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ORIGINAL PAPER

Ultrastructural and molecular characterization of *Glugea serranus* n. sp., a microsporidian infecting the blacktail comber, *Serranus atricauda* (Teleostei: Serranidae), in the Madeira Archipelago (Portugal)

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Abstract A new microsporidian infecting the connective tissue of the coelomic cavity of the blacktail comber *Serranus atricauda*, in the Madeira Archipelago (Portugal), is described on the basis of morphological, ultrastructural, and molecular features. The microsporidian formed large whitish xenomas adhering to the peritoneal visceral organs of the host. Each xenoma consisted of a single hypertrophic cell, in the cytoplasm of which mature spores proliferated within parasitophorous vacuoles surrounded by numerous collagen fibers. Mature spores were ellipsoidal and uninucleated, measuring an average of 6.5 ± 0.5 μm in length and 3.4 ± 0.6 μm in width. The anchoring disk of the polar filament was subterminal, laterally shifted from the anterior pole of the spore. The isofilar polar filament coiled in 18–19 turns, forming two rows that surrounded the posterior vacuole. The latter occupied about one third of the spore length. The polaroplast surrounding the apical and uncoiled portion of the polar filament displayed two distinct regions: a lamellar region and an electron-dense globule. Molecular analysis of the rRNA genes, including the internal transcribed spacer region, and phylogenetic analysis using maximum likelihood and neighbor joining demonstrated that this microsporidian parasite clustered with some *Glugea* species. Based on the differences found both at the morphological and molecular levels, to other members of the genus *Glugea*, the microsporidian infecting the blacktail comber is considered a new species, thus named *Glugea serranus* n. sp.

Keywords Fish parasite · Microsporidia · Fine structure · rRNA genes

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Introduction

Microsporidians (phylum Microsporidia Balbiani, 1882) are unicellular organisms, developing as obligatory intracellular parasites, in different animal hosts, in particular in arthropods and fish (Canning and Lom 1986; Lom and Dyková 1992; Sprague et al. 1992; Larsson 1999; Lom 2002). To date, about 187 genera have been described (Vávra and Lukeš 2013), of which 21 parasitize freshwater and marine fish worldwide, some causing diverse pathologies in commercially important species (Lom and Dyková 1992; Lom 2002; Lom and Nilsen 2003; Stentiford et al. 2013; Diamant et al. 2014). The genus *Glugea* Thélohan, 1891 (Microsporidia: Glugeidae) includes at least 33 species infecting numerous organs in the fish hosts (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). Their development in the host is characterized by the formation of large xenomas presenting sporogonic development within parasitophorous vacuoles with a

membrane-like wall. Mature xenomas display stratified organization, with developmental stages only at the periphery and the mature spores concentrated in the center of the xenoma. Nuclei remain isolated throughout the life cycle (Lom and Dyková 1992; Lom and Nilsen 2003).

Recently, the implementation of molecular procedures revealed that morphological features alone are insufficient for the reliable description of microsporidian parasites; rather, the taxonomic positioning of species must result from the combined use of both molecular and morphological criteria (Weiss and Vossbrinck 1999; Pomport-Castillon et al. 2000; Lom and Nilsen 2003; Vossbrinck and Debrunner-Vossbrinck 2005). Currently, GenBank database provides 27 nucleotide sequence entries for the genus *Glugea*, of which 16 are ribosomal RNA (rRNA) sequences. According to Lom and Nilsen (2003), fish microsporidia cluster into five different groups and *Glugea* rRNA sequences have been shown to cluster together with *Tuzetia weidneri* Canning, Curry, and Overstreet, 2002 on group 2. Presently inserted on group 1, there is a new clade sister to the one containing *Ichthyosporidium* Caullery and Mesnil, 1905; *Loma* Morrison and Sprague, 1981; and *Pseudoloma* Matthews, Brown, Larison, Bishop-Stewart, and Kent, 2001 species (Azevedo et al. 2016). The present paper describes a new microsporidian species infecting the viscera of the blacktail comber, *Serranus atricauda* Günther, 1874 (Teleostei: Serranidae), a marine benthopelagic fish species, using both morphological and molecular data for the discernment of its taxonomic positioning and phylogenetic relationships.

