

Ortholinea saudii sp. nov. (Myxosporea: Ortholineidae) in the kidney of the marine fish *Siganus rivulatus* (Teleostei) from the Red Sea, Saudi Arabia

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ABSTRACT: Myxozoans, a diverse group of microscopic obligate endoparasites, can cause diseases in a number of economically important fish, including the marbled spinefoot *Siganus rivulatus*. To date, only 1 myxozoan, *Zschokkella helmii*, has been reported to infect *S. rivulatus*. Here we describe another myxozoan detected in *S. rivulatus*. Investigations of 40 marbled spinefoot fish caught from the Red Sea coast, Jeddah, Saudi Arabia, revealed clusters of parasitic spores in the kidney. Light microscope examination of the native spores revealed sub-spherical, mature spores with smooth shell valves. The 2 polar capsules were equal in size, and the polar filament was perpendicular to the longitudinal axis of the polar capsules. Histopathological examinations of the infected kidneys demonstrated the presence of both spores and developmental stages in the lumen of the renal tubules without any pathological effect. Electron microscopy investigations showed maturing spores composed of 2 valvogenic cells, each with a prominent nucleus. The valvogenic cells enclosed 2 polar capsules containing 3 filament coils as well as a binucleated sporoplasm cell filling the space between and beneath the 2 polar capsules. BLAST search analysis of the amplified sequence from the detected parasite indicated a high percent of identity to the 18S rDNA genes of different myxosporean species. Phylogenetic analysis placed the detected parasite within a clade of *Ortholinea* sp. (AL-2006). Based on the results of the light and electron microscopy, histopathological, and molecular investigations, the detected parasite was identified as a myxosporean parasite belonging to the genus *Ortholinea* and designated as *O. saudii* sp. nov.

KEY WORDS: Marine fish · Red Sea fish · Myxospores · Endoparasites · Marbled spinefoot

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INTRODUCTION

Myxozoa is a diverse phylum comprising more than 2180 described species assigned to 62 genera, several of which can cause diseases in a number of economically important fish (Lom & Dyková 2006). Myxozoa belonging to the genus *Ortholinea* Shul-

man, 1962 are coelozoic parasites that inhabit the urinary system of marine and freshwater fish (Lom et al. 1992, Lom & Dyková 2006). The genus *Ortholinea* is represented by more than 12 species (Lom & Dyková 2006), of which only 2 were described from freshwater hosts (Abdel-Ghaffar et al. 2008b). Myxospores of this genus are spherical to sub-spherical

with a prominent sutural ridge and polar capsules that are subspherical to pyriform (Lom & Dyková 2006). Most members of this genus are characterized by the presence of external striations or ridges on the myxospore shell valves (Ali 2000). The marbled spinefoot *Siganus rivulatus* Forsskål & Niebuhr, 1775, is an economically important marine herbivorous fish that is suitable for aquaculture (Juario et al. 1985, Hara et al. 1986, El-Dakar et al. 2007, 2011). It inhabits shallow waters in the Red Sea not exceeding 60 m and travels in schools of up to 100 individuals over algae-covered and rocky bottoms (Randall 1992). As in the Indo-Pacific region, *S. rivulatus* is caught in large quantities by gill and trammel nets for commercial purposes. According to the available literature, only 1 myxozoan species, *Zschokkella helmii*, has been reported to infect *S. rivulatus* in Egypt (Abdel-Ghaffar et al. 2008a).

Here we describe the morphology and molecular characterization of a distinct ortholinid parasite from the renal tubules of *S. rivulatus* from the Red Sea in Saudi Arabia.

MATERIALS AND METHODS

Fish specimens

In total, 40 freshly caught marbled spinefoot, ranging from 16 to 20 cm in total length, were obtained from fishermen at Jeddah on the Red Sea coast (21° 31' N, 39° 13' E). Fish were necropsied and organs and body fluids examined for myxosporean infection using microscopy. Fresh myxospores were first photographed and then measured using a calibrated ocular micrometer on an Olympus microscope. Myxospores were described according to Lom & Arthur (1989). Measurements were made on 30 spores and are given in micrometers as arithmetic mean \pm SD (range).

