little information was available about microsporidia of the genus *Heterosporis* worldwide. This genus was erected by Schubert [11]. All species belonging to this genus are characterized by the presence of a dense wall of sporophocyst enclosing all the developmental stages (meronts, sporonts, sporophorous vesicles with sporoblasts, and spores) of the parasite. To the best of our knowledge, this genus includes eight recognized species infecting fresh- and saltwater fish of Africa, Europe (*H. finki*, *H. cichlidarum*, *H. schuberti* [12,13]), Japan, Taiwan (*H. anguillarum* [14]), USA (*Heterosporis* sp. [15]), the Arabian Gulf in Saudi Arabia (*H. saurida* [16]), the Great Lakes Region in USA (*H. sutherlandae* [4]), and Sunshine Coast region and Fraser Island in Queensland in Australia (*Heterosporis* sp. [17]).

Identification of microsporidians is based largely on the ultrastructural features of the spores and/or on the characteristic cell structure of the developmental stages [18]. Other peculiarities were observed within certain species of microsporidia, include the ability to form a thin surrounding membrane at an early infection stage (sporophorous vesicle) or to induce hypertrophy of the infected host cell as an adaptation where both entities assume a symbiotic co-existence [19]. Traditionally, the classification of microsporidia was nearly based on morphology alone, but this has been challenged more recently with the application of molecular techniques [20]. Morphological plasticity among microsporidia is a recently recognized phenomenon [17] and can lead to conflicting results in speciation when phylogenetic and ultrastructural characteristics are used. The use of SSU rDNA-based phylogenetic has identified ‘clades’ within the phylum Microsporidia [21]. It has even been proposed that the classes Terresporidia, Aquasporidia and Marinospordia could be recognized based on the host habitat colonization [22]. The use of SSU rDNA based phylogenetic is currently proposed as the main discriminator for relatedness amongst microsporidia [23], yet its use as a sole method for taxonomic classification is limiting and often confined to sequence data from one region of the genome. Thus, the most robust method for describing novel microsporidia is achieved with the integration of a range of features including host type, ecology, pathology, ultrastructural morphology and phylogenetic [24,25].

Therefore, the aim of the present study is to report the natural occurrence and prevalence of microsporidian infections in the lizardfish *Saurida lessepsianus* applying of light and transmission electron microscopic studies as well as to clarify the actual systematic and phylogenetic position of the studied parasite by molecular analysis of its 16SSU rDNA.

2. Materials and methods

2.1. Fish collection

A total of 130 freshly caught specimens of the lizardfish *Saurida lessepsianus* were collected monthly during the period of January–December 2016 from the boat landing sites of Hurghada City (27°15'26"N, 33°48'46"E) at the Red Sea Governorate, Egypt. The collected fish were immediately examined for any infection signs and then transported to the laboratory of Parasitology Research at Zoology Department, Faculty of Science, Cairo University, Egypt; for parasitological examination.

2.2. Gross and light microscopic examination

Skin surface, fins, and gills were examined for the presence of any

attached parasites, lesions and/or external changes. After dissection, internal organs, body cavity, fluids, etc ... were examined carefully for microsporidian infection. The collected parasite cysts were examined and photographed by using Nikon microscope (H600L, Japan) equipped with a Canon digital camera (Nikon, DS, Ri2). Parasite prevalence (total number of infected fish/total number of fish hosts examined × 100) was calculated according to Bush et al. [26]. Measurements were carried out by using a calibrated ocular micrometer according to the guidelines of Lom and Dyková [2].

2.3. Transmission electron microscopic examination

After light microscopic examination, isolated cysts were fixed in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate [pH 7.4] at 4 °C for 24 h, and then post-fixed in 1% aqueous OsO₄ at 4 °C for 1 h. After dehydration through graded ascending series of ethanol [50%, 70%, 80%, 90%, 95%, and 100%, staying 2 h in each stage] and in propylene oxide [two changes for 3 h each], cysts were embedded in Epon–Spurr's resin before being polymerized at 60 °C for 48 h. Embedded samples were trimmed and sectioned using Leica UC6 Ultramicrotome (Leica Micosystems, Vienna, Austria). Semithin sections were stained with toluidine blue and examined with a light microscope. Ultrathin sections were contrasted with 4% uranyl acetate and 0.25% lead citrate for 5 min; then examined under a JEOL transmission electron microscope (JEOL Ltd., Tokyo, Japan) in the Regional Centre for Mycology and Biotechnology (RCMB), Al-Azhar University, Egypt.