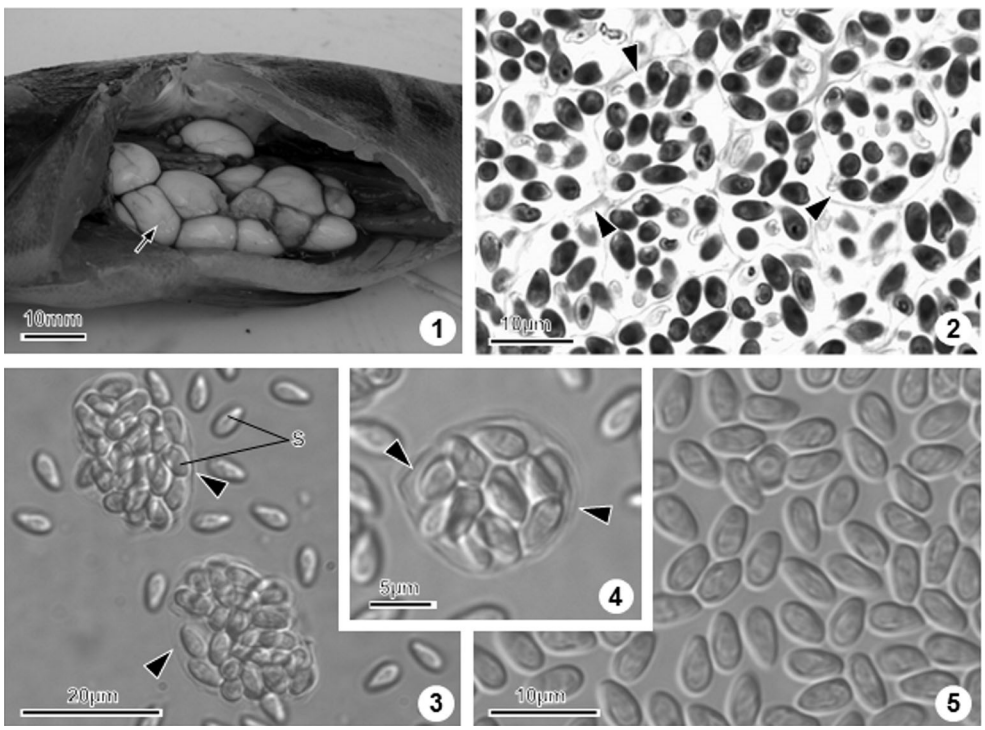
Material and methods

Host and parasite sampling

Forty-one specimens of the marine fish, blacktail comber, *S. atricauda* Günther, 1874 (Teleostei: Serranidae), ranging in length from 17 to 36 cm ($n = 23$) were caught by line fishing during October 2010 and October 2015, at Selvagens Islands, Madeira Archipelago, Atlantic Ocean (30° 09′ N/16° 03′ W), Portugal. Necropsy revealed the presence of large whitish xenomas in the connective tissue of the coelomic cavity, attached to the viscera. Preliminary microscopic analysis of smear preparations confirmed infection by a microsporidian parasite. Fresh isolated spores were observed and measured using Nomarski differential interference contrast (DIC) optics.

Transmission electron microscopy

For transmission electron microscopy (TEM), small fragments of the xenomas were fixed in 3 % glutaraldehyde

