

Glugea jazanensis sp. nov. infecting *Lutjanus bohar* in the Red Sea: ultrastructure and phylogeny

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ABSTRACT: During a survey of the microsporean fauna of the two-spot red snapper *Lutjanus bohar* Forsskål, 1775, from the Red Sea off Jizan (Saudi Arabia), a species of *Glugea* Thélohan, 1891 was found that did not conform to any known species. The species is characterized by the presence of spherical xenomas (ca. 2–5 mm in diameter) in the host body cavity. Examination of the lifecycle stages and mature spores using light and transmission electron microscopy also revealed morphological characteristics typical of species of the genus *Glugea*. Spores were elongated-ovoid with a posterior vacuole surrounded by the polar filament coils. Mature spores were 4.5 (4.0–4.8) µm long and 2.5 (2.0–2.5) µm wide. The polar filament was isofilar with 28 to 30 coils, although in most cases 29 coils, organized in 3 rows. Phylogenetic study based on the partial sequence of the small subunit (SSU) rRNA gene clustered the new microsporidia within the clade grouping species of the genus *Glugea*. The comprehensive analysis of the parasite's ultrastructural characteristics, together with molecular data for the SSU rDNA gene, suggests that this parasite is a new species of the genus *Glugea*, for which the name *Glugea jazanensis* sp. nov. is proposed.

KEY WORDS: Parasite · Microsporea · *Glugea* · Ultrastructure · SSU rDNA gene · Microsporidia · Glugeidae · Red snapper

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INTRODUCTION

Microsporidia are obligate intracellular parasites comprising some 1500 species which parasitize a wide variety of invertebrate and vertebrate hosts (Vávra & Lukeš 2013). These organisms have long been known to be causative agents of economically important diseases in insects (silk worms and honey bees), fish and mammals, and they also emerged as important opportunistic pathogens when AIDS became pandemic (Mathis et al. 2005). Classification of microsporidia is primarily based on structural characters observed under light and electron microscopy (Issi 1986, Sprague et al. 1992); however, small sub-

unit ribosomal DNA (SSU rDNA) gene sequences are now often used as a supporting, and sometimes even the principal, tool in defining taxa (Vávra & Lukeš 2013).

The fish family Lutjanidae contains 17 genera and 109 species, which are mainly confined to tropical and subtropical seas (Kumar et al. 2014). *Lutjanus* Bloch, 1790 is thus far the largest genus with 70 species, including at least 43 species from the Indo-West Pacific region (Allen et al. 2013). Of these species, the two-spot red snapper or red bass *L. bohar* Forsskål, 1775, is a large tropical reef fish that has a widespread distribution throughout the Indo-West Pacific, occurring from the Marquesas and Line Islands to

East Africa, and from Australia northward to the Ryuku Islands (Marriott & Mapstone 2006). This species is commercially important and is also prized as a game fish (Allen 1985). Despite the diversity of the family Lutjanidae, only 1 species of microsporidians has been described from this family. This species is *Microfilum lutjani*, which was described from *L. fulgens* by Faye et al. (1991). In the present study, we used morphological, ultrastructural and molecular approaches to describe *Glugea jazanensis* sp. nov., which was found to infect the abdominal cavity of *L. bohar* from the Red Sea off the coast of Saudi Arabia at Jizan.

MATERIALS AND METHODS

Light and transmission electron microscopy

From April 2013 to April 2014, 360 specimens of *Lutjanus bohar* (Teleostei: Lutjanidae) known by the Saudi common name 'Bohar', were collected from the Red Sea (16° 53' 21" N, 42° 32' 3" E), Jizan City, Saudi Arabia. The fish were necropsied and all organs were examined for microsporean infection. Fresh spores were measured and photographed using an Olympus BX51 microscope with an Olympus DP71 camera. Measurements are based on 30 fresh spores, and data are presented as mean \pm SD (range). For ultrastructural studies, xenomas and a small part of the surrounding tissues were excised and fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 24 h at 4°C, washed in the same buffer overnight at 4°C and post-fixed in 2% OsO₄, buffered with the same solution for 4 h at the same temperature. After dehydration in an ascending ethanol series followed by 2 changes of propylene oxide (4 h in each), the samples were embedded in Epon. Semi-thin sections were cut with a Leica ultracut UC7 and stained with toluidine blue for examination by light microscopy. Meanwhile, ultra-thin sections were contrasted with uranyl acetate and lead citrate to be examined with a JEOL-JSM-1011 transmission electron microscope at 80 kV.