2.4. Molecular analysis

2.4.1. DNA extraction and PCR amplification

Several cysts recovered from the peritoneal cavity of the infected fish were homogenized to isolate the spores and subsequently stored in 80% ethanol at 4 °C until further use. Genomic DNA was extracted from approximately 5 × 10⁶ spores using a QIAamp DNA MiniKit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA concentration and purity were determined spectrophotometrically by measuring absorbance at wavelengths of 260 and 280 nm. The 16 small subunit ribosomal (SSU) DNA gene cluster was targeted for amplification using specific PCR primers were HG4F (5'-GCC GCT TAA TTT GAC TCA AC-3') and HG4R (5'-TCT CCT TGG TCC GTG TTT CAA-3') designed by Gatehouse and Malone [27]. PCR amplification was carried out in 50 µl reaction mixture comprising 10 pmol of each primer, 10 nmol of each deoxyribonucleotide triphosphates (Finnzymes Products), 2 mM MgCl₂, 5 µl of 10 × Taq polymerase buffer, 1.50 units of Taq DNA polymerase (2U) (Finnzymes Products), 5 µl of genomic DNA, and 26 µl of distilled water. The PCR cycle consisted of an initial denaturation step of 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 72 °C for 2 min, and finished with terminal extension at 72 °C for 10 min, then rested at 4 °C. The PCR products were electrophoresed in 1.0% agarose gel in 1 × Tris-acetate-EDTA (TAE) buffer gel stained with 1% ethidium bromide and visualized with UV transilluminator.

2.4.2. Sequencing and phylogenetic analysis

Purified PCR products were sequenced in both directions using an ABI Prism Dye Terminator Cycle Sequencing Core Kit (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA) with 310 Automated DNA Sequencer (Applied Biosystems, USA) using the same primers for annealing. To evaluate the relationship of the present parasite with other microsporidia species, a homology search was performed on GenBank using BLASTn searches of the National Centre of Biotechnology Information non-redundant nucleotide (nr/nt) database. Data of 16S rDNA gene were aligned using CLUSTAL-X multiple sequence alignment [28] with some of previously recorded data from GenBank to analyze intra-specific differences. The alignment was corrected manually using the alignment editor of software BioEdit 4.8.9 [29]. The data analyzed with Maximum Composite Likelihood (MCL)

