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Ultrastructural and phylogenetic description of *Zschokkella auratis* sp. nov. (Myxozoa), a parasite of the gilthead seabream *Sparus aurata*

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ABSTRACT: A new myxosporean, *Zschokkella auratis* sp. nov., infecting the gall bladder of the gilthead seabream *Sparus aurata* in a southern Portuguese fish farm, is described using microscopic and molecular procedures. Plasmodia and mature spores were observed floating free in the bile. Plasmodia, containing immature and mature spores, were characterized by the formation of branched glycostyles, apparently due to the release of segregated material contained within numerous cytoplasmic vesicles. Mature spores were ellipsoidal in sutural view and slightly semicircular in valvular view, with rounded ends, measuring 9.5 ± 0.3 SD (8.7–10.3) µm in length and 7.1 ± 0.4 (6.5–8.0) µm in width/thickness. The spore wall was composed of 2 symmetrical valves united along a slightly curved suture line, each displaying 10 to 11 elevated surface ridges. Two equal subspherical polar capsules, 3.7 ± 0.3 (3.0–4.1) µm long and 3.0 ± 0.2 (2.6–3.2) µm wide, were located separately at the spore's extremities. Each polar capsule contained a polar filament forming 4 to 5 coils. The sporoplasm was binucleate and contained numerous sporoplasmosomes. Morphological data, tissue tropism, and molecular analysis of the small subunit rDNA gene identified this parasite as a new species of *Zschokkella*. Maximum parsimony, neighbor-joining, and maximum likelihood inferences clustered the parasite in a subclade containing other *Zschokkella* species parasitizing the gall bladder of brackish and marine fish hosts, located within the coelozoic clade of the major freshwater clade; this supports the existence of a marine subclade within the 'freshwater' clade, as well as the existence of a correlation between tissue tropism and myxosporean phylogeny.

KEY WORDS: *Zschokkella auratis* sp. nov. · Myxosporean · Parasite · Ultrastructure · SSU rDNA gene · Phylogeny · *Sparus aurata* · Fish farm · Portugal

INTRODUCTION

The gilthead seabream *Sparus aurata* Linnaeus, 1758, is the most important mariculture species in the Mediterranean Sea, thus constituting one of the most valuable commercial fish for southern Portuguese fish farms. Eight myxosporean species, *Kudoa iwatai*, *Enteromyxum leei* (formerly *Myxidium leei*), *Ceratomyxa sparusaurati*, *Sphaerospora sparidarum* (formerly *Leptotheca sparidarum*), *Sphaerospora sparis* (formerly *Polysporoplasma sparis*), *Henneguya* sp., *Ceratomyxa* sp.1 ex *S. aurata*, and *Ceratomyxa* sp. 2 ex *S. aurata*, have been reported infecting the gilthead seabream in Mediterranean fish farms; some of

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these species were associated with increased morbidity and mortality rates (Diamant et al. 1994, 2005, Alvarez-Pellitero et al. 1995, Sitjà-Bobadilla et al. 1995, Bahri et al. 1996, Sakiti et al. 1996, Palenzuela et al. 1999, Sitjà-Bobadilla & Alvarez-Pellitero 2001, Cuesta et al. 2006, Alama-Bermejo et al. 2011). Most of these species are known to occur in southern European sparid-growing farms, but no further knowledge has been acquired concerning other potentially harmful myxosporeans.

During the first parasitological survey for myxosporean parasites infecting sparid fishes in a southern Portuguese fish farm, a new species of *Zschokkella* Auerbach, 1910 was detected in the gall bladder of *Sparus aurata*, and is described here based on ultrastructural and molecular data. The genus *Zschokkella* of the family Myxidiidae Thélohan, 1892 comprises about 68 species, most of which are coelozoic and, less frequently, histozoic, in freshwater and marine fishes worldwide, with a few representatives described from amphibians and reptiles (Lom & Dyková 1992, 2006). *Zschokkella* spores are ellipsoidal in sutural view and slightly semicircular in valvular view, with rounded or bluntly pointed ends, containing 2 subspherical polar capsules that open subterminally, both to one side (Lom & Dyková 1992, 2006).

The similarity of these morphological characteristics to those displayed by species belonging to the genus *Myxidium* Bütschli, 1882 makes the distinction difficult between these 2 genera. The application of molecular methodologies to the description of *Zschokkella* species is therefore crucial, despite the fact that it does not resolve every phylogenetic issue (Fiala 2006, Gunter & Adlard 2008). GenBank provides only 16 *Zschokkella* sequences of the small subunit ribosomal DNA (SSU rDNA) gene: 5 from freshwater fish hosts (Holzer et al. 2004, Fiala 2006, Bartošová & Fiala 2011) and 11 from marine fish hosts (Palenzuela et al. 2002, Diamant & Palenzuela 2005, Fiala 2006, Freeman et al. 2008, Holzer et al. 2010, Yemmen et al. 2013). Of these, only the sequence of *Z. nova* has been linked to its correspondent actinosporean stage (Uspenskaya 1995). A 17th SSU rDNA sequence termed *Z. mugilis* Sitjà-Bobadilla & Alvarez-Pellitero, 1993 remains in GenBank; however, this species was recently revised to *Ellipsomyxa mugilis* (Køie & Karlsbakk 2009), having also been linked to its correspondent actinosporean stage developing in an estuarine polychaete (Rangel et al. 2009, 2012). Overall, the molecular information regarding Myxosporea has revealed the existence of 2 main clades dividing freshwater and marine species (Kent et al. 2000, 2001, Fiala 2006, Bartošová et al. 2009, Holzer et al. 2010), for which myxosporean parasites infecting anadromous hosts, as well as some species of *Henneguya, Chloromyxum*, *Ceratomyxa*, *Myxobolus*, *Myxidium*, *Parvicapsula*, and *Sphaeromyxa*, constitute exceptions (Diamant et al. 2004, Fiala 2006, Rocha et al. 2013). Phylogenetic analyses have also shown that most myxosporeans cluster according to their host tissue tropism rather than by spore morphology or host species (Eszterbauer 2004, Holzer et al. 2004, Fiala 2006, Bartošová et al. 2009, Fiala & Bartošová 2010), and the genus *Zschokkella* is no exception (Holzer et al. 2010). Considering this overview, we sought to accurately describe the ultrastructural and phylogenetic features of the parasitic stages found in the gall bladder of *Sparus aurata*.

