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Ultrastructural description and phylogeny of a novel microsporidian, *Glugea eda* n. sp. from the striated fusilier, *Caesio striata*, in the Red Sea off Saudi Arabia

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ABSTRACT

Glugea eda n. sp. is described from the mesenteries of the striated fusilier, *Caesio striata*, collected from the Red Sea coast off Yanbu' al Bahr, Saudi Arabia. Numerous blackish xenomas, ranged from 3 to 5 mm, were found in the body cavity associated with the mesenteries. Mature spores are monomorphic, ellipsoidal with an average size of 5(4–6) μm in length and 2.2 (2–3) μm in width. Observations of the ultrastructure revealed that the development was asynchronous and that the nuclei were isolated throughout the life cycle with uninucleate meronts. Sporoblasts were uninucleated and existed together with sporonts in a fully formed parasitophorous vacuole. The polar filament of the mature spore was isofilar with 24–28 coils, arranged in three rows. Phylogenetic analysis placed the current microsporidia within the clade grouping *Glugea* species and close to the species described from the Red Sea and Arabian Gulf. The morphometric and molecular comparison with other members of the genus *Glugea* evidenced the taxonomic novelty of the present form, suggesting that it should be considered as a new species. To the best of our knowledge, the parasite here described represents the first occurrence of microsporidian infection in the fish of the family Caesionidae.

1. Introduction

Microsporidia are obligatory intracellular, spore-forming parasites that infect a wide range of hosts, including humans and economically important fish and insects (Dean et al., 2016). The members of phylum Microsporidia are allocated to about 187 genera (Vávra and Lukeš, 2013), 21 of whom, containing more than 160 species, have been identified in marine and freshwater fish worldwide (Vavrá and Lukeš, 2013; Su et al., 2014; Phelps et al., 2015; Casal et al., 2016). The genus *Glugea* Thélohan, 1891 contains more than 34 species, mainly infecting various fish organs (Abdel-Baki et al., 2015a,b; Azevedo et al., 2016; Casal et al., 2016), and sometimes reported as causing significant mortality in both wild and farmed economically important fish, e.g. smelts and flatfish reviewed in Kent et al. (2014) and Ryan and Kohler (2016).

For a long time, the identification of microsporidian species was to a great extent dependant on ultrastructural aspects of the mature spores and/or their developmental stages (Lom and Dyková, 1992; Abdel-Baki et al., 2015a, b). Increasingly, however, molecular SSU rRNA analysis has come to be seen as a valid technique for providing significant data about phylogenetic relationships and, in the past decade, a number of studies have been conducted that combine both morphological and comparative rDNA gene sequences for the precise characterization of novel microsporidian species (Lovy et al., 2009; Morsy et al., 2012; Casal et al., 2016).

Fish of the family Caesionidae mainly inhabit coral reefs in tropical and sub-tropical areas. They are fast swimmers, streamlined, commonly school together near coral reef, and feed by picking zooplankton from near the surface to depths of 60 m (Carpenter, 1987; Holleman et al., 2013). The striated fusilier, *Caesio striata* Rüppell, 1830 is member of the family Caesionidae and has a fusiform body, which is green to blue green in colour with four blackish stripes. Geographically it is restricted