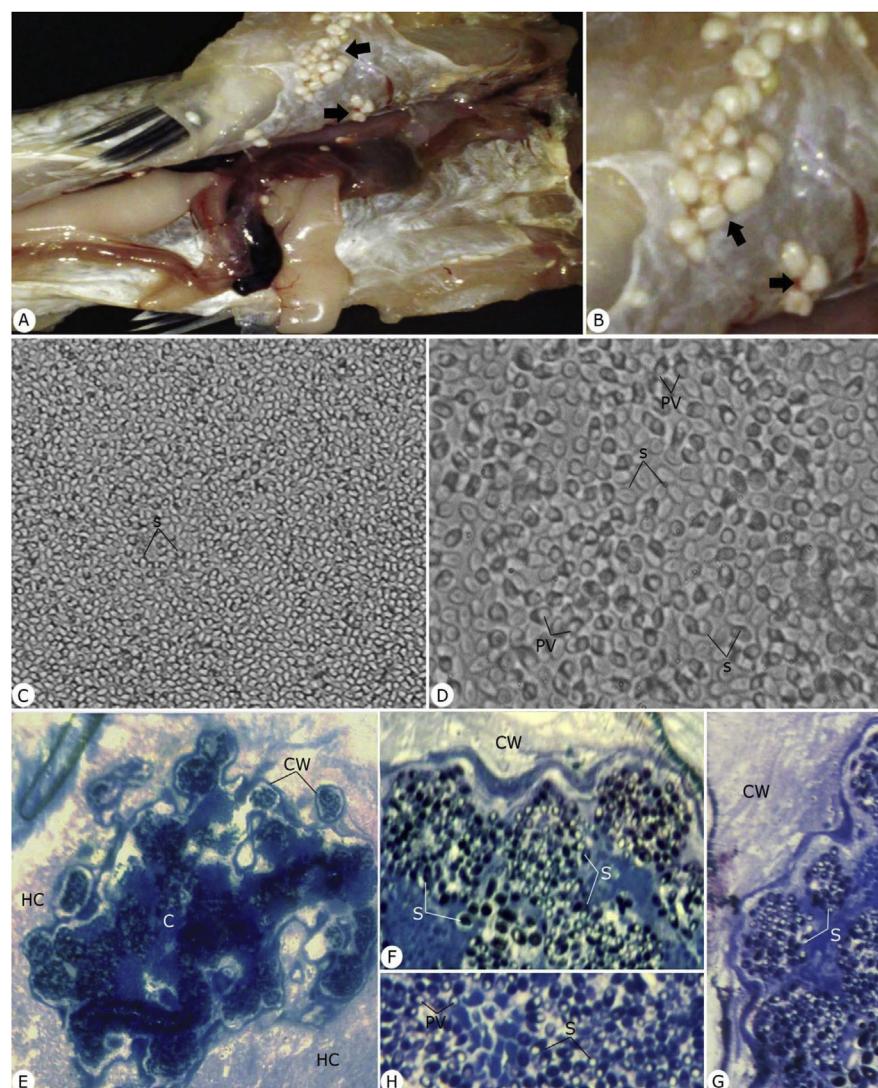


Fig. 1. (A,B) Photographs of the lizardfish *Saurida lessepsianus* infected with microsporidian parasite *Heterospisidium lessepsianum* sp. n. and the infection appeared in the form of whitish cysts (arrows) embedded in the peritoneum. (C,D) Photomicrographs of unfixed fresh spores (S) released after rupture of cysts. A posterior vacuole (PV) is observed situated away from the spore apex, $\times 400$. (E) Photomicrograph of semithin section stained with toluidine blue through parasite cyst (C) surrounded externally with thick cyst wall (CW) which is seen to encapsulate the parasite and spores (S) in various degrees of development. The cyst embedded within host cells (HC) surrounded by connective tissue, $\times 1.000$. (F–H) High magnifications of semi-thin section showing: (F,G) Periphery of the cyst showing cyst wall (CW) enclosing spores (S) in various degrees of development, $\times 1.200$. (H) Microsporidian spores (S) and each mature spore contains a posterior vacuole (PV), $\times 2.000$.

approach. Phylogenetic and evolutionary analyses conducted using MEGA version 6 [30]. Tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Also, The GC content was calculated using oligo calculator available at <http://www.sciencelauncher.com/oligocalc.html>.

3. Results

3.1. Gross and light microscopic examination

Twenty three out of 130 of the examined fish were found to be naturally infected with microsporidian parasites with a percentage of 17.69%. Infections were recorded as numerous macroscopic whitish cysts embedded in the abdominal cavity, skeletal muscle and mesenteric tissues of the infected fish (Fig. 1A and B). The observed cysts were up to 30 per infected fish which develop tumor-like masses reached 3.8–6.5 mm in diameter and inducing an enormous hypertrophy to the infected tissues. Concerning the seasonal prevalence of the microsporidian parasites, it was noted that they were generally higher during winter reaching 29.23% (19/65) and lower to 6.15% (4/65) in summer. In wet mounts, fresh spores appeared mostly ovoid to pyriform in shape and had a large vacuole at the posterior end (Fig. 1C and D). Two spore types (micro- & macrotype) were identified. The microspores reaching a size of 2.9–3.5 (3.1 ± 0.2) \times 1.7–2.8 (2.1 ± 0.1) μm , and

macrospores measured 4.2–5.3 (4.6 ± 0.2) \times 2.9–3.7 (3.4 ± 0.1) μm in size. Histological observations showed that parasitic foci were encapsulated by a host-derived fibrous membrane contained different developmental stages of the present microsporidian parasite (Fig. 1E–H).

3.2. Transmission electron microscopic examination

All developmental stages of the recorded microsporidian species were found within the cyst isolated from the infected fish (Fig. 2A). It was found that the smooth membranes of the sarcoplasmic reticulum forming a thick-walled coat (Fig. 2B). This amorphous coat surrounded the various developmental stages of the parasite (Fig. 2A and B). The first observed recognizable stage of parasite was the uninuclear meront with its spherical nucleus (Fig. 2C), which divided to produce binucleated meronts with two spherical nuclei (Fig. 2D). Additional multi-nucleated meronts (Fig. 2E) with three to six irregularly shaped nuclei arose by plasmotomy constantly producing new sporonts were also recorded (Fig. 2F). As development proceeded, advanced stages of sporoblasts contained already most of the typical structures of spores including the electron pale exospore (Fig. 2G). It became separated from the sporoblast to build a dense exospore around the mature spore (Fig. 2J). An evidence for spore morphogenesis is the presence of large membrane-bound vacuoles containing electron dense material named as the polar tube primordial or paramural bodies distributed in the