Molecular studies

DNA extraction was carried out from individual xenomas using the Qiagen DNeasy kit. A partial sequence of the SSU rRNA gene was amplified by PCR using the universal primers V1f 5'-CAC CAG GTT GAT TCT GCC TGA C-3' and 1942R 5'-GGT

TAC CTT GTT ACG ACT T-3'. Amplifications were performed in a final volume of 30 μ l of PCR mixture containing 1 \times *Taq* DNA polymerase buffer (MBI, Fermentas), 0.2 mmol of mixed dNTP, 1.5 mmol of MgCl₂, 0.2 pmol of each primer, 1 U of *Taq* DNA polymerase, 50–100 ng of DNA and ultra-pure water. The amplification was done in a thermocycler apparatus (Techne TC-Plus Satellites). The PCR program used was composed of an initial denaturation stage 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 60 s, and a final extension stage at 72°C for 5 min. Positive and negative controls were included in all PCR amplifications. Subsequently, PCR products were separated in 1% agarose gel electrophoresis in a Tris-borate-EDTA buffer (0.045 M Tris-borate, 0.001 M EDTA pH 8.0), stained with ethidium bromide and visualized on a UV transilluminator using a gel documentation system (BioRad Gel225 Doc™XR+). PCR products were sequenced by MacroGen Inc., using the same primers employed for PCR amplification.

Phylogenetic analysis

The newly obtained SSU rDNA was aligned with several other sequences of closely related genera obtained from a BLAST query of the GenBank database (Altschul et al. 1990) using ClustalX 2.1.0.12, for which the default parameters were applied (Larkin et al. 2007). MEGA software version 5 was used to generate phylogenetic trees based on the obtained alignment by both maximum likelihood (ML) and neighbour joining (NJ) methods (Tamura et al. 2011). The parameters for the ML analyses were general time reversible model, gamma distribution with invariant sites (G+I) and 1000 bootstrap replications. The NJ tree was constructed using the Kimura 2-parameter model with a gamma distribution (Kimura 1980), for a total of 1190 positions in the final dataset. All positions containing gaps and missing data were eliminated.

RESULTS

Light microscopy

The infection was reported as dark brownish spherical xenomas in the body cavity attached to the mesenteries. These xenomas ranged from about 2 to 5 mm. The semi-thin sections revealed that parasite xenomas were encircled by a fibrous layer produced

by the host (Fig. 1a). If a xenoma was squeezed, numerous mature spores were released (Fig. 1b). These spores were ovoid with a posterior vacuole occupying nearly half of the spore. The fresh mature spores were 4.5 ± 0.3 (4.0–4.8) μm long and 2.5 ± 0.2 (2.0–2.5) μm wide (Fig. 1b).

Ultrastructure

All lifecycle stages had isolated nuclei. Merogony was recognized as cylindrical binucleated (Fig. 1c) and multinucleated meronts with several unpaired nuclei. Meronts were surrounded by cisternae of the host cell's endoplasmic reticulum, and their cytoplasm was rich in endoplasmic reticulum (Fig. 1c). Sporonts were characterized by having a layer of amorphous materials secreted externally to their outer cell membrane, and by an increase in their cytoplasmic density (Fig. 1d). The sporonts then grouped together to form sporoblast cells (Fig. 1e). The sporoblasts gradually differentiated to the typical organelles of the spores (Figs. 1f & 2). Mature spores were elongate-ovoid, with a double-layered wall composed of an electron-dense exospore and an electron-lucent endospore of approximately the same thickness and displaying some protuberances (Figs. 1f & 2a,b). The polaroplast was lamellar at the apical part and vesicular and tubular at the end of the straight part of the polar tube (Fig. 1f). The posterior vacuole was located in the third posterior part and was filled with a membranous network and fine vesicles reminiscent of the posterosome structure (Fig. 2b). The nucleus was irregular to spindle shaped and placed in the centre of the spores (Fig. 2). The anchoring disc appeared like a mushroom cap located in the centre of the anterior pole of the spore and attaching to the polar tube (Fig. 1f). The polar filament itself extended from the anchoring disc obliquely backwards and then formed the polar filament coils in the posterior half of the spore (Fig. 1f). The polar filament isofilar possessed 28 to 30 coils, but in most cases 29 coils, organized in 3 rows at the posterior pole, winding from the basal to the anterior zone of the spore (Fig. 2).