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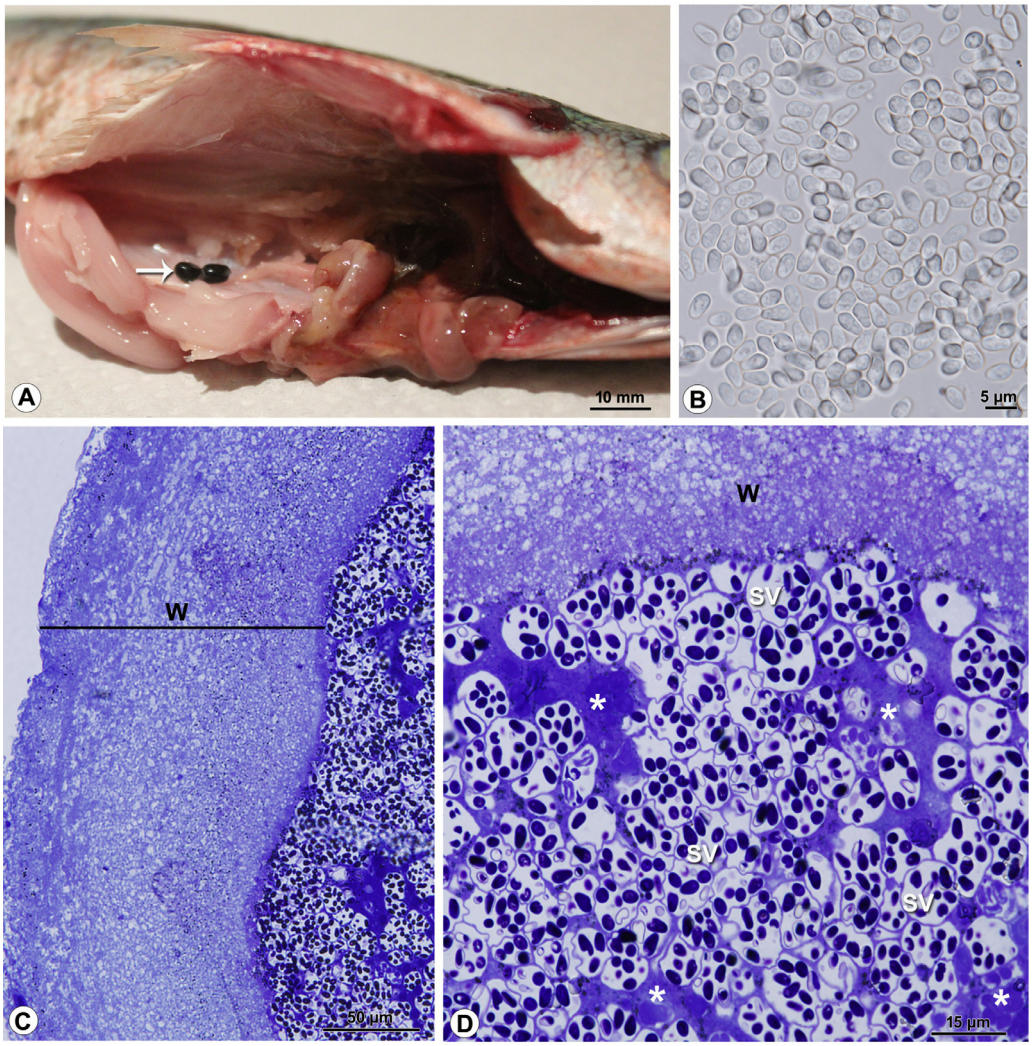

Fig. 1. *Glugea eda* n. sp. infecting *Caesio striata*. A. Xenomas (arrow) in the mesenteries of infected *Caesio striata*. B. Fresh spores of *Glugea eda* n. sp.. C. Semithin section of part of a xenoma showing the xenoma wall (W). D. Enlarged part of the xenoma near the wall (W) showing some sporophorous vesicles containing mature spores (SV) and some developmental stages (*).

to the Red Sea (Randal, 1983; Holleman et al., 2013). Thus far, no microsporidian species have been described in fish from the family Caesionidae.

The following represents a description of a new species of *Glugea* that was identified from the mesenteries of the striated fusilier, *Caesio striata*, collected from Red Sea off Saudi Arabia. We combined the morphological characteristics and SSU rRNA gene sequences to define the taxonomic placement and phylogenetic relationships of this new species.

2. Materials and methods

Altogether, 20 *Caesio striata* fish, 19 to 25 cm in length, were collect at the vessel arrival site off the shoreline of Yanbu' al Bahr (24° 5′ 0″ N, 38° 0′ 0″ E) on Saudi Arabia's Red Sea coast. The fish were dissected, and all organs were investigated to determine the presence of parasitic infection. Xenomas and fresh spores were examined using an Olympus BX51 microscope and photographed with a DP71 digital camera (Olympus, Japan). Fresh spores (n = 30) were measured and described according to the criteria established by Lom and Nilsen (2003).