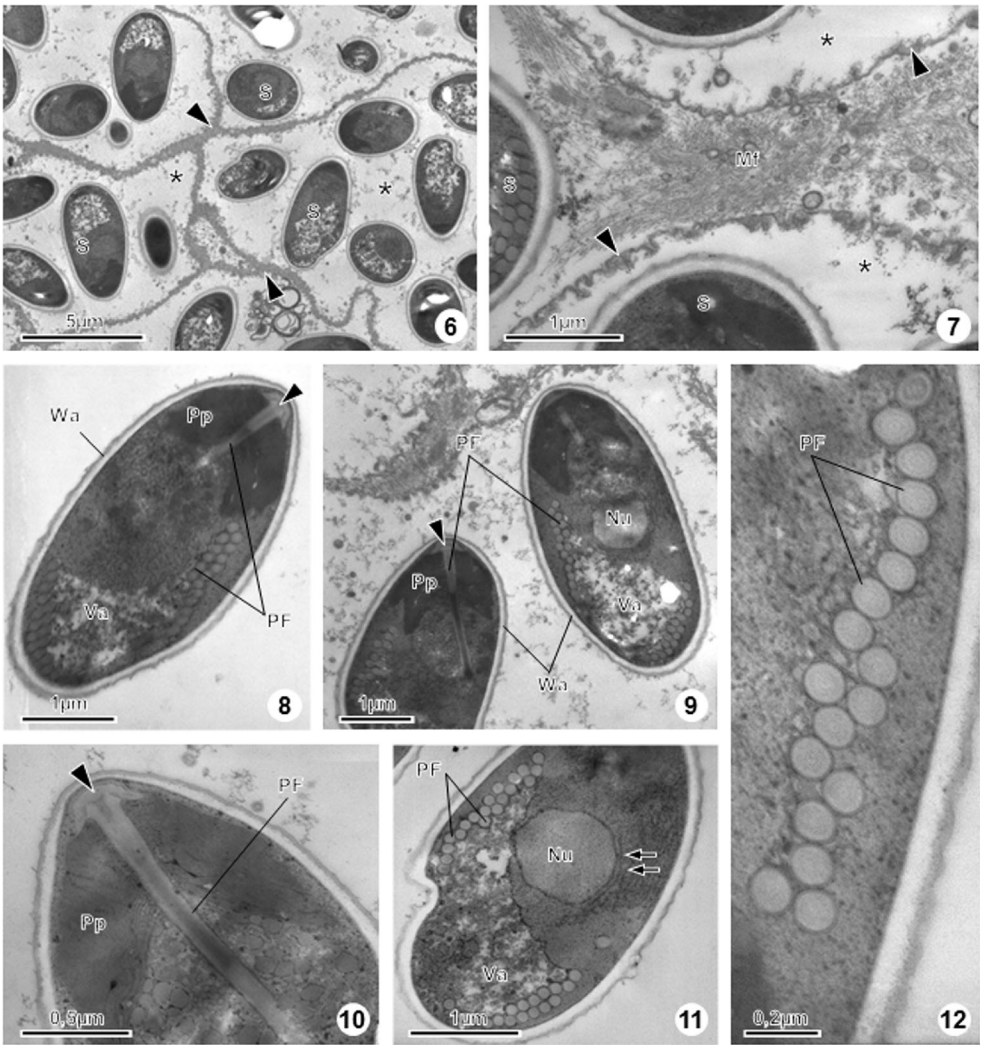


Figs. 1–5 Macrograph, light micrographs of *Glugea serranus* n. sp. from the abdominal cavity of *Serranus atricauda*, in the Madeira archipelago. **1** Several large whitish xenomas (*arrow*) located within the abdominal cavity, adjacent to the visceral organs. **2** Semithin section of a xenoma showing numerous mature spores contained within parasitophorous vacuoles (*arrowheads*). **3** Parasitophorous vacuoles (*arrowheads*) containing numerous mature spores (*S*), which also appear free in the preparation, observed in DIC optics. **4** DIC micrograph evidencing the membrane (*arrowheads*) of a parasitophorous vacuole. **5** Free fresh mature spores observed in DIC optics

buffered in 0.2 M sodium cacodylate (pH 7.2) at 4 °C for 24 h, washed overnight in the same buffer at the same temperature, and post-fixed in 2 % osmium tetroxide with the same buffer for 3 h at 4 °C. After dehydration in an ascending ethanol series and propylene oxide, the fragments were embedded in EPON. Semithin sections were stained with methylene blue-Azure II for light microscopy. Ultrathin sections were contrasted with both aqueous uranyl acetate and lead citrate and observed and photographed using a JEOL 100CXII TEM operated at 60 kV.

DNA isolation and PCR amplification

Small fragments of the xenomas were homogenized and subsequently stored in absolute alcohol at 4 °C. The genomic DNA of approximately 5×10^6 spores, estimated using a Neubauer counting chamber, was extracted using a GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma) following the manufacturer's instructions for animal tissue, but with a 12-h period of incubation. The DNA was stored in 50 μl of TE buffer at −20 °C. The majority of the region coding for the small subunit (SSU)



Figs. 6–12 Transmission electron micrographs of *Glugea serranus* n. sp. from the abdominal cavity of *Serranus atricauda*, in the Madeira Archipelago. **6** Xenoma displaying numerous mature spores (*S*) contained within parasitophorous vacuoles (*asterisks*). Notice the membranes (*arrowheads*) comprising the wall of the latter. **7** Ultrastructural aspect of some collagen microfibrils (*Mf*) comprising the membranes (*arrowheads*) that surrounded the parasitophorous vacuoles (*asterisk*) containing mature spores (*S*). **8** Longitudinal section of a spore showing its double-layered wall (*Wa*), the anchoring disk (*arrowhead*) from which the polar filament (*PF*) extends, the polaroplast (*Pp*) located at the anterior pole, and the vacuole (*Va*) at the posterior pole. **9** Spores evidencing its double-layered wall (*Wa*), the subterminal and laterally shifted anchoring disk (*arrowhead*) from which the polar filament (*PF*) extends, the polaroplast (*Pp*) comprised of a lamellar region and an electron-dense globule, the vacuole (*Va*) at the posterior pole, and the central nucleus (*Nu*). **10** Anterior pole of a spore, displaying the anchoring disk (*arrowhead*) in continuation with the polar filament (*PF*), which is enveloped by the polaroplast (*Pp*). **11** Transverse section of a spore evidencing the nucleus (*Nu*) surrounded by endoplasmic reticulum (*arrows*), as well as the polar filament (*PF*) coiled around the vacuole (*Va*). **12** Ultrastructural detail, depicting the organization and composition of the polar filament (*PF*)

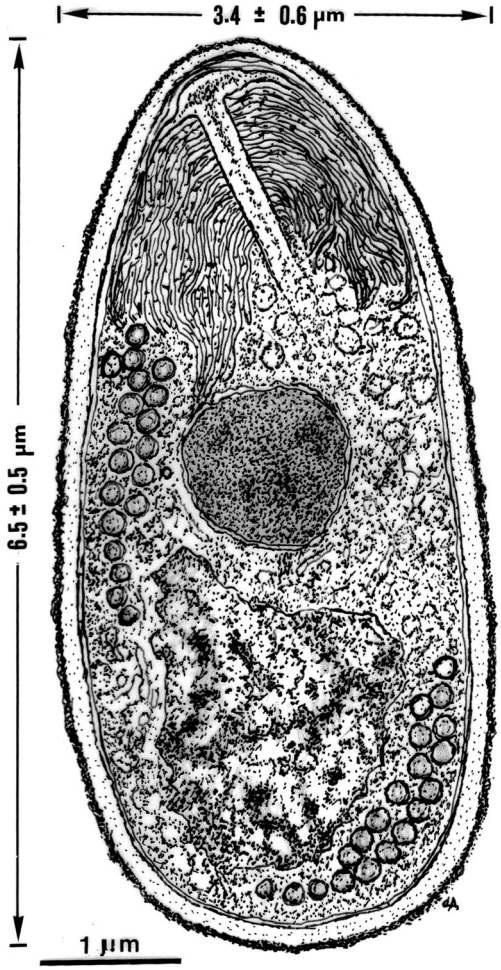


Fig. 13 Schematic drawing of the longitudinal section of a spore of *Glugea serranus* n. sp. from the abdominal cavity of *Serranus atricauda*, in the Madeira Archipelago, depicting the organization of the anchoring disk and the polar filament forming two rows coiled around the posterior vacuole, as illustrated in the micrographs