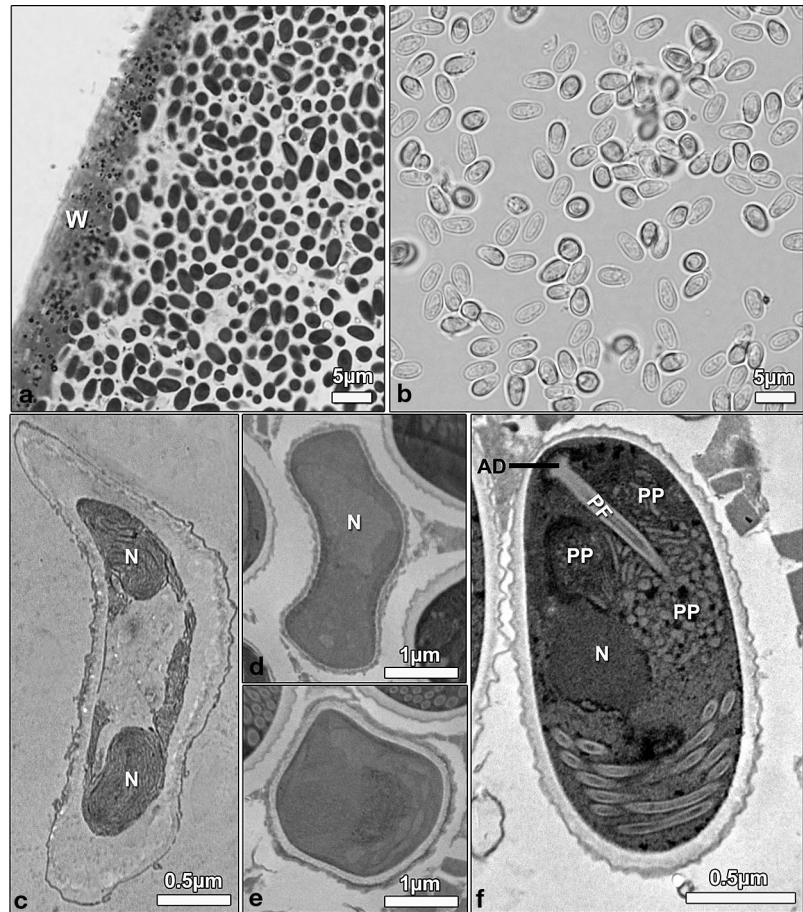


Fig. 1. Light and ultrastructural aspects of *Glugea jazanensis* sp. nov., a parasite in the body cavity of the two-spot red snapper *Lutjanus bohar*. (a) Semithin section through xenoma surrounded by a wall of fibrous tissue (W) and containing aggregates of spores. (b) Fresh mature spores. (c) Meront with 2 nuclei (N). (d) Sporont with nucleus (N). (e) Sporoblast. (f) Mature spore showing anchoring disc (AD), polar filament (PF), polaroplast (PP) and nucleus (N)

Description of *Glugea jazanensis* sp. nov.

Systematic position

Phylum: Microsporidia Balbiani, 1882

Class: Haplophasea Sprague, Becnel & Hazard, 1992

Order: Glugeida Issi, 1986

Family: Glugeidae Thélohan, 1892

Genus: *Glugea* Thélohan, 1891

Species: *Glugea jazanensis* sp. nov.