To study the ultrastructure, fragments of isolated xenomas were fixed in 3% glutaraldehyde buffered in 0.1 M phosphate solution at 4 C for 24 h and then rinsed in the same buffer. The post-fixed specimens were processed in 2% OsO4 with the same buffer. After dehydration, the specimens were embedded in pure resin. Ultrathin sections were acquired with a Leica ultracut UC7 microtome and stained with uranyl acetate and lead citrate, before being photographed using a JEOL-JSM-1011 electron microscope at 80 kV (Abdel-Baki et al., 2015a).

2.1. DNA extraction and sequencing

Genomic DNA was extracted from ethanol preserved xenomas using the Qiagen DNeasy Kit (Qiagen, Germany), according to the protocol recommended by the manufacturer for use with animal tissue. The sequences of the small subunit (SSU) rDNA and ITS region were amplified using the universal primer pair, V1f 5`-CACCAGGTTGATTCTGCCT GAC-3` (Nilsen, 2000) and the HG5F_rev (5′-TCACCCCACTTGTCG TTA-3′) (Abdel-Baki et al., 2015a) in a 30 µl reaction mixture that comprised of 1 x × Taq DNA polymerase buffer (MBI, Fermentas), 0.2 mmol of mixed dNTP, 1.5 mmol of $MgCl_2$, 0.2 pmol of each primer, 1 U of Taq DNA polymerase and 50–100 ng of DNA, and ultra-pure water. Amplification conditions were an initial denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 90 s, with a terminal extension at 72 °C for 5 min. Positive and negative controls were included in all the PCR amplifications. Finally,

the PCR products were electrophoresed in 1% agarose gel in a Tris-borate-EDTA buffer (0.045 M Tris-borate, 0.001 M EDTA pH 8.0), stained with 1% ethidium bromide, and visualized on an UV transilluminator with the aid of a gel documentation system (BioRad Gel225 Doc™XR+). The PCR products were sequenced by Macrogen Inc. (Seoul, South Korea) using the same primers employed for PCR amplification.

2.2. Phylogenetic analysis

The newly prepared SSU rDNA sequence was aligned with sequences of closely related microsporidians obtained from a BLAST query of the GenBank database (Altschul et al., 1997) using the ClustalX 2.1.0.12, applying the default parameters (Larkin et al., 2007). amongst the twenty-seven selected sequences for phylogenetic analysis, five belong to the genus *Glugea* Thélohan, 1891, eight to the family of pleistophoridae Doflein, 1901 and six to the family of Sprageueidae Weissenberg, 1976. *Brachiola algerae* (Vavra et Undeen, 1970) Lowman, Takvorian et Cali, 2000 was used as outgroup. A phylogenetic tree based on the resulting alignment was constructed using maximum likelihood and neighbour joining analyses that were run on MEGA 7 software (Tamura et al., 2011). The parameters for the maximum likelihood analyses were General Time Reversible model, 1000 bootstrap replications, Gamma distributed with Invariant sites ($G + I$), number of discrete gamma rates of 5 and complete deletion. The rate variation amongst sites was modelled with a gamma distribution (shape parameter = 5). The genetic distance matrix was calculated with aid of the Kimura 2-parameter model distance for transition and transversion (Kimura, 1990) for a total of 1096 positions in the final dataset. All positions containing gaps and missing data were eliminated.

3. Results

Of the twenty fish examined, two (10%) were found to be infected with microsporidian parasites. Infection was encountered as deep brownish and predominately uniformly sized xenomas, ranging from 3 to 5 mm attached to the mesentery (Fig. 1A). When the a xenoma was crushed, huge numbers of monomorphic ellipsoidal mature spores were released. Spores were 5 ± 0.2 (4–6) μm long and 2.2 ± 0.3 (2–3) μm wide (Fig. 1B) with features typical of microsporidia which including: an extrusion apparatus consisting of a polar tube that is attached to the anchoring disc, and depending on the species, forms from 4 to approximately 30 coils.