Light and electron microscopy

For light microscopy, infected kidney samples were fixed in 10% neutral buffered formalin. The fixed tissues were then processed for histological examination by being sectioned and stained with hematoxylin and eosin (H&E). For electron microscopy, the infected kidney samples were immediately fixed in 3% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.3) for 24 h and then washed in the same buffer. Post fixation was done in 2% OsO₄ in the same buffer

for 2 h at 4°C, followed by dehydration in an ethanol series. Samples were then processed to be embedded in araldite. Semi-thin sections were stained with toluidine blue and safranin. Ultra-thin sections were contrasted with uranyl acetate and lead citrate and then examined by transmission electron microscopy (TEM; Zeiss EM 109)

DNA extraction

Kidney tissues were collected from samples that revealed myxosporean infection by light microscopy examination and preserved in 95% ethanol for DNA extraction. DNA was extracted using a QIAamp DNA mini kit, following the tissue protocol (Qiagen). Kidney tissues were processed by thorough grinding in liquid nitrogen with a mortar and pestle. Subsequently, 25 mg of the tissue powder was placed into a liquid nitrogen-cooled 2 ml micro-centrifuge tube and incubated with Proteinase K and lysis buffer at 56°C until complete lysis of the tissues. DNA extraction was then completed as per the manufacturer's instructions and eluted in 100 μ l elution buffer. A negative extraction control (a sample containing sterile water) was performed to control for contamination during the extraction process. Quality and quantity of the purified DNA were assessed by measuring the optical density at 260 and 280 nm. DNA samples were stored in aliquots at -20°C until required.

PCR amplification

The 18S rDNA gene of the suspected myxosporean parasite was amplified by nested polymerase chain reaction (nPCR) using universal eukaryotic primers (ERIB1 and ERIB10) and myxosporean-specific 18S rDNA primers (Myxospec-F and Myxospec-R) according to Barta et al. (1997) and Fiala (2006), respectively, with some modifications. Amplification was performed in a 25 μ l reaction volume with 2 \times Reddy Mix PCR Master mix (Thermo Scientific) which contained 75 mM Tris-HCl (pH 8.8), 20 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 0.01% Tween-20, 0.2 mM each nucleotide triphosphate, 1.25 U Thermoprime Plus DNA polymerase, red dye for electrophoresis, 3 μ l of DNA template, and 10 pmol each of ERIB1 (5'-ACC TGG TTG ATC CTG CCA G-3') and ERIB10 (5'-CTT CCG CAG GTT CAC CTA CGG-3') primers. The amplification was carried out in a Mastercycler Gradient thermocycler (Eppendorf) with the following cycling profile: 95°C for 3 min, then 35 PCR cycles of

95°C for 1 min (denaturation), 48°C for 1 min (annealing), and 72°C for 2 min (extension), with a final extension step of 72°C for 10 min. Two µl from the initial PCR products were used as a template for the nested PCR using 10 pmol each of Myxospec-F (5'-TTC TGC CCT ATC AAC TWG TTG-3') and Myxospec-R (5'-GGT TTC NCD GRG GGM CCA AC-3') primers. PCR conditions were the same as in the first round with an annealing temperature of 52°C.

Detection of PCR products

PCR amplification products were subjected to electrophoresis analysis on 1.5% agarose in Tris acetate-EDTA buffer (0.04 M Tris acetate, 1 mM EDTA), stained with ethidium bromide and visualized on a UV transilluminator. A DNA molecular weight marker (Biozym) was used to comparatively determine the molecular size of the PCR amplicons.

Cloning and sequencing of PCR products

For sequencing, the PCR products were separated from the agarose gel, excess primers, and unincorporated nucleotides using the MinElute gel extraction kit (Qiagen) as per the manufacturer's instructions, and then cloned into the pDrive Cloning Vector using a Qiagen PCR Cloning plus Kit according to the manufacturer's instructions. Recombinant plasmids were purified from *Escherichia coli* using a QIAprep Miniprep kit (Qiagen) following to the manufacturer's instructions. Plasmid concentrations were determined with a Biophotometer (Eppendorf). Cloned PCR products were sequenced in a commercial sequencing laboratory (LGC Genomics).

Phylogenetic analysis

Sequenced products were subjected to Basic Local Alignment Search Tool (BLAST) analysis to search for sequence similarity in the GenBank database according to Altschul et al. (1997). Sequences were downloaded from GenBank for phylogenetic analysis, and *Buddenbrockia plumatellae* (FJ981824) was used as an outgroup species. Multiple sequence alignments were generated using Clustal X software (Thompson et al. 1997). A phylogenetic tree based on 18S rDNA gene sequences was created using the neighbor-joining (NJ) algorithm of the DNASTAR software with the default setting of the program

according to Saitou & Nei (1987). Bootstrap confidence values were calculated with 1000 repetitions and random number generator seed = 111.

RESULTS

Light microscopy

All investigated organs were normal without any pathological alterations. Two out of 40 marbled spinefoot fish examined were found to be infected with Myxosporea. The infection was reported as clusters of myxospores in fresh squash preparations of the kidney.