MATERIALS AND METHODS

Eighty-nine specimens of the gilthead seabream, *Sparus aurata* Linnaeus, 1758 (Teleostei, Sparidae) were collected between June 2012 and March 2013 from a fish farm facility on the Alvor estuary, near the Atlantic coast (37° 08′ N, 08° 37′ W), Portimão, Algarve, Portugal. Upon necropsy, microscopic analysis of several organs and tissues was performed for the detection of myxosporean parasites. Preliminary observations revealed myxosporean plasmodia and spores parasitizing the gall bladder of several specimens. The bile of the parasitized fish, as well as small fragments of the infected gall bladders, were collected and prepared for observation by light microscopy (LM), including differential interference contrast (DIC) optics (Leitz DM RBE, Leica), transmission electron microscopy (TEM), and scanning electron microscopy (SEM), as well as for molecular procedures.

TEM and SEM

For TEM, free spores and plasmodia isolated from the bile were fixed in 5% glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.4) at 4°C for 24 h, washed overnight in the buffer at the same temperature, and post-fixed in 2% osmium tetroxide with the same buffer for 3 h at 4°C. Dehydration in an ascending ethanol series and propylene oxide was followed by inclusion in epoxy embedding medium (EPON). Semi-thin sections were stained with methylene blue-Azure II for LM. Ultrathin sections were contrasted with uranyl acetate and lead citrate, observed and photographed using a JEOL 100CXII TEM operated at 60 kV.

For SEM, free spores isolated from the bile were fixed in 5% glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.4) at 4°C for 24 h, washed in the same buffer at the same temperature, dehydrated in an ascending ethanol series, critical point dried, coated with a gold-palladium alloy (60%), and observed and photographed in a JSM-6301F SEM operated at 15 kV.

DNA isolation and PCR amplification

Free spores and plasmodia obtained from the bile of several specimens were fixed and preserved in absolute ethanol at 4°C. Genomic DNA extraction was performed using a GenElute™ Mammalian Genomic DNA Miniprep Kit, following the manufacturer's instructions. The DNA was stored in 50 µl of TE buffer at −20°C until further use. The SSU rDNA gene was amplified using both universal primers and myxosporean-specific primers: the 5′-end with the primers 18e (5′-CTG GTT GAT CCT GCC AGT-3′; Hillis & Dixon 1991) and R1 (5′-CCT TCC GTC AAT TCC TTT AAG-3′; Azevedo et al. 2009); and the 3′-end with the primers MyxospecF (5′-TTC TGC CCT ATC AAC TTG TTG-3′; Fiala 2006) and 18r (5′-CTA CGG AAA CCT TGT TAC G-3′; Whipps et al. 2003). The sequence region between MyxospecF and R1 overlaps, allowing complete amplification of the SSU rDNA gene. PCRs were performed in 50 µl reactions using 10 pmol of each primer, 10 nmol of each dNTP, 2.5 mM $MgCl_2$, 5 µl of 10× *Taq* polymerase buffer (Finnzymes), 1.5 units of *Taq* DNA polymerase, and 3 µl (approximately 100–150 ng) of genomic DNA. The reactions were run on a Hybaid PxE Thermocycler, with initial denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 45 s, 53°C for 45 s, and 72°C for 90 s. The final elongation step was performed at 72°C for 7 min. Aliquots (5 µl) of the PCR products were electrophoresed through a 1% agarose 1× tris-acetate-EDTA buffer (TAE) gel stained with ethidium bromide. PCR products were purified using a single-step enzymatic cleanup that eliminates unincorporated primers and dNTPs.

DNA sequencing, and distance and phylogenetic analyses

The PCR products from different regions of the SSU rDNA gene were sequenced directly. The sequencing reactions were performed using a BigDye Terminator v1.1 from the Applied Biosystems Kit, and were run on an ABI3700 DNA analyzer (Perkin-Elmer, Applied Biosystems).

In order to determine the phylogenetic position of the new *Zschokkella* species amongst its closest relatives sequenced to date, namely other members of the genus *Zschokkella*, 58 myxosporean SSU rDNA sequences from GenBank were obtained and analyzed, according to highest similarity score. *Tetracapsuloides bryosalmonae* and *Buddenbrockia plumatellae* were selected as outgroup species. The alignment was performed with ClustalW in MEGA 5 software (Tamura et al. 2011), with an opening gap-penalty of 10 and a gap extension of 4 for both paired and multiple alignments, resulting in a total of 2380 positions in the final dataset. Subsequent phylogenetic and molecular evolutionary analyses were conducted using MEGA 5. Phylogenetic analyses were performed using maximum parsimony (MP), neighbor-joining (NJ), and maximum likelihood (ML) methodologies. For MP, the close neighbor interchange heuristic option with a search factor of 1 and random initial tree addition of 500 replicates was performed. For NJ, a Kimura 2-parameter substitution model with gamma distribution (shape parameter = 1.4) was performed. For ML, the general time reversible substitution model with 4 gamma-distributed rate variation among sites was performed. All positions with less than 95% site coverage were eliminated from all trees, resulting in a total of 725 positions in the final dataset, and the analysis was run using 500 bootstrap replicates in order to test the robustness of the resulting tree.

After a second alignment only for *Zschokkella* sequences (resulting in a total of 2194 positions in the final dataset) the distance estimation was carried out in MEGA 5, using the Kimura 2-parameter model distance matrix for transitions and transversions, with all ambiguous positions removed for each sequence pair.