rRNA gene was amplified by PCR using the primers V1f (5′-CACCAGGT TGATTCTGCC-3′) (Nilsen 2000) and HG5F_rev (5′-TCACCCCACTTGTCGTTA-3′) (Abdel-Baki et al. 2015a). To amplify the 3′ end of the SSU, internal transcribed spacer (ITS), and 5′ end of the large subunit (LSU) rRNA gene, the HG4F (5′ GCGGCTTAATTTGACTCAAC-3′) and HG4R (5′-TCTCCTTGGTCCGTGTTTCAA-3′) primers were used (Gatehouse and Malone 1998). PCRs were carried out in 50-μl reactions using 10 pmol of each primer, 10 nmol of each dNTP, 50 mM of $MgCl_2$, 5 μl 10× Taq polymerase buffer, 1.5 units Taq DNA polymerase (Nyztech), and 3 μl of the genomic DNA. The reactions were run on a Hybaid PxE Thermocycler (Thermo Electron Corporation, Milford, MA), with initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min. The final elongation step was performed at 72 °C for 10 min. Five-microliter aliquots of the PCR products were electrophoresed through a 1 % agarose 1× tris-acetate-EDTA buffer (TAE) gel stained with ethidium bromide. The PCR products for the SSU gene and ITS region, with an approximate size of 900 and 1100 bp, respectively, were sequenced directly. The sequencing reactions were performed using a BigDye Terminator v1.1 kit from the Applied Biosystems kit, and were run on an ABI3700 DNA analyzer (Perkin-Elmer, Applied Biosystems, Stabvida, Co., Oeiras, Portugal).

Fig. 14 Maximum likelihood tree showing the relationships *of Glugea serranus* n. sp. and other microsporidians based on the SSU rDNA sequences. The *numbers* on the branches are bootstrap confidence levels on 500 replicates for ML/NJ trees, and the *scale* is given under the tree. The tree was generated using 40 microsporidia selected sequences with *Vairimorpha necatrix* as the outgroup. GenBank accession numbers in *parentheses* after the species name

Distance and phylogenetic analysis

To evaluate the phylogenetic position of the parasite here described among its closest relatives sequenced to date, 40 microsporidian ribosomal DNA (rDNA) sequences were extracted from GenBank, according to the BLAST homology score (Altschul et al. 1990). The sequence and NCBI accession number data obtained from GenBank are as follows: *Dasyatispora levantinae* (GU183263); *Glugea anomala* (AF044391); *Glugea arabica* (KT005391); *Glugea atherinae* (U15987); *Glugea epinephelusis* (AY090038); *Glugea hertwigi* (GQ203287); *Glugea jazanensis* (KP262018) *Glugea nagelia* (KJ802012), *Glugea pagri* (JX852026); *Glugea plecoglossi* (AJ295326); *Glugea stephani* (AF056015); *Glugea* sp. GS1 (AJ295325); *Heterosporis anguillarum* (AF387331); *Heterosporis* sp. PF (AF356225); *Heterosporis* sp. NBDP-2013a (KC137548); *Ichthyosporidium weisii* (JQ062988), *Ichthyosporidium* sp. (L39110); *Loma acerinae* (AJ252951), *Loma embiotocia* (AF320310); *Loma psittaca* (FJ843104); *Loma salmonae* (U78736); *Loma* sp. SVB-PE3 (HM626217); *Microsporidium cerebralis* (JQ316511); *Ovipleistophora mirandellae* (AF356223); *Ovipleistophora ovariae* (AJ252955); *Myosporidium merluccius* (AY530532); *Nucleospora salmonis* (U78176); *Pleistophora ehrenbaumi* (AF044392); *Pleistophora finisterrensis* (AF044393); *Pleistophora hippoglossoideos* (AJ252953); *Pleistophora hyphessobryconis* (GU126672); *Pleistophora typicalis* (AF044387); *Pleistophora* sp. 1 (AF044394); *Pleistophora* sp. 2 (AF044389); *Pleistophora* sp. 3 (AF044390); *Pleistophora* sp. KB-2011 (HQ703580); *Pleistophora* sp. LM-2014 (KF830721); *Potaspora morhaphis* (EU534408); *Pseudoloma neurophilia* (AF322654); and *Spraguea gastrophysus* (GQ868443). *Vairimorpha necatrix* (Y00266) was used as the outgroup.