Type host: *Lutjanus bohar* Forsskål 1775

Locality: Red Sea off Jizan City (16° 53' 21" N, 42° 32' 3" E), Saudi Arabia

Site of infection: Skeletal muscles of the abdominal cavity and mesenteric tissues

Prevalence of infection: 14.7% (53/360)

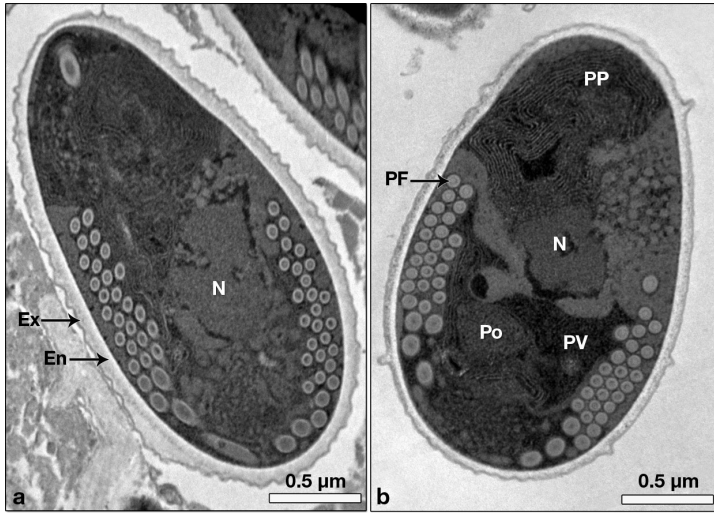


Fig. 2. Details of the ultrastructural morphology of *Glugea jazanensis* sp. nov. spores from the body cavity of the two-spot red snapper *Lutjanus bohar*. (a) Spore ultrastructure showing the spore wall, which is composed of the exospore (Ex) and endospore (En). N: nucleus. Polar filament (PF), polaroplast (PP), nucleus (N) and posterior vacuole (PV) with posterosome (Po)

Type specimen: Two slides containing semi-thin sections of xenomas containing spores at different developmental stages of the hapantotype were deposited in the parasitological collection of the Hungarian Natural History Museum under the inventory number HHNM- 70640.

Etymology: The specific epithet '*jazanensis*' derives from the host locality Jizan City.

Molecular analysis

An SSU rDNA sequence of 1234 bp was generated for the new microsporidian and deposited in GenBank under accession number KP262018. Multiple sequence alignment showed strong similarity with microsporidia belonging to the genus *Glugea* (Table 1). The highest percentages of similarities were 99.62 and 99.56% observed, respectively, with *G. nagelia* Abdel-Baki, Al-Quraishy, Rocha, Dkhil, Casal & Azevedo, 2015, from *Cephalopholis hemistiktos* (Actinopterygii: Serranidae) off the Red Sea and *Glugea* sp. Wu, Wu, Wu & Wang, 2002 from *Epinephelus awoara*. Similarity with other *Glugea* species varied between 91.95 and 90.3% (Table 1). Species of the genus *Pleistophora* had similarities varying between 87.6 and 85.4%. ML and NJ yielded trees with similar topology. The present species forms a highly supported subclade (bootstrap = 100%) with *G. nagelia* and the unidentified *Glugea* species hav-

Table 1. Evolutionary distances calculated as a percentage of similarity based on the Kimura 2-parameter model obtained after pairwise analysis of the small subunit (SSU) rDNA of the selected microsporidia. Percentages of similarities between *Glugea jazanensis* sp. nov. and the other apparent microsporidia are shown in **bold**