RESULTS

Collected and analyzed gilthead seabream did not present external symptoms of infection or disease. Microscopic analysis of 12 different organs revealed the presence of both plasmodia and mature spores floating free in the bile of several specimens. The morphological and ultrastructural features of the mature spores identified the parasite as a member of the genus *Zschokkella*, according to the classification provided by Lom & Dyková (2006). A new species, named *Zschokkella auratis* sp. nov., is described

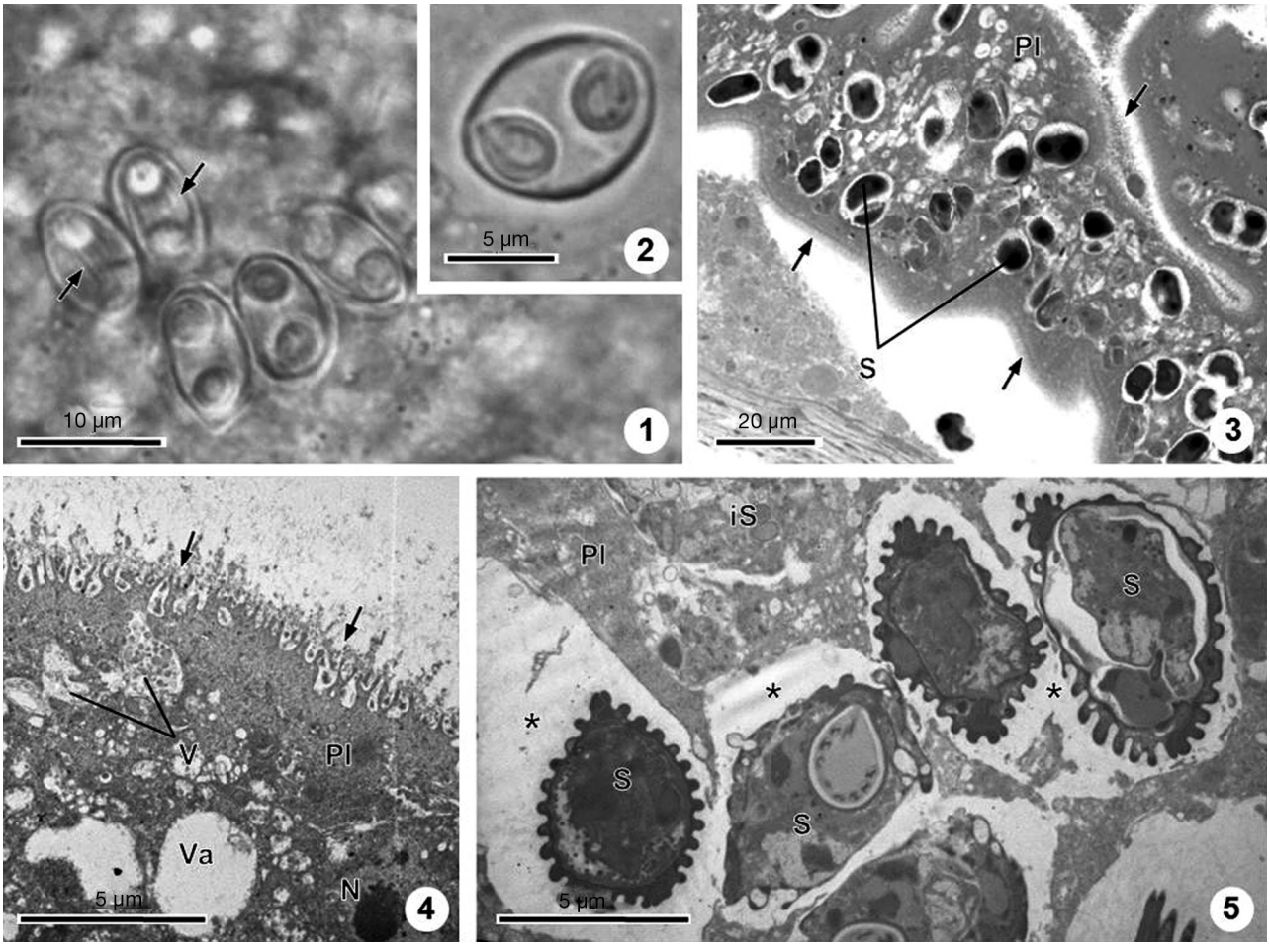
based on ultrastructural and molecular features, with the following taxonomic position:

Phylum: Myxozoa Grassé, 1970
Class: Myxosporea Bütschli, 1881
Order: Bivalvulida Shulman, 1959
Family: Myxidiidae Thélohan, 1892
Genus: *Zschokkella* Auerbach, 1910

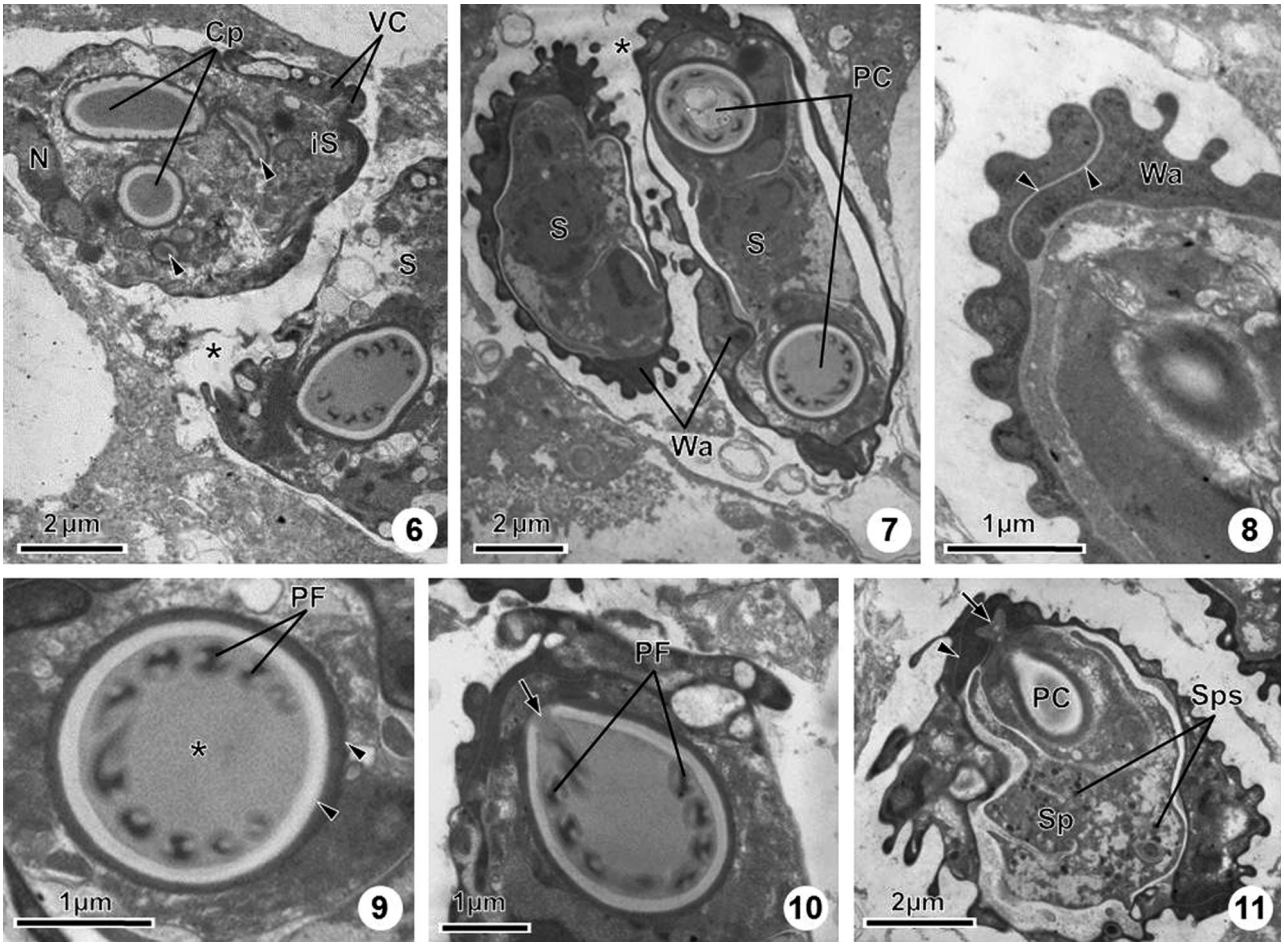
Zschokkella auratis sp. nov. (Figs. 1 to 14)