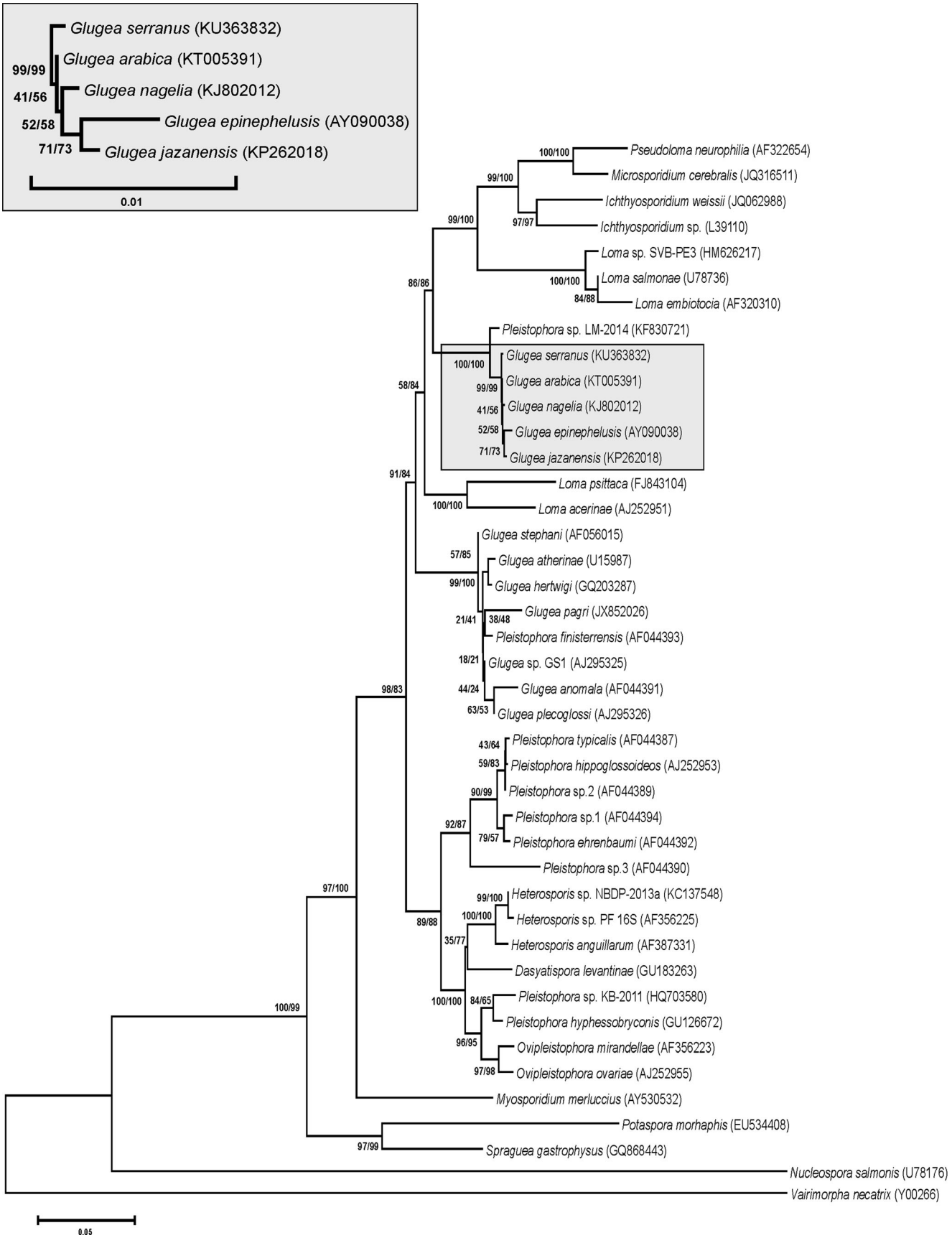

Glugea serranus (KU363832)
Glugea arabica (KT005391)
99/99
Glugea nagelia (KJ802012)
41/56
Glugea epinephelusis (AY090038)
52/58
Glugea jazanensis (KP262018)
71/73
0.01
100/100
Pseudoloma neurophilia (AF322654)
99/100
Microsporidium cerebralis (JQ316511)
Ichthyosporidium weissii (JQ062988)
99/100
97/97
Ichthyosporidium sp. (L39110)
Loma sp. SVB-PE3 (HM626217)
100/100
Loma salmonae (U78736)
86/86
84/88
Loma embiotocia (AF320310)
Pleistophora sp. LM-2014 (KF830721)
100/100
Glugea serranus (KU363832)
58/84
99/99
Glugea arabica (KT005391)
41/56
Glugea nagelia (KJ802012)
52/58
Glugea epinephelusis (AY090038)
71/73
Glugea jazanensis (KP262018)
91/84
Loma psittaca (FJ843104)
100/100
Loma acerinae (AJ252951)
Glugea stephani (AF056015)
57/85
Glugea atherinae (U15987)
99/100
Glugea hertwigi (GQ203287)
21/41
38/48
Glugea pagri (JX852026)
Pleistophora finisterrensis (AF044393)
18/21
Glugea sp. GS1 (AJ295325)
98/83
44/24
Glugea anomala (AF044391)
63/53
Glugea plecoglossi (AJ295326)
43/64
Pleistophora typicalis (AF044387)
59/83
Pleistophora hippoglossoideos (AJ252953)
90/99
Pleistophora sp.2 (AF044389)
92/87
Pleistophora sp.1 (AF044394)
79/57
Pleistophora ehrenbaumi (AF044392)
Pleistophora sp.3 (AF044390)
97/100
99/100
Heterosporis sp. NBDP-2013a (KC137548)
89/88
100/100
Heterosporis sp. PF 16S (AF356225)
35/77
Heterosporis anguillarum (AF387331)
Dasyatispora levantinae (GU183263)
100/100
84/65
Pleistophora sp. KB-2011 (HQ703580)
100/99
Pleistophora hyphessobryconis (GU126672)
96/95
Ovipleistophora mirandellae (AF356223)
97/98
Ovipleistophora ovariae (AJ252955)
Myosporidium merluccius (AY530532)
Potaspora morhaphis (EU534408)
97/99
Spraguea gastrophysus (GQ868443)
Nucleospora salmonis (U78176)
Vairimorpha necatrix (Y00266)
0.05