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 <i>Glugea jazanensis</i> n. sp	-																
2 <i>Glugea nagelia</i>	99.62																
3 <i>Glugea sp.</i>	99.56	99.53															
4 <i>Glugea hertwigi</i>	91.95	91.95	91.42														
5 <i>Glugea atherinae</i>	91.6	91.69	91.16	99.8													
6 <i>Glugea plecoglossi</i>	91.6	91.69	91.16	99.3	99.6												
7 <i>Glugea anomala</i>	91.6	91.69	91.16	99.8	100	99.6											
8 <i>Glugea stephani</i>	91.1	91.19	90.66	99.3	99.6	99.1	99.6										
9 <i>Glugea pagri</i>	90.3	90.45	89.91	97.5	97.7	97.3	97.7	97.3									
10 <i>Pleistophora mirandellae</i>	87.6	87.53	86.97	87.4	87.7	87.7	87.7	87.1	86.3								
11 <i>Ovipleistophora mirandellae</i>	87.6	87.53	86.97	87.7	88	88	88	87.4	86.6	98							
12 <i>Heterosporis anguillarum</i>	86.8	86.18	85.61	87.9	88.2	88.2	88.2	87.6	87.4	93.7	93.9						
13 <i>Pleistophora mulleri</i>	86	86.7	86.13	86.5	86.8	86.8	86.8	86.3	85.5	91.9	92.4	92.4					
14 <i>Pleistophora sp. 2</i>	86	86.18	85.61	86.5	86.8	86.8	86.8	86.3	85.5	91.9	92.4	100					
15 <i>Pleistophora sp. 3</i>	86	86.18	85.61	86.5	86.8	86.8	86.8	86.3	85.5	91.9	92.4	100					
16 <i>Pleistophora hippoglossoides</i>	86	86.18	85.61	86.5	86.8	86.8	86.8	86.3	85.5	91.9	92.4	100	100				
17 <i>Pleistophora typicalis</i>	85.4	85.62	85.04	86	86.3	86.3	86.3	85.7	84.9	91.4	91.9	91.9	99.6	99.6	99.6	99.6	-

ing a GenBank entry AY090038 (direct submission by Wu et al. in 2002) from *E. awoara* within the large clade grouping all selected *Glugea* species (Fig. 3).

DISCUSSION

According to available literature, 17 species of the genus *Glugea* have so far been described from fresh and marine fish (Su et al. 2014), although some of these species might be synonymous, since their classification is based mainly on spore morphology and host origin (see Vagelli et al. 2005). Of all currently described species of the genus *Glugea*, 7 species have been reported from freshwater hosts, and these can be regarded as separate species which do not need to be compared with the present species. Of the remaining species, *G. berglax* Lom & Laird, 1976; *G. plecoglossi* Takahashi & Egus, 1977; and *G. athe-*

rinae Berrebi 1979 differ in having longer spores (6.4, 5.8 and 5.7 μm , respectively, vs. 4.5 μm ; see Lom & Dyková 1992). Although a notable feature of the members of the genus *Glugea* is the presence of a single mature spore type, as in the present species, the existence of *Glugea* species with 2 types of spores has been reported previously (e.g. *G. vincentiae*, Vagelli et al. 2005; and *G. pagri*, Su et al. 2014). The number of polar filament coils in mature spores is also a common diagnostic for microsporidian species (Lovy et al. 2009). Our present species showed 29 to 34 polar filament coils in 3 rows inside the spore, which greatly exceeds the range of coils in any previously described *Glugea* spp. (see Su et al. 2014). The previously described microsporidian from fish of the family Lutjanidae was *Microfilum lutjani*, which was found in the gill filaments of *Lutjanus fulgens* (Faye et al. 1991). This species differs from our present species in having a very characteristic uncoiled polar filament that has not been described previously in the Microsporidia. It is worth mentioning that the present new species is similar to *G. nagelia* (the only species described for this genus in Saudi Arabia; see Abdel-Baki et al. 2015) in terms of host habitat and spore shape, although it differs in terms of site of infection (body cavity vs. intestinal wall), spore size (4.5 \times 2.5 μm vs. 5.1 \times 2.2 μm) and the number of the filament coils (28–30 vs. 26–29).