Diagnosis. Plasmodia and mature spores were observed floating free in the bile (Figs. 1 to 3).

Description. Plasmodia were surrounded by a highly irregular cell membrane, entirely covered by fine projections, from which ramified strands of mucus extended, forming glycostyles (Figs. 3 & 4). The surface membrane revealed marked aspects of exocytosis, rather than pinocytic vesicles or canals. The ectoplasmic margin displayed numerous vesicles containing granular material, as well as some dense granules and mitochondria (Fig. 4). The endoplasm further displayed vegetative nuclei, several large vacuoles (Figs. 4 & 5), and numerous disporoblasts simultaneously containing developing and mature spores (Figs. 3, 5 to 7). The earliest sporogenic stages were not observed. The final stages of sporogenesis, namely immature spores, were recognized by their valvogenic, capsulogenic, and sporoplasmogenic cells. In maturing spores, each capsulogenic cell formed a

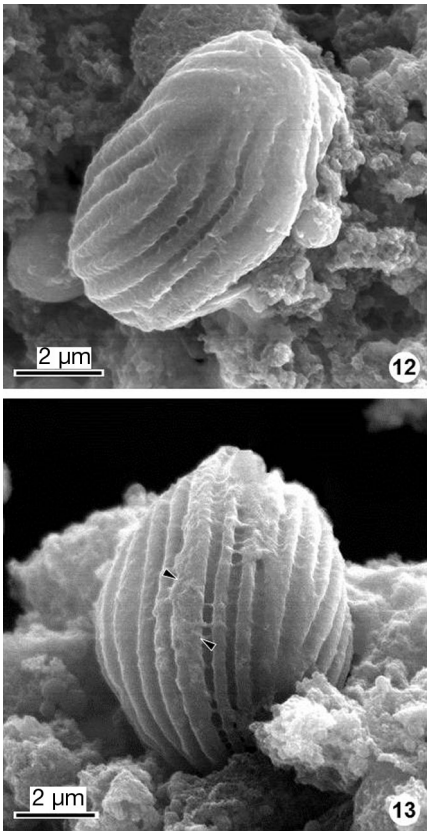


Figs. 1–5. Light and transmission electron micrographs of *Zschokkella auratis* sp. nov. infecting the gall bladder of *Sparus auratus*. Fig. 1. Free fresh mature spores observed in differential interference contrast optics. Notice the surface ridges (arrows) present in the spore wall. Fig. 2. Free fresh mature spore, slightly semi-circular in valvular view and containing 2 subspherical polar capsules. Fig. 3. Semi-thin section of a plasmodium (Pl) displaying irregular periphery (arrows) and containing numerous spores (S). Fig. 4. Ultrathin section of a plasmodium (Pl) containing vegetative nuclei (N), numerous segregating vesicles (V) and some large vacuoles (Va), and displaying a highly irregular cell membrane, apparently due to the release of segregated material (arrows). Fig. 5. Ultrathin section of a plasmodium (Pl) showing an immature spore (iS) and several mature spores (S) contained within disporoblasts (*)



Figs. 6–11. Transmission electron micrographs of *Zschokkella auratis* sp. nov. infecting the gall bladder of *Sparus auratus*. Fig. 6. Disporoblast (*) simultaneously containing an immature spore (iS) and a mature spore (S). Notice the 2 capsulogenic cells, each exhibiting a globular capsular primordium (Cp) extending into an external tubule (arrowheads), as well as the nucleus (N) of 1 of the valvogenic cells (VC). Fig. 7. Disporoblast (*) containing 2 mature spores (S), 1 of which is in transverse section, allowing visualization of the 2 valves comprising its wall (Wa), as well as the 2 polar capsules (PC) located at the same level. Fig. 8. Detailed aspect of the 2 valves uniting along a slightly curved suture line (arrowheads) to comprise the spore wall (Wa). Fig. 9. Transverse section of a polar capsule, displaying its double-layered wall (arrowheads) containing a homogeneous matrix (*) and the polar filament (PF). Fig. 10. Longitudinal section of a polar capsule, evidencing the cap-like structure (arrow) located at the apex of this structure, and from which the polar filament (PF) extends. Fig. 11. Oblique section of a spore showing the extrusion pore (arrow) of a polar capsule (PC) located near the suture line (arrowhead), as well as the sporoplasm (Sp) containing numerous sporoplasmosomes (Sps)