Table 1 Comparison of some rDNA sequences: percentage of identity (top diagonal) and pairwise distance (bottom diagonal) obtained by p-distance

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
(1) *Glugea serranus* n. sp. (KU363832)	–	99.6	99.6	99.5	99.4	98.2	93.2	91.7	92.1	90.4	90.2	90.1	89.5	79.5
(2) *Glugea nagelia* (KJ802012)	0.004	–	99.7	99.4	99.3	89.1	93.0	91.6	91.9	89.9	89.7	89.6	89.1	78.6
(3) *Glugea jazanensis* (KP262018)	0.004	0.003	–	99.8	99.5	98.0	92.6	91.5	91.8	91.8	92.1	92.2	92	78.7
(4) *Glugea arabica* (AY900038)	0.005	0.006	0.002	–	99.4	89.2	93.1	91.7	92.0	90.0	89.7	89.6	89.1	78.1
(5) *Glugea epinephelusis* (AY090038)	0.006	0.007	0.005	0.006	–	89.1	92.7	91.6	91.9	92.1	92.3	92.3	92.1	76.8
(6) *Pleistophora* sp. LM-2014 (KF830721)	0.018	0.019	0.020	0.018	0.019	–	93.0	91.8	92.5	92.3	92.4	92.5	92.4	78.9
(7) *Glugea stephani* (AF056015)	0.068	0.070	0.074	0.069	0.073	0.070	–		97.7	97.5	98.2	98.3	98.1	71.0
(8) *Glugea pagri* (JX852026)	0.083	0.084	0.085	0.083	0.084	0.082	0.026	–		99.5	99.2	99.3	99.1	98.0
(9) *Glugea atherinae* (U15987)	0.079	0.081	0.082	0.080	0.081	0.075	0.005	0.023	–		88.9	99.2	88.8	86.1
(10) *Glugea hertwigi* (GQ203287)	0.096	0.101	0.082	0.100	0.079	0.077	0.003	0.025	0.005	–		99.5	88.8	98.7
(11) *Glugea plecoglossi* (AJ295325)	0.098	0.103	0.079	0.103	0.077	0.076	0.007	0.018	0.008	0.011	–		99.0	98.7
(12) *Glugea* sp. GS1 (AJ295325)	0.099	0.104	0.078	0.104	0.076	0.075	0.005	0.017	0.007	0.008	0.005	–		98.7
(13) *Pleistophora finisterrensis* (AF044393)	0.105	0.109	0.080	0.109	0.079	0.076	0.008	0.019	0.009	0.012	0.012	0.010	–	
(14) *Glugea anomala* (AF044391)	0.205	0.214	0.213	0.219	0.231	0.212	0.081	0.029	0.180	0.139	0.013	0.013	0.013	

The alignment was performed using Clustal W (Thompson et al. 1994) in MEGA 5.05 software (Tamura et al. 2011), with an opening gap penalty of 10 and a gap extension penalty of 4 for both pairwise and multiple alignments. After trimming the LSU rRNA 3′ end, the resulting alignment comprised 2149 informative characters in the final dataset. Subsequent phylogenetic and molecular evolutionary analyses were conducted in MEGA 5.05, using maximum likelihood (ML) and neighbor-joining (NJ) methodologies. For ML, the general time reversible substitution model with 4 gamma distributed rate variation among sites was performed. For NJ, a Kimura two-parameter substitution model with gamma distribution (shape parameter = 1.4) was performed. All positions with less than 75 % site coverage were eliminated from all trees, and the bootstrap consensus tree was inferred from 500 replicates for ML, NJ, and MP.

Distance estimation was carried out in MEGA 5.05, using the p-distance model distance matrix for transitions and transversions, with all ambiguous positions removed for each sequence pair.

Results

Morphological and ultrastructural description

Several whitish xenomas, the larger measuring about 10 mm in length, were macroscopically observed in the host coelomic cavity, adherent to the visceral organs (Fig. 1). Each xenoma consisted of a single hypertrophic cell, in the cytoplasm of which monomorphic mature spores proliferated within parasitophorous vacuoles (Figs. 2–5). The latter displayed a dense and irregular single membrane; the interspaces between parasitophorous vacuoles were filled with numerous collagen fibers (Figs. 6 and 7). Mature spores were ellipsoidal and uninucleated, averaging 6.5 ± 0.5 μm in length and 3.4 ± 0.6 μm in width (Figs. 3–5). The spore wall, 115–125 nm thick, was double layered, composed of a thin electron-dense exospore and a thick electron-lucent endospore. The anchoring disk of the polar filament was subterminal, laterally shifted from the anterior pole of the spore (Fig. 8). The manubrium of the polar filament measured about 210–215 nm in diameter, and was surrounded by the polaroplast, the latter displaying two distinct regions: a lamellar region and an electron-dense globule. The isofilar polar filament was composed of three concentric layers, measuring about 120–125 nm in diameter, obliquely arranged to the spore longitudinal axis at an angle of 42–48°, and forming 18–19 coils organized in two rows (Figs. 9, 10, 11, 12, and 13) surrounding the posterior vacuole. The latter occupied about one third of the spore length and contained small irregular granular masses randomly dispersed in the vacuolar matrix (Fig. 9). The posterosome was not observed. The nucleus occupied a central position between the polaroplast and the posterior vacuole (Fig. 11).