Semithin sections of mature xenomas showed a wall of amorphous material ~ 150 μm thick (Fig. 1C) encapsulating numerous sporophorous vesicles containing the spores of different maturity and sporoblasts (Fig. 1D). The thick xenoma wall contains tubule -like structures and several lamellar formations (Fig. 1C). In respect to these developmental stages, the nuclei appeared isolated throughout the life cycle. Merogonial stages were not encountered. The most recognizable stage was the multinuclear sporogonial plasmodium which separate into fragments containing one or several nuclei. After plasmotomy, uninucleated sporoblasts and sporonts both with irregular shapes were produced within the fully-formed sporophorous vesicles (Fig. 2C). It was observed that the episporontal cavity containing a large number of tubular inclusions which involving in the formation of exospores of the future spore wall (Fig. 2B).

The mature spores, meanwhile, were monomorphic and almost elliptical, with a thick wall composed of jagged electron-dense exospore and an electron-lucent endospore (Figs. 2D, 2E). The exospore measured ~ 60 nm wide while endospore was ~ 130 nm wide. The anchoring disc was situated at the anterior pole and the polar filament expanded from this (Fig. 2F). The well-developed bipartite polaroplast was composed of two distinct lamellar and vesicular parts (Fig. 2F). In the anterior part, the thinnest lamellae were tightly packed. The posterior polaroplast was composed of vesicles exhibited a tubular structure (Fig. 2F). The polar filament was isofilar and formed 24–28 coils coordinated in three layers at the posterior part of the spores (Figs. 2D, 2E, 2G). The nucleus, with few chromatins, was located between the polaroplast and the posterior vacuole (Fig. 2E).

3.1. Taxonomic summary

Fish host: English name: Striated fusilier, Latin name: *Caesio striata* Rüppell, 1830

Fish Family: Caesionidae

Locality: Off Yanbu' al Bahr (24° 5′ 0″ N, 38° 0′ 0″ E), Red Sea coast of Saudi Arabia.

Site of infection: Mesenteries

Prevalence of infection: 2/20 (10%)

Type-material: One microscope slide with semithin sections of mature xenomas was deposited in the parasitological collection of the Zoology Department Museum, College of Science, King Saud University, Saudi Arabia, with number (G/01/2019). In addition, the SSU rRNA gene and ITS region sequences were deposited in GenBank with accession number (MK568064).

Etymology: The specific epithet is given after the common name of the fish host.

3.2. Molecular and phylogenetic analysis

A sequence of 1564 bp of the SSU rRNA gene and ITS region of *Glugea eda* n. sp. was obtained and deposited in the GenBank database under the accession number (MK568064). The GC content was 52.8%. Pairwise analysis showed that the sequence of *G. eda* n. sp. exhibited the highest similarity score with five previously-described *Glugea* species. This similarity varied between 99.47% in the case of *G. serranus* and 99.32% in the case of *G. nagelia*. The similarity with other *Glugea* species was lower, however, varying between 92.9% and 90.6%. Phylogenetic trees based on the SSU rDNA gene and generated with maximum likelihood and neighbour joining methods showed similar topology and indicated that the new species clusters closely with previously identified *Glugea* species with high bootstrap support (Fig. 3). The genus *Glugea* forms two sister subclades, G1 and G2. The new species appears close to the subclade G2, formed by *G. jazanensis, G. epinephelusis, G. nagelia, G. serranus* and *G. arabica*. Subclade G1, meanwhile, contains other species including the type species *G. anomala, G. sardinellensis, G. pagri, G. atherinae, G. plecoglossi* and *G. hertwigi*.

4. Discussion

The morphological and ultrastructural aspects of the present microsporidian in terms of the formation of large xenomas, with developmental stages at the periphery and mature spores within sporophorous vesicles in the centre, basically coincide with those for the genus Glugea (Larsson, 1999; Lom and Nilsen, 2003; Casal et al., 2016). In addition, the molecular analysis of the rRNA genes, including the ITS region, supports the contention that the microsporidian described here belongs to the genus Glugea. According to the available literature, and although xenomic microsporidia lack clear host specificity (Lom and Dyková, 2005; Casal et al., 2016), *Glugea eda* sp. n. represents the first occurrence of a microsporidian species infecting a fish of the family Caesionidae. So far, 34 *Glugea* species have been described from both marine and freshwater fish in different geographic locations, with xenoma formation in the peritoneal cavity, musculature and throughout the visceral organs (Lom, 2002; Vagelli et al., 2005; Wu et al., 2005; Voronin and Iukhimenko, 2010; Su et al., 2014; Abdel-Baki et al., 2015a, b; Azevedo et al., 2016, Casal et al., 2016). If these species are compared with *G. eda* sp. n., however, it is evident that differ quite clearly in several aspects. For example, *G. fennica* and *G. pimephales* differ in having spores that measure more than 7 μm in length (see