globular primordium that extended into an external tubule. The valvogenic cells appeared less dense than the mature valves and displayed remnant nuclei (Fig. 6). Mature spores were ellipsoidal in sutural view and slightly semi-circular in valvular view, with rounded ends, measuring 9.5 ± 0.3 SD (8.7–10.3) µm in length and 7.1 ± 0.4 (6.5–8.0) µm in width/thickness (n = 40; Figs. 1 & 2). Spore wall was thick, composed of 2 symmetrical valves united along a slightly curved suture line (Figs. 7 & 8). DIC and TEM revealed the presence of surface ridges projecting from the spore wall (Figs. 1, 5, 7 & 8). SEM showed each valve bearing 10 to 11 elevated surface ridges, parallel to the suture line, and forming a distinct pattern along the entire spore body (Figs. 12 & 13). Two equal-sized subspherical polar capsules, 3.7 ± 0.3 (3.0–4.1) µm long and 3.0 ± 0.2 (2.6–3.2) µm wide (n = 30), were located subterminally and at the same level within the spores, opening at nearly opposite positions (Figs. 1, 2 & 7). The polar capsules displayed a double-layered wall comprised of an outer electron-dense layer and an inner electron-lucent layer, containing a dense and homogeneous matrix, and along which the polar filament coiled in 4 to 5 turns, terminating in a cap-like structure (Figs. 9 & 10). Upon extrusion, each polar filament exits the



Figs. 12 & 13. Scanning electron micrographs of *Zschokkella auratis* sp. nov. infecting the gall bladder of *Sparus auratus*. Fig. 12. Spore in slightly oblique valvular view, allowing recognition of the surface pattern formed by the ridges that extend throughout the spore body. Fig. 13. Spore in sutural view, showing the surface ridges organized parallel to the suture line (arrowheads)

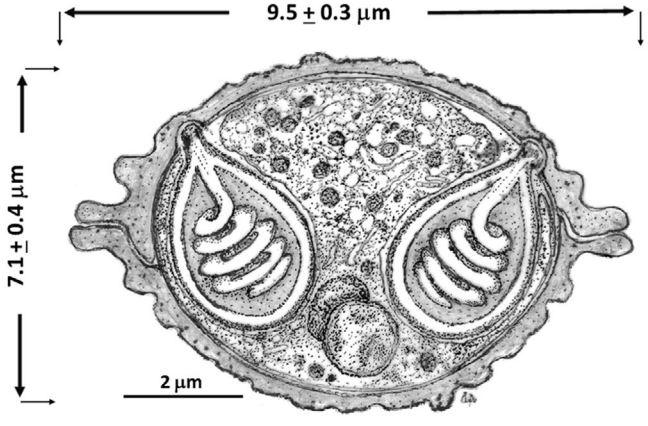


Fig. 14. *Zschokkella auratis* sp. nov. Schematic drawing of a spore, depicting the internal and external organization described in the 'Results'

spore body through its respective extrusion pore, located near the suture line (Fig. 11). A large binucleate sporoplasm surrounded the 2 polar capsules and contained numerous electron-dense sporoplasmosomes (Fig. 11). The ultrastructural features observed are represented in a schematic drawing (Fig. 14), providing a better view of the spore's morphology.

Type host. Gilthead seabream *Sparus aurata* Linnaeus, 1758 (Teleostei, Sparidae).

Type locality. Alvor estuary, near the Atlantic coast (37° 08′ N, 08° 37′ W), Portimão, Algarve, Portugal.

Site of infection. Gall bladder, floating free in the bile.

Prevalence. 11 specimens out of 89 (12.4%) were infected with *Zschokkella auratis* sp. nov.

Type specimens. One glass slide with semi-thin sections displaying a plasmodium containing several mature spores of the hapantotype was deposited in the Type Slide Collection of the Laboratory of Pathology, at the Interdisciplinary Centre of Marine and Environmental Research, Porto, Portugal, under CIIMAR 201.3.

Etymology. The specific epithet '*auratis*' derives from the specific epithet of the host species.

Molecular analysis

The resulting consensus DNA sequence of the SSU rDNA gene, composed of 1996 bp, was deposited in GenBank (accession number KC849425). In total, 58 SSU rDNA sequences, including those with the higher BLAST scores, were aligned with the SSU rDNA sequence obtained for *Zschokkella auratis* sp. nov. There were a total of 725 positions in the final dataset. MP/NJ/ML analyses of the SSU rDNA sequences placed the parasite within the coelozoic clade of the major freshwater clade, clustering together with the sequences of 5 other *Zschokkella* spp. parasitizing the gall bladder and *Myxobolus spirosulcatus* parasitizing the bile ducts of brackish and marine fish hosts, with a bootstrap support value of 85% for MP, 92% for NJ, and 92% for ML. The sequence of *M. spirosulcatus* (AB530261) obtained from the spinal cord of *Seriola quinqueradiata* also clusters within the coelozoic clade of *Zschokkella auratis* sp. nov., to join its other sequence (AB530263) obtained from the bile ducts of *Seriola dumerili* (Yokoyama et al. 2010). The remaining 6 sequences belonging to *Zschokkella* species infecting marine hosts cluster within the major marine clade: 3 together with *Sinuoelinea* sp. forming a clade of species isolated from the excretory system (kidney and urinary bladder); and the other 3 together

with *Ellipsomyxa mugilis* and *E. gobii* forming a clade of species infecting the gall bladder, sister to the marine histozoic clade represented by *Enteromyxum* and *Kudoa* species. The SSU rDNA sequences belonging to freshwater *Zschokkella* species all cluster within the major freshwater clade: those infecting the gall bladder among the subclades of the coelozoic clade (biliary and urinary tract), and *Zschokkella* sp. AH2003 with other species from the excretory system (kidney and urinary bladder). The histozoic freshwater clade is represented by *Myxobolus* species (Fig. 15).

Pairwise comparisons among the SSU rDNA sequences showed that the 5 *Zschokkella* sequences comprising this subclade are also those presenting a higher percentage of identity to *Zschokkella auratis* sp. nov., i.e. *Zschokkella* sp. PS030203 (93.8%), *Z. solaea* (92.8%), *Z. icterica* (92.7%), *Zschokkella* sp. 1 IF-2006 (92.6%), and *Zschokkella* sp. 3 IF-2006 (91.8%; Table 1).