Overall, therefore, based on these morphometric comparisons, the present species can be considered a new species. Although careful morphological study continues to be indispensable for proper identification and subsequent reorganization of existing and new species (Lom & Nilsen 2003), this should be supported by the comparison of SSU rDNA sequences, especially when describing a new species (Vávra & Lukeš 2013). Molecular analyses based on the SSU rDNA gene, either using the evolutionary genetic distance or the phylogenetic analyses, are especially informative. In this study, the new species was shown to possess the highest percentages of similarity with other species of the genus *Glugea*. The highest percentages of similarity were observed with *G. nagelia* (99.62%) and with the unidentified *Glugea* species (99.56%). The phylogenetic tree shows clustering of the 3 species in the same subclade with a boot-

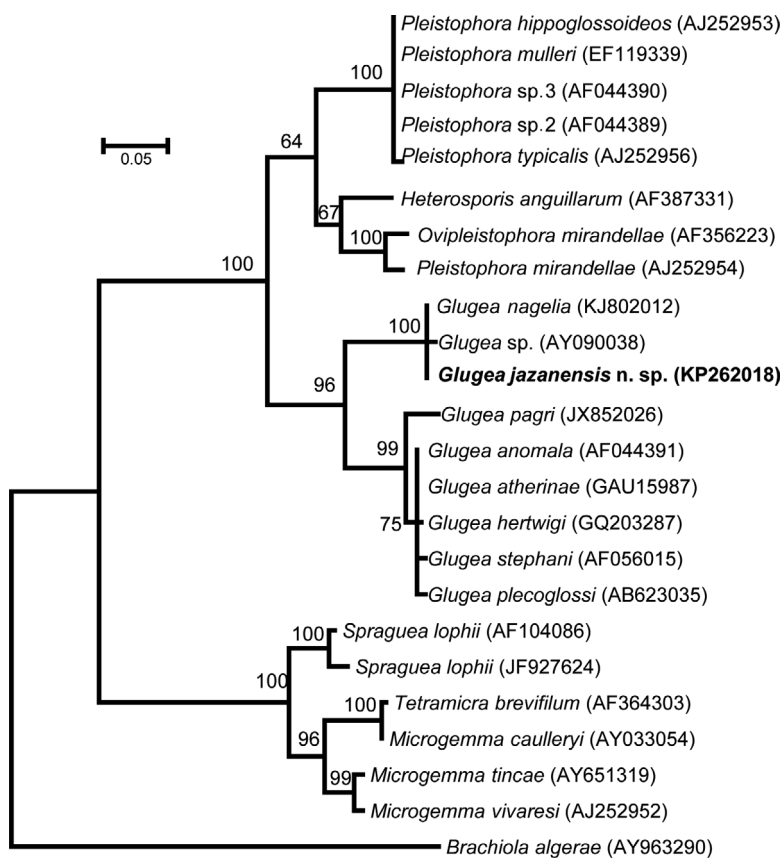


Fig. 3. Maximum likelihood (ML) phylogenetic tree based on the small subunit (SSU) rDNA data set of selected microsporidian species showing the position of *Glugea jazanensis* sp. n. Bootstrap values from ML analysis are indicated at each node. GenBank accession numbers for each species are reported in parentheses. Bootstrap supports based on 1000 replicates are given in branches. *Brachiola algerae* was used as the outgroup. Scale bar shows the number of changes per site

strap values were observed with *G. nagelia* (99.62%) and with the unidentified *Glugea* species (99.56%). The phylogenetic tree shows clustering of the 3 species in the same subclade with a boot-

strap value of 100%. The second subclade in the clade of *Glugea* is formed by *G. anomala* (Pomport-Castillon et al. 2000), *G. atherinae*, *G. hertwigi* (Lovy et al. 2009), *G. stephani* (Pomport-Castillon et al. 2000) and *G. plecoglossi* (Pomport-Castillon et al. 2000). It is worth noting that the percentages of similarities between these 5 species vary between 97.7% (*G. pagri* vs. *G. anomala*) and 99.8% (*G. anomala* vs. *G. hertwigi*), while *G. anomala* and *G. atherinae* appear identical with 100% similarity. A high percentage of similarity was also observed within the genus *Pleistophora*; for example, *P. aegyptiaca* showed 99.8% similarity with *P. anguillarum* (Abdel-Ghaffar et al. 2012). Thus, considering the evolutionary genetic distances and the phylogenetic analysis, we believe that the present SSU rDNA study, along with the structural and ultrastructural data, supports the establishment of *G. jazanensis* as a new species distinct from any other sequenced *Glugea* species.

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