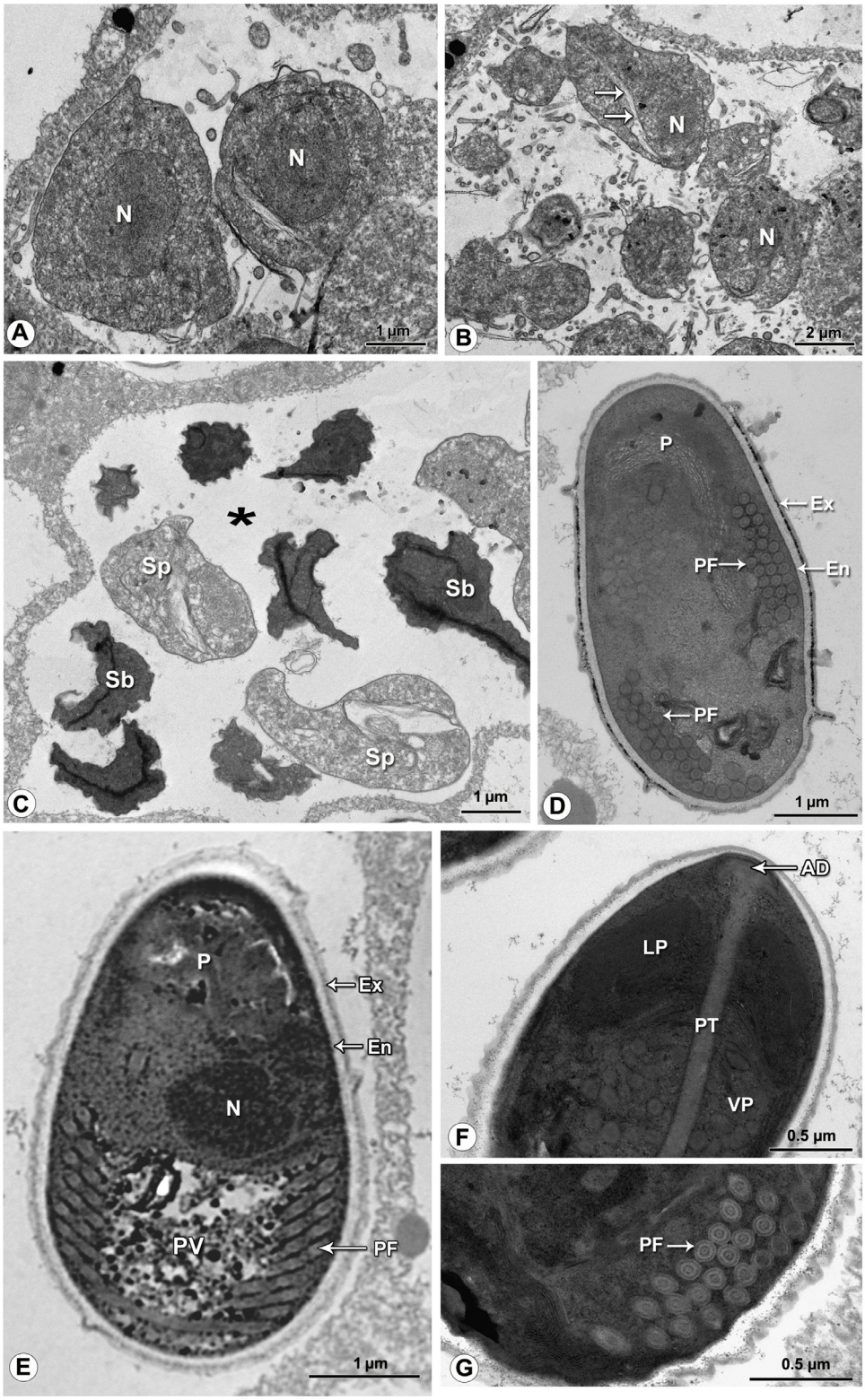

Fig. 2. Transmission electron micrographs of *Glugea eda* n. sp. infecting *Caesio striata*. A-B. Uninucleated meronts in division (Me) by plasmotomy (arrows) or by multiple fission (arrowheads) showing the nucleus (N). C. Parasitophorous vesicles (*) containing some early sporoblasts (Sb) and sporonts (Sp). D-E. Longitudinal sections of mature spores showing the exospore (Ex), endospore (En), polar filament (PF), polaroplast (P), nucleus (N), and posterior vacuole (PV). F. Detail of the anterior part of the spore showing the anchoring disc (AD), lamellar polaroplast (LP), vesicular polaroplast (VP) and the polar tube (PT). G. Detail of the spore posterior part showing the arrangement of polar filament (PF) in three layers.

Lom and Dyková, 1992). *Glugea vincentiae* and *G. pagri* also differ since they have two types of spore (see Vagelli et al., 2005; Su et al., 2014). In terms of polar filament coils, *G. anomala, G. stephani G. hertwigi* and *G. plecoglossi* (see Lom et al., 1995; Takvorian and Cali, 1996; Lovy et al., 2009; Zhou et al., 2017, respectively) showed a rather lower number of polar filament coils than the present species (11–16 vs 24–28). *Glugea atherinae* differs in having two types of xenoma and oval spores, a posterior vacuole that occupies half of the spore and a lower number of polar filament turns (9–10 vs 24–28) (see Berrebi, 1979). In the same way, the lack of parasitophorous vesicles, and pyriform spores with s lower number of filament coils (13–14 vs 24–28) clearly differentiate *G. sardinellensis* from the new species described here (see Mansour et al., 2016).

Molecular data based on the SSU rRNA sequence with ITS showed a high similarity score with *Glugea* species, mainly those infecting fish from the Red Sea and Arabian Gulf, like *G. jazanensis, G. nagelia* and *G.*