DISCUSSION

The morphology of the myxosporean species described here is consistent with the characteristics defined for the genus *Zschokkella* by Lom & Dyková (2006), differentiated from the genus *Myxidium* based mainly on the position and organization of the polar capsules. Considering that the gilthead seabream inhabits marine and brackish habitats, and further acknowledging that *Zschokkella* species infect both freshwater and marine hosts, all *Zschokkella* records from the gall bladder, freshwater and marine, were considered for morphological comparison. Several studies have reported surface ridges as a reliable feature for morphological comparison (Lom & Dyková 1993, 2006, Bartošová & Fiala 2011); therefore, species with smooth valves were not considered. Among the ca. 68 known *Zschokkella* species, 13 species infecting the gall bladder and presenting spores characterized by the presence of surface ridges are relatively similar to *Z. auratis* sp. nov. but present significantly different morphometrics (Table 2). Morphological comparison between these species reveals the lack of truly differentiating characteristics between *Zschokkella* species, once more acknowledging the importance of molecular based taxonomic studies for this genus.

Table 1. Comparison of some small subunit ribosomal DNA sequences: percentage of identity (top diagonal) and nucleotide difference (bottom diagonal) obtained by Kimura-2 parameter analysis. The alignment resulted in a total of 2194 positions in the final dataset. For distance estimation, all ambiguous positions were removed for each sequence pair. GenBank accession numbers given in parentheses after the species names

Zschokkella species	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)
(1) *Z. auratis* sp. nov.	–	93.8	92.8	92.7	92.6	91.8	90.0	90.0	89.5	89.2	88.8	84.0	74.4	73.6	70.9	70.8	67.4	60.7	53.6
(2) *Z.* sp. PS030203 (DQ333435)	113	–	91.5	97.1	91.1	90.5	88.8	88.0	88.3	88.1	87.5	84.6	75.6	74.7	72.2	70.5	69.2	63.2	54.7
(3) *Z. solaea* (JX271832)	132	149	–	90.7	95.6	96.6	90.8	91.5	90.8	87.6	89.9	83.1	75.4	73.8	72.2	71.0	67.5	61.2	56.0
(4) *Z. icterica* (DQ333434)	131	54	163	–	90.8	90.0	88.3	88.4	88.2	88.1	87.3	84.3	75.5	74.1	71.4	69.8	68.8	62.7	54.5
(5) *Z.* sp. 1 IF-2006 (DQ377696)	66	79	40	81	–	95.1	88.8	88.0	89.3	86.9	88.3	86.4	75.7	74.1	71.8	74.1	72.5	66.9	50.9
(6) *Z.* sp. 3 IF-2006 (DQ377704)	129	140	55	148	45	–	89.3	90.1	90.0	87.9	89.6	82.4	75.6	73.7	73.3	73.6	69.4	60.4	56.5
(7) *Z. nova* (DQ377690)	184	196	170	205	98	169	–	97.7	89.5	98.0	89.7	82.3	75.1	73.3	71.3	69.8	67.2	58.7	53.4
(8) *Z. nova* (DQ377688)	184	194	159	204	96	161	45	–	89.5	99.9	89.8	83.0	75.1	73.3	71.1	69.7	67.7	57.9	53.3
(9) *Z.* sp. SA-2005 (DQ118776)	193	204	170	205	92	159	197	197	–	87.0	94.4	82.5	74.6	74.3	70.9	70.9	68.1	59.9	54.3
(10) *Z. nova* (GU471266)	71	79	82	79	86	80	14	1	84	–	86.1	85.0	72.3	68.7	64.4	69.1	69.7	60.9	56.1
(11) *Z. parasiluri* (DQ377689)	204	216	185	220	101	168	194	196	108	90	–	82.5	75.1	76.2	71.3	70.8	67.7	59.2	53.7
(12) *Z.* sp. DDI-2008 (FJ361238)	256	237	273	240	108	243	284	275	282	90	281	–	72.7	73.9	70.1	69.8	66.9	58.0	55.3
(13) *Z. neopomacentri* (DQ516379)	350	334	333	333	157	276	343	343	347	126	341	342	–	89.0	92.4	64.2	80.2	77.7	65.8
(14) *Z.* sp. 2 IF-2006 (DQ377700)	176	170	175	173	173	176	178	178	172	148	161	167	77	–	84.9	70.1	77.7	75.3	60.4
(15) *Z.* sp. IE-2005 (DQ452716)	392	376	376	384	191	302	388	390	392	170	386	376	113	108	–	62.5	78.5	75.6	62.1
(16) *Z.* sp. AH2003 (AJ581918)	378	382	376	388	198	312	390	391	375	174	376	365	389	194	424	–	58.8	55.8	48.7
(17) *Z. lophii* (DQ301509)	446	412	437	415	180	551	448	443	438	142	442	418	278	149	296	440	–	85.2	65.7
(18) *Z.* sp. 4 IF-2006 (DQ377705)	558	500	547	507	217	487	589	606	579	182	591	546	312	166	337	484	228	–	60.9
(19) *Z. hildae* (FM957569)	608	573	591	577	320	519	609	611	598	260	605	545	406	240	443	563	426	511	–