Spore description

Mature myxospores are subspherical in the frontal view, slightly wider than long, with a length of 10 ± 0.4 (9–11) µm and a width of 12 ± 0.5 (11–13) µm (Fig. 1). Shell valves were smooth and the sutural line was indistinct. The 2 polar capsules were equal, spherical, and occupied nearly half of the myxospore length. They measured 4.5 ± 0.3 (4.0–5.0) µm in diameter. The polar filament with 3 coils was perpendicular to the longitudinal axis of the polar capsules. Sporoplasm was binucleated, single-celled, and filled the entire extracapsular myxospore cavity.

Histopathology

Examination of the histological sections revealed that all infected kidneys harbored both myxospores and various developmental stages in the lumen of the renal tubules and rarely in the glomeruli (Fig. 2). Infected tubules were often completely occluded by parasites in various stages of development (Fig. 2).

Electron microscopy

The infection appeared as many plasmodia completely filling the lumen of the infected renal tubules. The earliest developmental stages observed, within the plasmodia, were 1-cell stages (generative cells). These cells were nearly spherical with a diameter of about 3 µm. The cytoplasm was bounded by a single-unit membrane and contained a number of mitochondria. The centric nucleus was large and contained a prominent nucleolus (Fig. 3a). The next

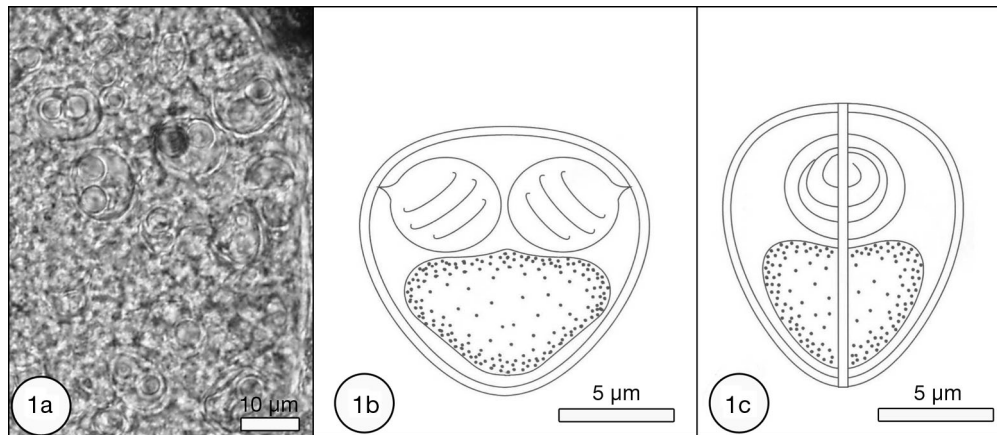


Fig. 1. Spores of *Ortholinea saudii* sp. nov. infecting the kidney of marbled spinefoot *Siganus rivulatus* from the Red Sea in Saudi Arabia. (a) Fresh spores in squash preparation of the kidney, and schematics of a mature spore in (b) frontal and (c) sutural views

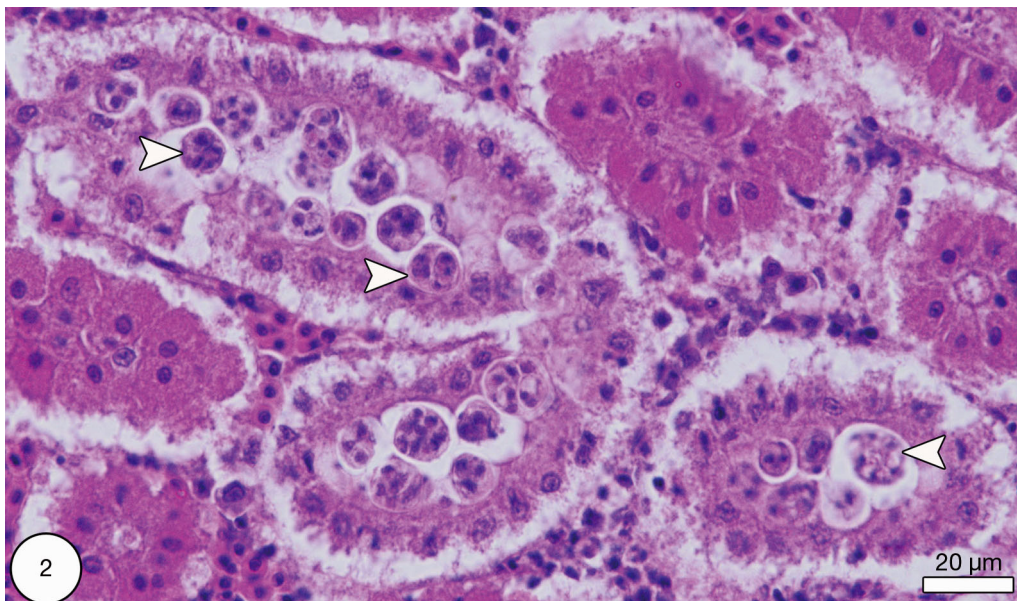


Fig. 2. Histology of marbled spinefoot *Siganus rivulatus* kidney infected with *Ortholinea saudii* sp. nov. Infected kidney tubules were often completely occluded by spores and parasites in various stages of development (arrowheads; see Fig. 3 for details)