Taxonomic summary

Phylum Microsporidia Balbiani, 1882

Class Marinosporidia Vossbrinck and Debrunner-Vossbrinck 2005

Order Glugeida Issi, 1986

Family Glugeidae Thélohan, 1892

Genus *Glugea* Thélohan, 1891

Species *Glugea serranus* n. sp.

Host: Blacktail comber, *S. atricauda* Günther, 1874 (Teleostei: Serranidae) ranging in length from 17 to 36 cm

Table 2 Comparative measurements (in µm) from *Glugea* spp. phylogenetically related

Glugea sp.	Host and location of infection	Habitat	Xenoma	Spore			Polar filament			References
		Countries		Shape	Length	Width	Coils	Rows	Type	
G. epinephelusis	*Epinephelus akaara* Abdominal cavity	Marine South China	Blackish	Ellipsoidal	5.5 (4.6–7.2)	3.1 (2.8–3.5)	14–16	–	–	Wu et al. 2005
G. nagelia	*Cephalopholis hemistiktos* Intestinal wall	Marine Red Sea	Whitish	Ovoid to pyriform	5.1 (4.3–6.0)	2.2 (1.8–2.9)	26–29	3	Isofilar	Abel-Baki et al. 2015
G. jazanensis	*Lutjanus bohar* Abdominal cavity	Marine Red Sea	Dark brownish	Elongate-ovoid	4.5 (4.0–4.8)	2.5 (2.0–2.5)	28–30	3	Isofilar	Abel-Baki et al. 2015
G. arabica	*Epinephelus polyphekadion* Intestinal wall	Marine Red Sea	Blackish	Ellipsoidal to pyriform	6.3 (5.9–6.6)	3.3 (2.9–3.7)	27–29	3–4	Isofilar	Azevedo et al. 2016
G. serranus n. sp.	*Serranus atricauda* Connective tissue of abdominal cavity	Marine Madeira, Portugal	Whitish	Ellipsoidal	6.5 ± 0.5	3.4 ± 0.6	18–19	2	Isofilar	This study

Type locality: Selvagens Islands, Madeira Archipelago, Atlantic Ocean (30° 09′ N/16° 03′ W), Portugal

Site of infection: Large whitish xenomas juxtaposed to the connective tissue of the coelomic cavity, adherent to the visceral organs

Prevalence of infection: 21 out of 41 blacktail combers were infected ($P = 51.2$ %).

Specimens deposited: One glass slide with semithin sections containing mature spores of the hapantotype was deposited in the Type Slide Collection of the Laboratory of Animal Pathology at the Interdisciplinary Centre of Marine and Environmental Research, Porto, Portugal, reference CIIMAR 2016.10.

Etymology: The specific epithet *serranus* derives from the specific epithet of the host species.

Molecular characterization and phylogeny

The complete SSU rRNA gene sequence + ITS region and partial LSU rRNA gene, composed of 1820 bp, was obtained and deposited in GenBank (accession number KU363832). In total, 40 SSU rDNA sequences, including those with the higher BLAST scores, were aligned with the SSU rDNA sequence obtained for *G. serranus* n. sp. After the trimming of the 3′ end SSU rDNA, the alignment resulted in 2149 bp.

All phylogenetic analyses (MP, NJ) placed *G. serranus* n. sp. clustering alongside *G. arabica* (KT005391), *G. epinephelusis* (AY090038), *G. nagelia* (KJ802012), *G. jazanensis* (KP262018), and a *Pleistophora* sp. (KF830721) to form a clade with a bootstrap of 100 % (Fig. 14). Pairwise comparisons between the SSU rDNA sequence of *G. serranus* n. sp. and all other sequences from the genus *Glugea*, as well as to the other two from the genus *Pleistophora*, revealed the parasite presenting significant similarity to *G. nagelia* (KJ802012), *G. jazanensis* (KP262018), *G. arabica* (KT005391), and *G. epinephelusis* (AY090038). The percentage of similarity to all others was lower than 98.2 % (Table 1).

Discussion

The phylum Microsporidia encompasses 21 genera recognized as fish parasites, infections of which can elicit severe pathological effects, including malformations of the body, impairment of swimming, and increased mortality rates (Rodriguez-Tovar et al. 2004; Brown et al. 2010; Stentiford et al. 2013; Diamant et al. 2014). In particular, members of the genus *Glugea* typically produce large xenomas that often lead to atrophy of visceral organs (Lom and Dyková 1992; Lom 2002). With basis on the morphological and ultrastructural

features of the xenomas and spores, namely the formation of large xenomas presenting stratified development and containing mature ovoid spores within parasitophorous vacuoles, the microsporidian here described is identified as a member of the genus *Glugea* (Canning et al. 1982; Lom and Dyková 1992; Larsson 1999; Lom and Nilsen 2003). This is corroborated by the rDNA sequence analysis and by the site of infection, which is also a characteristic used for the differentiation between genera. In the case of *Glugea* spp., xenomas are usually subcutaneous and adherent to several visceral organs (Lom and Dyková 1992; Wu et al. 2005; Su et al. 2014; Abdel-Baki et al. 2015a, b; Azevedo et al. 2016), as it was also observed for *G. serranus* n. sp. Further comparing host specificity, habitat, and the morphological and ultrastructural traits of the parasite to other *Glugea* (namely to those presenting the highest molecular similarity), it is suggested that the parasite here described is a new species.