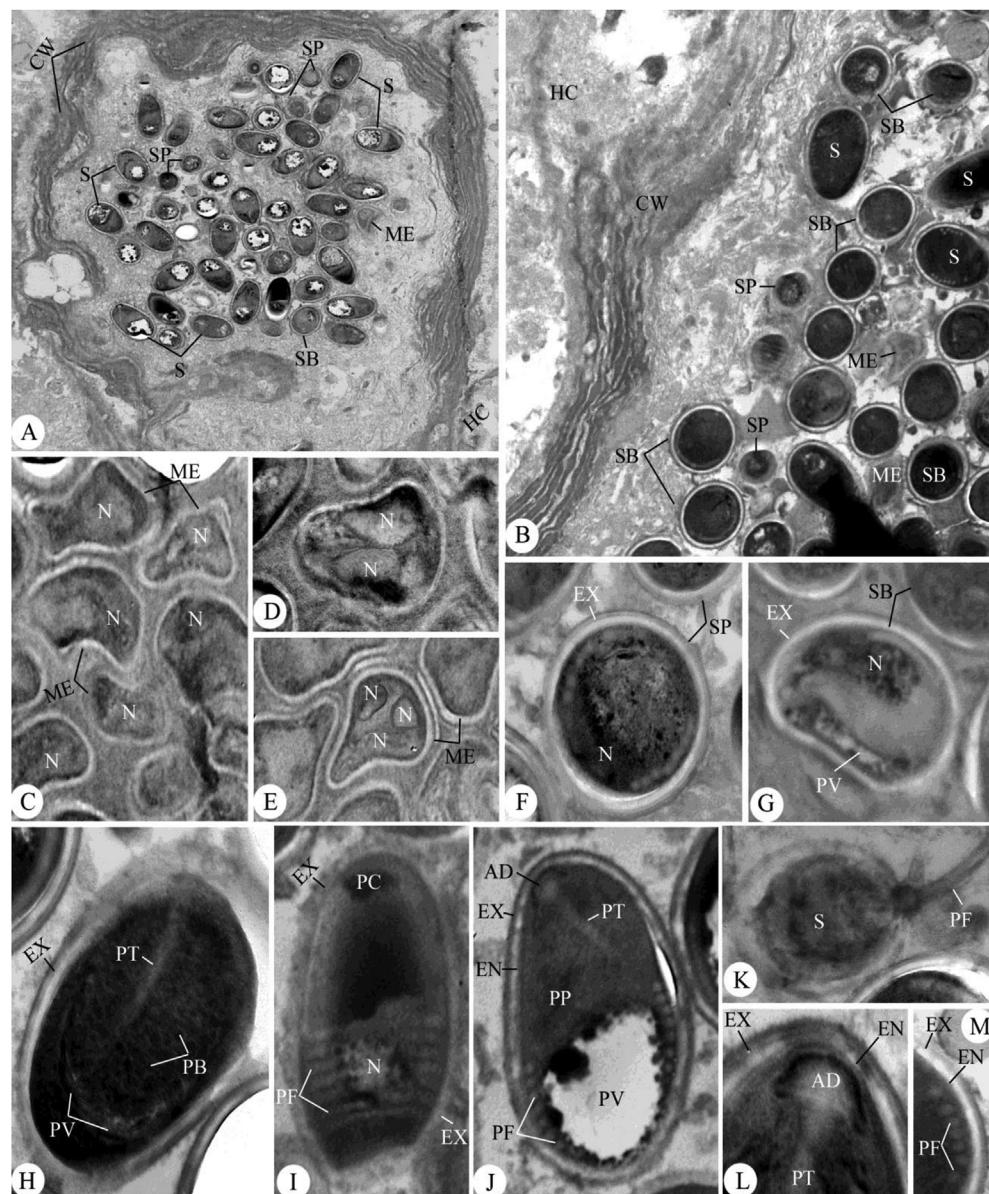


Fig. 2. (A–M) Transmission electron micrographs the cyst and different developmental stages of *H. lessepsianus* sp. n. inside the host cell, showing: (A) Cyst containing meronts (ME), sporonts (SP), sporoblasts (SB), spores (S), embedded in the host cells (HC) surrounded by the thick cyst wall (CW), $\times 3,400$. (B) Periphery of the cyst showing surrounding walls (CW) followed by various developmental stages as meronts (ME), sporonts (SP), sporoblasts (SB), spores (S), $\times 4,800$. (C) Uninucleated meronts (ME), $\times 6,100$. (D) Binucleated meronts (ME) with 3–4 nuclei (N), $\times 3,400$. (E) Multinucleated meronts (ME) with 3–4 nuclei (N), $\times 3,400$. (F) Sporont with nucleus (N) and surrounded by exospore (EX), $\times 6,000$. (G) Sporoblast surrounded by exospore (EX) and contained nucleus (N) and characteristic posterior vacuole (PV), $\times 6,000$. (H) Immature spore surrounded by the exospore (EX) showing paramural bodies (PB) that appeared in the matrix of the spore, posterior vacuole (PV) and the polar tube (PT), $\times 12,000$. (I) A nearly mature uninucleate (N) spore with the polaroplast (PP), polar cap (PC), and polar filament (PF), $\times 12,400$. (J) Mature uninucleate spore showing spore wall of two layers of exospore (EX) and endospore (EN), anchoring disc (AD), polar tube (PT), polar filament (PF) with 7–8 turns, and the posterior vacuole (PV). Note that some of the paramural bodies (PB) begin to move toward the membrane being ready to fuse with the membrane for the formation of the endospore (EN), $\times 14,000$. (K) Mature spore with extruded polar filament (PF) in the host cell, $\times 16,000$. (L) The anchoring disk (AD) at the spore apex showing the lucent layer of the shaft of the future polar tube (PT), $\times 18,000$. (M) Polar filament coils of a spore with a long polar filament (PF), $\times 18,000$.

spore cavity and represented as the precursors of the polar tube and endospore formation (Fig. 2H and I). The spores were lined by a thick and amorphous wall (Fig. 2J). The spore wall was thick except for the anterior end where the central zone of the anchoring disc contacted the wall (Fig. 2J), which was thick and consisted of an electron lucent endospore and a thin electron-dense exospore (Fig. 2J,L,M). The anchoring disc was located in the apical region of the spore in an eccentric position in relation to the spore axis, producing a bilateral asymmetry (Fig. 2J,L). The polaroplast consisted of an anterior region of closely packed membranes and a posterior region comprising a series of loosely packed membranes (Fig. 2J). The posterior vacuole situated at the basal part of the spore between the polar filament coils, was irregular and contained light material (Fig. 2J). The polar filament was isofilar, and it was coiled 7–8 times within the posterior vacuole and organized into a single row (Fig. 2J,M). Spores were able to eject spontaneously their filament (Fig. 2K). Table 1 showed a comparison between the present microsporidian parasite with other previously described species regarding to the habitat, shape and size of the mature spores and the number of polar filament coils.