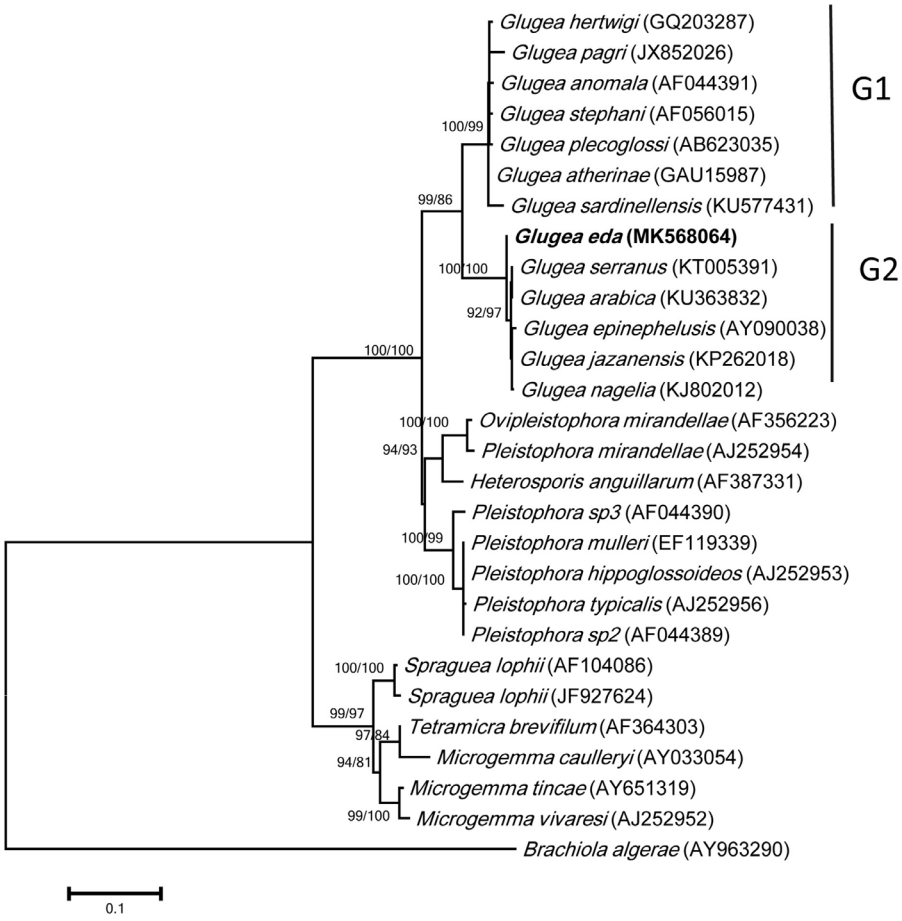

Fig. 3. Maximum likelihood tree with 27 microsporidia 16S rDNA sequences showing the position of*Glugea eda* n. sp. amongst *Glugea* spp. Bootstrap support for maximum likelihood/neighbour joining after 1000 replicates are presented on the branches. GenBank accession numbers are shown in parenthesis after the species name. *Brachiola algerae* is used as outgroup. G1 and G2 represent two sister related groups of the genus *Glugea*.

arabica. Two other species, G. *epinephelusis*, identified in *Epinephelus akaara* in China, and G. *serranus*, infecting *Serranus atricauda*, in Portugal are also genetically close to *G. eda* sp. n. (see Abdel-Baki et al., 2015a; b, Azevedo et al., 2016, Wu et al., 2005, Casal et al., 2016 respectively). All these species cluster phylogenetically in the same subclade, G2, defined by Mansour et al. (2016), which is close to a second subclade that comprises the other *Glugea* species, mainly the Mediterranean ones, including the type species *G. anomala*. In addition to sequence data, morphological comparison with the genetically more closely-related species in subclade G2 revealed significant morphological differences in terms of xenomas, spore size and polar filament turns. In this regard, *G. epinephelusis* differs in having larger spores (5.5 × 3.1 vs 5 × 2.2) with fewer polar filament turns (18–19 vs 24–28). Also, *Glugea jazanensis* has relatively smaller spores and multinucleated cylindrical meronts compared to the uninucleated, irregular to nearly round, ones in *G. eda* . sp. n.. Although *G. nagelia* and *G. eda* n. sp. have similar host habitat and spore measurements, they differ in terms of site of infection (intestinal wall vs mesentery), spore shape (pyriform vs elliptical) and the number of the filament coils (26 – 29 vs 24 – 28). *G. arabica*, meanwhile, differs in having larger spores (6.3 × 3.3 vs 5.0 × 2.2), a