recognizable stage was the 3-cell stage which was composed of 2 secondary cells enclosed in a primary cell (Fig. 3b). This was followed by a series of divisions of the former which gave rise to pansporoblasts (Fig. 3c). Each pansporoblast produced 1 or 2 myxospores (Fig. 3d). The mature myxospores were composed of 2 valvogenic cells with a prominent nucleus enclosing 2 polar capsules with 3 filament coils and a binucleated sporoplasm filling the space between the 2 polar capsules (Fig. 3d).

Molecular analysis

Myxosporean-specific 18S rDNA primers amplified a single 831 bp amplicon from each sample examined. No amplification products were detected from the negative extraction or no-template controls. Alignment of these sequences together revealed 100% similarity between each other. A BLAST search of the amplified sequence against the GenBank database revealed 90% similarity (with 79%

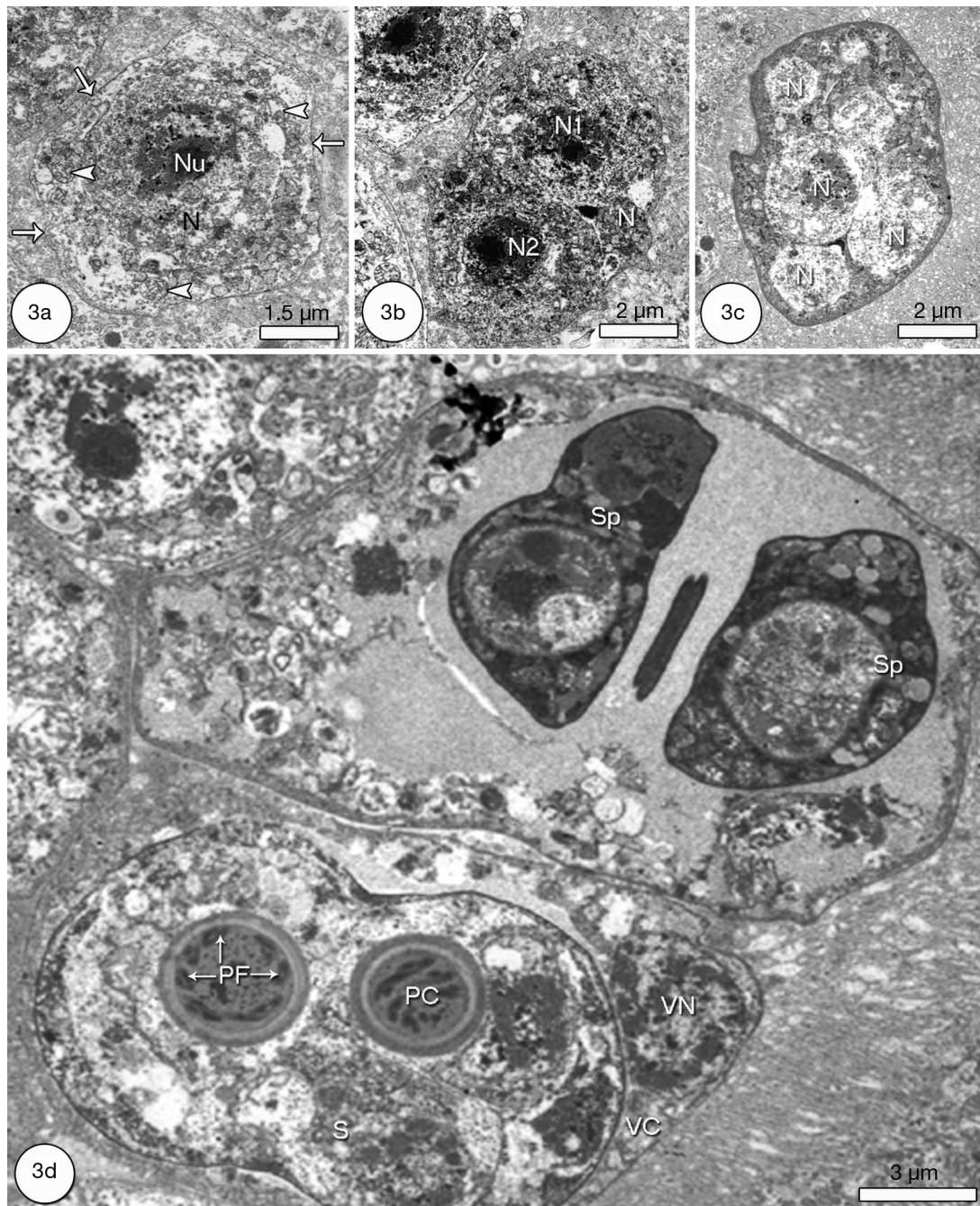


Fig. 3. Electron micrographs through a part of the kidney of marbled spinefoot *Siganus rivulatus* infected with *Ortholinea saudii* sp. nov., showing the different stages of development. (a) Unicellular