3.3. Molecular analysis

In order to get a further, independent line of evidence for the identity and the systematic position of the isolated microsporidian parasite infecting the lizardfish *S. lessepsianus*, a phylogenetic analysis based on 16S rDNA gene was performed. The sequence of 615 bp length with a GC content of 56.26% for the partial sequence of 16S rDNA gene of the studied species was sequenced and deposited in the GenBank database under the accession number MF769371. Multiple sequence alignment calculated the percentages of identities between the present novel sequence and a range of other microsporidia predominantly from aquatic hosts which demonstrated a high degree of similarity ($> 82\%$), as shown in Table 2. Comparison of the nucleotide sequences and divergence showed that the 16S rDNA gene sequence of our microsporidian species yielded the highest BLAST scores with lowest divergence values for *Heterosporis saurida* (gb| JF745533), *Heterosporis anguillarum* (gb| AF387331), *Heterosporis sutherlandae* (gb| KC137553) (Tables 2 and 3). Based on 16S rDNA sequence data, the constructed dendrogram consisted of major lineage as shown in Fig. (3). This lineage was clustering fish-infecting microsporidia belonging to the *Heterosporis*, *Ovipleistophora*, *Pleistophora*, *Dasyatispora*, *Microsporidium*

Table 1
Comparative measurements (in μm) of *Heterosporis lessepsianus* sp. n. described in this study and other species described previously.

<i>Heterosporis</i> Species	Type host	Site of infection	Habitat/countries	Spore shape	Spore size (PF coils)	References
				Microspores	Macrospheres	
<i>H. anguillarum</i>	<i>Anguilla japonica</i> <i>Pterophyllum scalare</i>	Skeletal tissues Connective tissue, cells, myocytes	Euryhaline/Japan, Taiwan Freshwater/aquaria in Germany/ imported from South America	Elongated ovoid Ovoid, elongated, posterior flat	3.5 × 2.4 (–) 3.0 × 1.5 (8)	Hoshina [14] Schubert [11]
<i>H. cichlidarum</i>	<i>Hemichromis binotatus</i>	Gill filaments	Freshwater/aquaria in France/imported from West and Central Africa	Ovoid, slightly pyriform	–	Coste and Bouix [13]
<i>H. schuberti</i>	<i>Pseudocrenilabrus multicolor</i> , <i>Ancistrus cirrhosus</i>	Skeletal tissues	Freshwater/aquaria in Germany/ imported from East Africa and South America	Ovoid	3.4–4.9 × 2.4–3.4 (–)	Lom et al. [44]
<i>H. saurida</i>	<i>Saurida undosquamis</i>	Skeletal muscles, mesenteric tissues	Marine/Arabian Gulf, Saudi Arabia	Ovoid to pyriform	3.3 × 2.0 (5–6)	Al Quraishi et al. [16]
<i>H. anguillarum</i> <i>H. sutherlandae</i>	<i>Thamnophis sirtalis</i> <i>Perca fluviatilis</i> , <i>Esox lucius</i> , <i>Sander vitreus</i>	Muscle Skeletal muscles, mesenteric tissues	Euryhaline/Japan, Taiwan Euryhaline/Japan, Taiwan	Elongated ovoid Ovoid to pyriform	5.3 × 6.8–2.0–4.0 (29–42) –	Richter et al. [51] Phelps et al. [4]
<i>Heterosporis</i> sp. <i>H. lessepsianus</i>	<i>Hydrophis major</i> <i>Saurida lessepsianus</i>	Muscle Abdominal cavity, skeletal muscle and mesenteric tissues	Marine/Queensland, Australia Marine/Red Sea, Egypt	Round to ovoid Ovoid to pyriform	2.9–3.5 × 1.7–2.8 (7–8) –	Gillet et al. [17] Present study

genera. Phylogenetic analyses placed our novel sequence infecting *S. lessepsianus* within a clade containing other *Heterosporis* species with sequence similarity ranged between 98 and 93%. The most closely related sequence was provided by the GenBank entry JF745533 for *H. saurida* isolated from *S. undosquamis* with the highest percentage of identity (98%) and the lowest divergence value (0.9). In the constructed dendrogram, *Loma salmonae* (acc. no. HM626215) represented as out-group.

3.4. Taxonomic summary

Parasite name: *Heterosporis lessepsianus* sp. n. (Family Glugeidae [31]).