Presently, there are 33 *Glugea* species, predominantly reported adhering to organs of the digestive tract of freshwater and marine fishes (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). To our best knowledge, and despite fish microsporidia lacking clear host specificity (Lom and Dyková 1992; Lom et al. 1995; Nagasawa and Cruz-Lacierda 2004; Payghan et al. 2009), the parasite here described constitutes the first occurrence of a *Glugea* species infecting a marine fish of the family Serranidae in the Madeira Archipelago, and one of the few reports of microsporidians infecting serranids worldwide (Zhang et al. 2004, 2005; Wu et al. 2005; Marzouk et al. 2010; Jithendran et al. 2011).

The molecular analysis performed in this study revealed *G. serranus* n. sp. presenting the highest similarity to four *Glugea* infecting marine fish hosts: *G. epinephelusis*, *G. jazanensis*, *G. nagelia*, and *G. arabica* (Wu et al. 2005; Abdel-Baki et al. 2015a, b; Azevedo et al. 2016). Analyzing the morphological and ultrastructural features of the xenomas and mature spores, some differences were found (Table 2). Similarly to *G. serranus* n. sp., *G. nagelia* develops whitish xenomas, but its spores are ovoid to pyriform rather than ellipsoidal, present smaller measurements, and display the polar filament coiled in a higher number of polar filament coils and rows (Abdel-Baki et al. 2015a). *G. jazanensis*, *G. arabica*, and *G. epinephelusis* all differ from *G. serranus* n. sp. in forming brownish or darkish xenomas. Also, *G. jazanensis* and *G. epinephelusis* both present smaller spores, respectively displaying a higher and lower number of polar filament coils and rows (Wu et al. 2005; Abdel-Baki et al. 2015b). Of the four species, the spores of *G. arabica* are the most similar to those of *G. serranus* n. sp. in terms of shape and measurements; however, its spore wall is thinner (~75 nm) and the numbers of coils and rows of the polar filament are significantly higher. Also, the polaroplast of these two species displays some ultrastructural differences (Azevedo et al. 2016). Further morphological comparison of *G. serranus* n. sp. to its congeners revealed its spores as being one of the largest, with measurements that are similar to those of *Glugea fennica* Lom and Weiser, 1969 and *Glugea berlax* Lom and Laird, 1976, besides *G. arabica* as previously mentioned. Both of these species lack molecular data, being differentiated from *G. serranus* n. sp. on the basis of type locality, host specificity, and site of infection. *G. berlax* was reported from the musculature of the intestinal wall of *Macrourus berlax* in the sea at Grand Banks of Newfoundland, and the spores present a wider range in terms of size 6.4 μm (4.2 to 8.0) × 2.7 μm (2.4 to 3.1) (Lom and Laird 1976). The few data available for *G. fennica* report this parasite infecting the subcutaneous tissue of the body and fins of *Lota lota* in freshwaters of Finland and Northern Russia (Lom and Dyková 1992), thus suggesting that it has no relationship to *G. serranus* n. sp.

The current trend for the phylogenetic setting of microsporidia is the host habitat, according to which this parasitic group divides into three classes: Aquasporidia, Marinosporidia, and Terresporidia (Vossbrinck and Debrunner-Vossbrinck 2005). Microsporidia that parasitize fish are further subdivided in five groups (1 to 5), with most *Glugea* spp. clustering within group 2 (Lom and Nilsen 2003). In the past few years, several microsporidian species have been molecularly characterized, including some *Glugea* spp. that formed a new subclade within group 1, sister to the subclade containing *Pseudoloma neurophilia*, *Ichthyosporidium* spp., *Loma* spp., and *Microsporidium cerebralis* (Azevedo et al. 2016). The phylogenetic analyses here performed revealed *G. serranus* n. sp. clustering within the *Glugea* subclade of group 1, with a bootstrap support value of 100 %. Clustering within this subclade are other five marine species: *G. arabica*, *G. epinephelusis*, *G. jazanensis*, and *G. nagelia*, as well as a sequence of *Pleistophora*. The latter was reported infecting the marine host *Alepes djedaba*, a carangid fish from the Red Sea; nonetheless, is taxonomic classification remains dubious, since there is no publication associated with this submission on GenBank. A similar situation is observed with the sequence of *Pleistophora finisterrensis* (AF044393). Originally reported from the hypoaxial muscles of the fish host *Micromesistius poutassou* in the northwest coast of Spain (Leiro et al. 1996), this parasite forms one size of spore and consistently clusters within group 2, which comprises all other sequenced *Glugea* spp. (*G. stephani*, *G. atherinae*, *G. hertwigi*, *G. pagri*, *G. anomala*, *G. plecoglossi*, and *Glugea* sp. GS1). Acknowledging this phylogenetic positioning, Bell et al. (2001) addressed the possibility of this *Pleistophora* being misclassified. The taxonomic revision of microsporidians is not unusual, namely for the genus *Glugea*, for which the

reclassification of species has been performed. It is the case of *L. acerinae* (syn. *Glugea acerinae*), *Microgemma caulleryi* (syn. *Glugea caulleryi*), and *Spraguea americana* (syn. *Glugea americanus*) (Lom 2002). The future resolution of old taxonomic misconceptions is necessary to allow the discernment of the evolutionary characters driving *Glugea* phylogeny.

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