different number of filament coils (27 – 29 vs 24 – 28) that are arranged in four rows, compared to the three in our species, and also a different site of infection (intestinal wall vs mesentery). Finally, *G. serranus* differs in having a larger xenoma, up to 10 mm in length, and fewer polar filament coils (18–19 vs 24–28). Despite the high level of nucleotide similarity between species of the same subclade, it is evident that there are in fact significant morphological differences between these species that could be considered sufficient to distinguish them. This suggests the importance of combining molecular and morphological data when characterizing new microsporidian species. *Caesio striata* is apparently endemic in the Red Sea (Randall, 1983; Holleman et al., 2013) and no microsporidian infection is reported in the literature in this fish species. Accordingly, it appears that the present species does not conform with the morphological and genetic characters of any of the previously described species. Thus, it is suggested that the present species must be considered distinct and designated as a new species. The name *Glugea eda* sp. n. is proposed after the common local name of the fish host (Eda).

CRediT authorship contribution statement

Lamjed Mansour: Conceptualization, Methodology, Software, Writing - original draft. **J.Y. Zhang:** Conceptualization, Investigation, Writing - review & editing. **Heba M. Abdel-Haleem:** Conceptualization, Methodology, Writing - original draft. **Ahmed B. Darwish:** Conceptualization, Methodology, Writing - original draft. **Saleh Al-Quraishy:** Funding acquisition, Project administration, Writing - review & editing. **Abdel-Azeem S. Abdel-Baki:** Conceptualization, Visualization, Investigation, Writing - review & editing.

Declaration of Competing Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2020.105331.

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