Type Host: Lizardfish *Saurida lessepsianus* (Family Synodontidae [32]).

Type Locality: Hurghada City at the Red Sea Governorate, Egypt.

Type material: Permanent slides as well as 80% ethanol preserved samples were deposited in parasitological collection of the Hungarian Natural History Museum, Hungary, Budapest [deposition no. HNHM-50219].

Site of infection: Abdominal cavity, skeletal muscle and mesenteric tissues of infected fish.

Prevalence of infection: 17.69% (23 out of 130) were found to be naturally infected.

Etymology: Specific epithet ‘lessepsianus’ derived from the species epithet of the host species *Saurida lessepsianus*.

4. Discussion

Fish play a vital role as a source of animal protein worldwide and increasing role in solving human nutritional problems [33]. Fortunately, there are different and extensive water sources that are expected to yield tremendous amounts of fish in Egypt [34]. There are many factors affecting these sources which represent an important warning signal for fish health and human consumption [35]. In the present study, it was observed that the lizardfish *S. lessepsianus* was naturally infected by a single microsporidian parasite with 17.69% as a percentage of parasitic infection. Regarding the infection rate fluctuation which reached 29.23% in winter and fall to 6.15% in summer is in general agreement with Abdel-Ghaffar et al. [34] reported that *Pleistophora aegyptiaca* appeared as macroscopic cysts embedded in the peritoneal cavity of *Saurida tumbil* with 15.5% as a prevalence rate of infection and reaching during winter to 24.4% and fall to 4% in summer; followed by Morsy et al. [36] stated that *Pleistophora pagri* were found in the epithelial lining of the peritoneum and also in the intestinal epithelium of the common sea bream *Pagrus pagrus* with rate of infection 30.6% inducing an enormous hypertrophy to the infected organs in addition the infection with this parasite is generally increased during winter to 20% and fall to 10% in summer. The possible explanation for these fluctuations could due to fish suffered from high degrees of parasitic infections during hibernation in winter when they are in a state of exhaustion as stated by Abdel-Ghaffar et al. [34]. The infection in this study was reported in the abdominal cavity, skeletal muscle and mesenteric tissues of infected lizardfish *S. lessepsianus*. There has not been a prior report of microsporidia infection in this fish species in the Red Sea, Egypt. Negm-Eldin [37] has been reported the infection by the microsporidian *Pleistophora oolyticus* in *S. tumbil* on the Egyptian coast of the Red Sea. Additionally, Peyghan et al. [38] described a microsporidian species obtained from the lizardfish *S. undosquamis* that was caught in the Persian Gulf; however, light and electron microscopy showed that the spores were similar to those of the genus *Glugea*. Abdel-Baki et al. [39] indicated the prevalence of *Microsporidium* sp. infected *S. undosquamis* from the Arabian Gulf, Saudi Arabia. Abdel-Ghaffar et al. [34] demonstrated that *Pleistophora aegyptiaca* infecting *S. tumbil* inhabited the Red Sea, Egypt. Al Quraishi et al. [16] reported a microsporidian parasite *Heterosporis saurida*

Table 2Microsporidia species used in the phylogenetic analysis of the present *Heterosporis lessepsianus* n. sp.