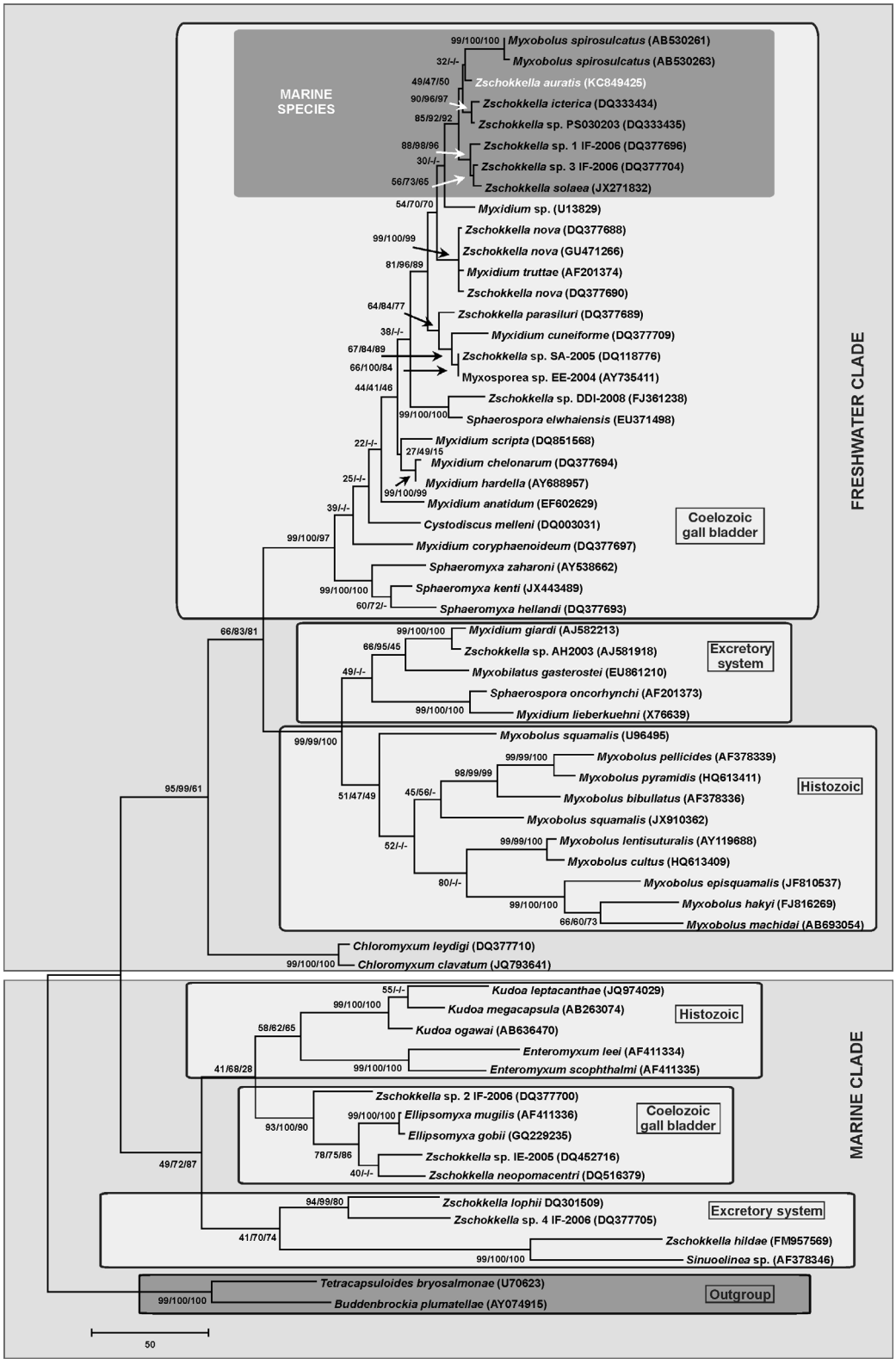


Fig. 15. Maximum parsimony tree of the small subunit ribosomal DNA (SSU rDNA) sequence of *Zschokkella auratis* sp. nov. and other selected myxozoan species. The numbers on the branches are bootstrap confidence levels on 500 replicates corresponding to the maximum parsimony, neighbor-joining, and maximum likelihood (MP/NJ/ML) trees. There were a total of 725 positions in the final dataset. GenBank accession numbers in parentheses given after the species name; scale is given under the tree

Table 2. *Zschokkella* spp. characterized by the presence of surface ridges and infecting the gall bladder of freshwater and marine fish. SL: spore length; SW: spore width; PCL: polar capsule length; PCW: polar capsule width; nFC: number of polar filament coils; nSR: number of surface ridges. Measurements are given in µm. n.g.: not given

Zschokkella spp.	Hosts	Location	SL	SW	PCL	PCW	nFC	nSR	Source
Zschokkella auratis sp. nov.	*Sparus aurata*	Portugal	9.5 ± 0.3 (8.7–10.3)	7.1 ± 0.4 (6.5–8.0)	3.7 ± 0.3 (3.0–4.1)	3.0 ± 0.2 (2.6–3.2)	4–5	10–11	Present study
Z. nova Klokacewa, 1914	*Carassius vulgaris*	Germany	9.5–11.5	6.5–7.0	3.0–3.5	3.0–3.5	n.g.	8–11	Klokacewa (1914)
Z. acheilognathi Kudo, 1916	*Acheilognathus lanceolatum*	Japan	10.0–14.0	6.0–7.0	2.0–3.0	2.0–3.0	n.g.	n.g.	Kudo (1916)
Z. parasiluri Fujita, 1927	*Parasilurus asotus*	Japan	11.94–14.0	4.0–6.0	3.7–5.0	3.5–4.5	6–7	5–6	Fujita (1927)
Z. ilishae Chakravarty, 1943	*Hilsa illisha*	Bay of Bengal	12.4	6.2	4.3	4.3	n.g.	n.g.	Chakravarty (1943)
Z. russelli Tripathi, 1948	*Gaidropsarus mediterraneus, Ciliata mustela*	Off England	13.2–16.6	8.6–9.9	2.5–3.5	2.5–3.5	n.g.	9–2	Tripathi (1948)
Z. striata Schulman, 1962	*Pseudogobio rivularis*	n.g.	12.9–14.0	6.3–7.0	4.2–5.6	3.8–4.2	n.g.	n.g.	Gong et al. (2003)
Z. heronensis Moser, Kent & Dennis, 1989	*Chaetodon plebeius, Choerodon venustus*	Off Australia	10.6 (10.0–11.0)	7.0	2.9 (2.5–3.0)	2.0	4–6	6–8	Moser et al. (1989)
Z. ganapatii Dorothy & Kalavati, 1992	*Liza macrolepis*	Bay of Bengal	12.8 (11.2–15.5)	9.8 (7.7–10.3)	3.4 (2.6–4.3)	3.4 (2.6–4.3)	n.g.	9–12	Dorothy & Kalavati (1992)
Z. leptatherinae Su & White, 1995	*Leptatherina presbyteroides; Atherinosoma microstoma; Kestratherina brevirostris; K. esox; K. hepsetoides*	Off Australia	15.3 (13.0–17.0)	11.8 (9.5–14.0)	3.9 (3.5–5.0)	3.4 (3.0–4.0)	4–5	10–12	Su & White (1995)
Z. saurogobionis Gong, Lu & Wang, 2003	*Saurogobio dumerili*	China	18.3 ± 1.0 (17.2–19.2)	9.8 ± 0.8 (9.0–10.8)	6.7 ± 0.5 (6.2–7.2)	6.7 ± 0.5 (6.2–7.2)	5	10–12	Gong et al. (2003)
Z. egyptica Ali, Abdel-Baki & Abdel-Ghaffar, 2007	*Plotosus lineatus, Upeneus tragula*	Red Sea	13.0±0.6 (12.2–15.4)	10.5±0.6 (9.5–11.0)	4.8±0.6 (4.2–5.2)	4.8±0.6 (4.2–5.2)	4	9 – 11	Ali et al. (2007)
Z. helmii Abdel-Ghaffar et al., 2008	*Siganus rivulatus*	Off Egypt	10.8 (10.0–11.0)	7.5 (7.0–8.0)	2.2 (2.0–3.0)	2.2 (2.0–3.0)	5	9 – 11	Abdel-Ghaffar et al. (2008)
Z. scomberosis Sarkar, 2012	*Scomberoides commersonianus*	Off India	17.8 ± 1.4 (16.5–19.5)	10.4 ± 1.4 (9.0–12.0)	4.9 ± 0.9 (3.7–5.2)	4.2 ± 0.6 (3.0–4.5)	n.g.	n.g.	Sarkar (2012)