Parasite species	Order/Family	Host species (Country)	Source	Accession no.	Percent identity (%)	Divergence value
<i>Heterosporis saurida</i>	Glugeida/Pleistophoridae	<i>Saurida undosquamis</i> (Saudi Arabia)	GenBank	JF745533	98	0.9
<i>Heterosporis anguillarum</i>	Glugeida/Pleistophoridae	<i>Anguilla japonica</i> (Japan)	GenBank	AF387331	96	2.6
<i>Heterosporis sutherlandae</i>	Glugeida/Pleistophoridae	<i>Sander vitreus</i> (USA)	GenBank	KC137553	94	4.5
<i>Ovipleistophora mirandellae</i>	Glugeida/Pleistophoridae	<i>Rutilus rutilus</i> (Finland)	GenBank	AJ295327	91	7.8
<i>Ovipleistophora diplostomuri</i>	Glugeida/Pleistophoridae	<i>Lepomis macrochirus</i> (USA)	GenBank	KY809102	90	8.9
<i>Pleistophora</i> sp.	Glugeida/Pleistophoridae	<i>Puntius tetrazona</i> (China)	GenBank	HQ703580	89	9.1
<i>Pleistophora hypessobryconi</i>	Glugeida/Pleistophoridae	<i>Leiarius marmoratus</i> (Brazil)	GenBank	KM458722	89	9.1
<i>Dasyatispora levantinae</i>	Glugeida/Pleistophoridae	<i>Dasyatis pastinaca</i> (Turkey)	GenBank	GU183263	88	9.5
<i>Pleistophora</i> sp.2	Glugeida/Pleistophoridae	<i>Zeugopterus punctatus</i> (Norway)	GenBank	AF044389	88	9.5
<i>Pleistophora mulieri</i>	Glugeida/Pleistophoridae	<i>Gammarus duebeni</i> (United Kingdom)	GenBank	EF119339	87	9.8
<i>Pleistophora typicalis</i>	Glugeida/Pleistophoridae	<i>Myoxocephalus scorpius</i> (Norway)	GenBank	AF044387	87	9.8
<i>Pleistophora ehenbaumi</i>	Glugeida/Pleistophoridae	<i>Anarhichas lupus</i> (Norway)	GenBank	AF044392	86	10.1
<i>Pleistophora</i> sp.1	Glugeida/Pleistophoridae	<i>Glyptocephalus cynoglossus</i> (Norway)	GenBank	AF044394	86	10.1
<i>Microsporidium cypselurus</i>	Microsporea/–	<i>Cypselurus pinnatibarbus</i> (Japan)	GenBank	AJ300706	85	10.4
<i>Pleistophora finisterrensis</i>	Glugeida/Pleistophoridae	<i>Micromesistius poutassou</i> (Norway)	GenBank	AF044393	84	10.8
<i>Glugea</i> sp. GS1	Glugeida/Glugeidae	<i>Gasterosteus aculeatus</i> (United Kingdom)	GenBank	AJ295325	84	10.8
<i>Glugea plecoglossi</i>	Glugeida/Glugeidae	<i>Plecoglossus altivelis</i> (China)	GenBank	KX814862	84	10.8
<i>Glugea hertwigi</i>	Glugeida/Glugeidae	<i>Osmerus mordax</i> (Canada)	GenBank	GQ203287	84	10.8
<i>Pleistophora</i> sp.3	Glugeida/Pleistophoridae	<i>Taurulus bubalis</i> (Norway)	GenBank	AF044390	84	10.8
<i>Glugea anomala</i>	Glugeida/Glugeidae	<i>Gasterosteus aculeatus</i> (Norway)	GenBank	AF044391	82	12.1

infecting *S. undosquamis* from the Arabian Gulf, Saudi Arabia. Microsporidia are currently classified on the basis of their ultrastructural features, including size and morphology of the spores, number and arrangement of coils in polar tube of mature spores which vary depending on the genus and species of microsporidia, developmental life cycle and host-parasite relationship [40]. The light and ultrastructural examinations of the single nuclei, sporophorocysts, sporophorous vesicles, asynchronism of the development cycle and spore morphologies of the parasite described here all revealed structures typical of parasites belonging to the phylum Microsporidia and the genus *Heterosporis*. In the present study, microsporidian parasite was recognized by the double sporogonic sequence leading to two types of spores (micro- and macrospores) which was the characteristic feature for the genus *Heterosporis*, these results are coincided with data obtained by Mladineo and Lovy [41] reported that the presence of both types of spores in the cyst of tuna microsporidium has been observed only in one representative of pyriform microsporidia group (e.g. *Heterosporis*), although present within phylogenetically distant genera like *Pleistophora*, *Ovipleistophora* [42] and *Spraguea* [43]. Concerning the main points of comparison as habitat, shape and size of the mature spores and the number of polar filament coils, the present microsporidian did not seem similar with other previously described species. It slightly similar to *H. saurida* Al Quraishi et al. [16] infecting *S. undosquamis* with the same host genus but differs from it in slightly smaller spore size and large number of filament coils. In addition, it differs from *Heterosporis finki* Schubert [11] infecting *Pterophyllum scalare* from South American rivers, *Heterosporis schuberti* Lom et al. [44] parasitized *Ancistrus cirrhosus* inhabited African and South American rivers, *Heterosporis cichlidarum* Coste and Bouix [13] from *Hemichromis bimaculatus* from west and central Africa, *H. anguillarum* Lom et al. [45] parasite of a euryhaline fish *Anguilla japonica* from Japan; in spore shape, larger spore size, contained more filament coils for both types of spores, and more numerous inside sporophorous vesicles; in addition to the site of infection of the skeletal muscles for them except for *Heterosporis cichlidarum* infecting the gill filaments of its host. 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 [10,12,39]. However, differences in the development, morphology, and ultrastructure can suggest possible taxonomic lines [46].

Recently, phylogenetic reconstruction based on comparison of SSU rDNA sequences has successfully been used to detect and classify various organisms [47]. As a result, a number of studies on microsporidia

are now including both ultrastructural and rDNA sequence characters [48]. Data obtained from the phylogenetic analysis of 16S rDNA sequence have revealed that some of the developmental features and ultrastructural characteristics used to designate microsporidian taxa were 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 [23,49]. In the present study, assignment of the present parasite to the genus *Heterosporis* is also strongly supported by the molecular data. The general structure of the phylogram obtained in this study is consistent with the previous data obtained by Diamant et al. [7], and Vossbrinck and Debrunner-Vossbrinck [22] whom reported that clade III within the Class Marinospordia included all *Heterosporis*, *Ovipleistophora*, *Dasyatispora*, and *Pleistophora* sequences and supported by higher bootstrap values. However, the addition of the new sequences from this study identified the ancestral marine origin of this species, and it strongly aided the understanding of the cladistics 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 showed 98% similarity to *H. saurida* isolated from *S. undosquamis*, with the lowest divergence value (0.9). Previous molecular phylogenetic studies by other authors as Diamant et al. [7], and Phelps et al. [4] whom demonstrated a high degree of sequence similarity between a subset of *Heterosporis* species, and these are here designated into the *Heterosporis* forming clade in the phylogenetic tree that distinct from other clades. Also, it was observed that all *Heterosporis* showed at least 94% similarity to our sequence, whilst parasites from other clades showed only 82–91% similarity as observed by Al Quraishi et al. [16]; our results clearly demonstrated the importance of using classical ultrastructural information in combination with molecular data when describing novel parasites [40]. In addition, the phylogenetic tree from MP showed that the sequences of *Heterosporis* species and *Dasyatispora levantinae* (GU183263) formed a cluster distant from the *Ovipleistophora* species and *Pleistophora* species. The genetic distances also indicated that there were similarities in the rDNA sequences among the species belonging to the *Heterosporis* and *Dasyatispora* genera, having percentage identities with *H. saurida*, *H. anguillarum*, *H. sutherlandae* and *D. levantinae* of 98%, 96%, 94%, and 88% respectively. These data are consistent with data obtained by Al Quraishi et al. [16] who indicated that *H. saurida* and *D. levantinae* are more closely related species; however, they are different ultrastructurally because the *Dasyatispora* sporophorocyst is absent, and the spore is monomorphic. Recognition of the monophyletic nature of the

Table 3

Sequence alignment of 16S rDNA gene of *Heterosporis lessepsianus* n. sp. with the most closely related species of the same genus. (Only variable sites are shown. Dots represent bases identical to those of the first sequences, and dashes indicate gaps).

genus *Heterosporis* has resulted in reclassification of many species into other genera [50].

5. Conclusion

No microsporidia have been observed or described with comparable spore morphology and picture of infection from freshwater and marine fish living in the same geographic area in addition to the presence of unique genetic sequence for this microsporidian parasite. Considering these data and the host specificity, we believe that this micro-organism represented a new species that should be included in genus *Heterosporis* and propose the name *Heterosporis lessepsianus* for it.

6. Compliance with ethical standards

All procedures involved in this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals and have been approved and authorized by the Institutional Animal Care and Use Committee (IACUC) in the Faculty of Science.

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Conflict of interest

The authors have indicated that they have no conflict of interest regarding the content of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.micpath.2019.02.025>.

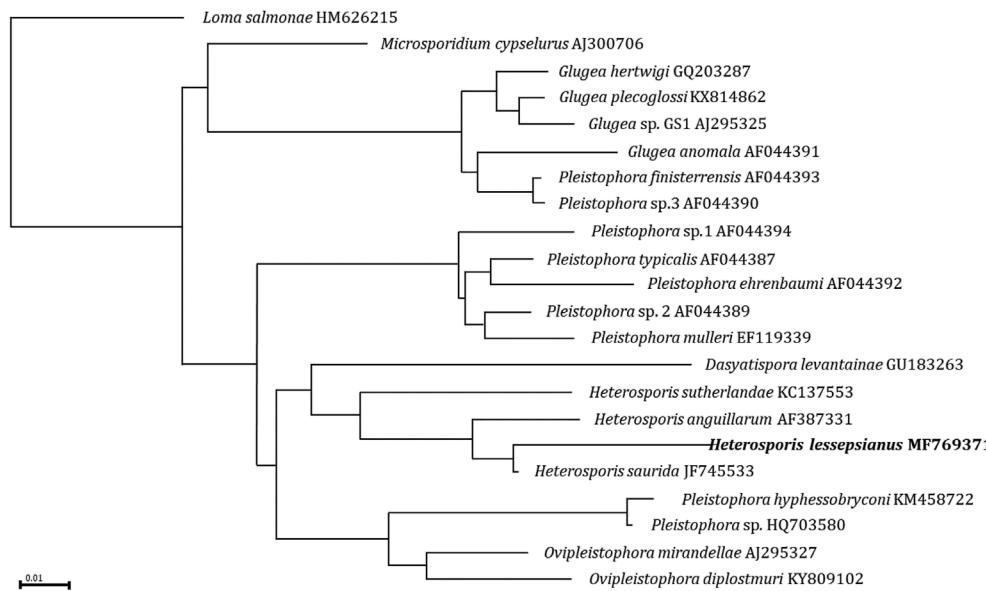


Fig. 3. Phylogenetic tree generated by maximum parsimony analyses of the partial SSU rDNA sequence of *Heterosporis lessepsianus* sp. n. and the microsporidian species with the highest BLAST matches. GenBank accession numbers are given after the species names, and a scale bar is provided under the tree. Microsporidian parasites examined in the present study are in bold.

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