Considering implications of the molecular analysis of *Zschokkella auratis* sp. nov. provided in this paper, it should first be noted that most myxosporean genera are poly-/paraphyletic, and this includes the species of *Zschokkella* (Kent et al. 2001, Freeman et al. 2008), which cluster within different subclades of the main freshwater and marine clades. Our molecular analysis of the SSU rDNA gene showed *Z. auratis* sp. nov. establishing phylogenetic relationships for MP, NJ, and ML in concordance with previously published cladograms (Fiala 2006, Freeman et al. 2008, Holzer et al. 2010). The formation of well-defined coelozoic and histozoic clades for both the freshwater and marine clades, and the fact that *Z. auratis* sp. nov. clusters together with other *Zschokkella* sequences from the gall bladder of marine and brackish fish hosts, as well as *Myxobolus spirosulcatus* from the bile ducts, strengthens the contention that tissue tropism constitutes an important criterion for the recognition of myxosporean phylogeny (Holzer et al. 2004, 2010, Fiala 2006, Bartošová et al. 2009, Fiala & Bartošová 2010).

Concerning the molecular based division of myxosporeans into the major freshwater and marine clades (Kent et al. 2000, 2001, Fiala 2006, Bartošová et al. 2009, Holzer et al. 2010), we note that the clade comprising *Zschokkella auratis* sp. nov. constitutes yet another exception to this main division. Several studies show that myxosporean parasites infecting anadromous hosts, as well as some species of *Henneguya, Chloromyxum*, *Ceratomyxa*, *Myxobolus*, *Myxidium*, *Parvicapsula*, and *Sphaeromyxa*, constitute exceptions to the major division into the freshwater and marine clades (Diamant et al. 2004, Fiala 2006, Azevedo et al. 2009, Rocha et al. 2013). However, 4 of the 6 species comprising the clade containing *Z. auratis* sp. nov. contradict this hypothesis: *Zschokkella* sp. PS030203, *Zschokkella* sp. 3 IF-2006, and *Myxobolus spirosulcatus* were sequenced from strictly marine fish hosts; and, although *Zschokkella* sp. 1 IF-2006 was sequenced from *Eugerres plumier*, a fish known to inhabit marine, freshwater, and brackish waters, the host specimens were captured from the Caribbean Sea, a clearly marine environment (Fiala 2006). Another possible explanation for the existence of a marine subclade within the major freshwater clade lies in the variability of the SSU rDNA gene regions that are expected to be conserved in myxosporean species. Freeman et al. (2008) considered this to be the reason preventing the design of truly 'universal' myxozoan primers. Furthermore, many sequences in GenBank, as well as in other molecular databases, constitute incomplete records or lack more vigorous pruning of the ambiguous regions in primary alignments, thus hindering the true acknowledgment of myxosporean phylogeny. Nevertheless, to assess these possibilities, additional information from the SSU rDNA gene of other myxosporeans, as well as of other genes, would be necessary.

Overall, no significant relationship between spore morphology and phylogenetic grouping was evident. The spore-based taxonomy is rather artificial between *Zschokkella* and *Myxidium*, as well as other genera, with the molecular approach enhancing the already vague boundaries between these genera (Fiala 2006, Lom & Dyková 2006, Freeman et al. 2008). Further research must strive to revaluate the taxonomy and phylogeny of these taxa.

Turning to consider the ultrastructure of the sporogenic development of *Zschokkella auratis* sp. nov., it is assumed that the lack of early sporogenic stages and prevalence of mature spores in the plasmodia indicate a somewhat synchronous development. The ultrastructural aspects of plasmodial development described here are not similar to those usually recognized in coelozoic myxosporeans, viz. variations in size and the differentiation of peripheral projections for nutritional intake (Sitjà-Bobadilla & Alvarez-Pellitero 1993, 2001, Rocha et al. 2011). In the case of *Z. auratis* sp. nov., the irregularity of the plasmodia surface appears to result from the exocytosis of segregated material contained within the numerous cytoplasmic vesicles, rather than from the formation and differentiation of peripheral projections. The contents of the vesicles appear to incorporate the mucus branches, forming the glycostyles that cover the entire surface of the plasmodia. The latter constitute a rather rare feature for myxosporean plasmodia, with a few species exhibiting similar structures (Current 1979, Current et al. 1979, Lom 2004). Curiously, spores of *Henneguya pilosa* display a similar glycostyle-like coating (Azevedo & Matos 2003). The factors interfering with the differentiation of cell coats in plasmodia, however, remain unknown, but may be involved with the physical and chemical strengthening of the cellular membrane towards the host immune response, as well as the physical and biological conditions of the organ of infection.

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