stages (generative cell) surrounded by single unit membrane (highlighted by arrows) with a large nucleus (N), a prominent nucleolus (Nu), and mitochondrial structures (arrow-heads). (b) Two secondary cells (N1, N2) enclosed in a primary cell (N). (c) Pansporoblast with many nuclei (N). (d) Disporic pansporoblast with 2 spores (Sp) and mature spores containing 2 polar capsules (PC) with 3 polar filament coils (PF) and sporoplasm (S) and surrounded by valvogenic cells (VC) with the valvogenic nucleus (VN)

query coverage) to the 18S rDNA gene of *Ortholinea* sp. (AL-2006) and an 86% similarity (with 70–73% query coverage) to 18S rDNA gene each of *Myxobilatus gasterostei*, *O. orientalis*, *Myxidium anatum*,

Aurantiactinomyxon pavinsis, *Chloromyxum* sp., *Neoactinomyxum eiseniellae*, and *Zschokkella* sp. A phylogenetic tree of these results is presented in Fig. 4. The sequence obtained from this study was

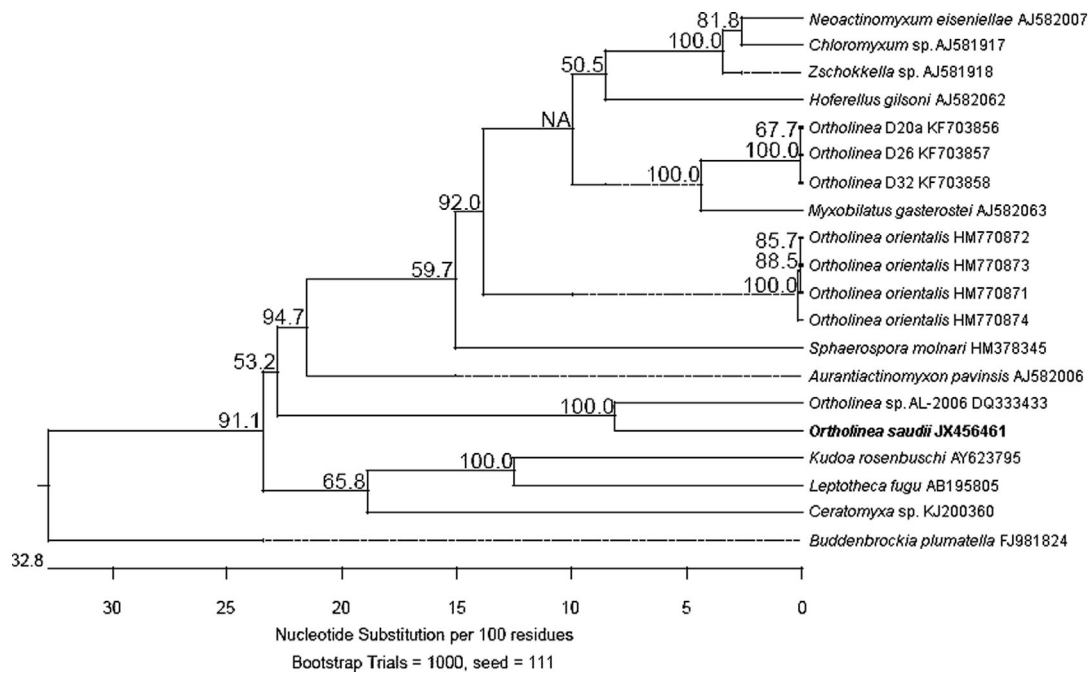


Fig. 4. Phylogenetic tree showing the relative positions of *Ortholinea saudii* sp. nov. to 18 other myxosporidian species. The tree is based on nucleotide sequences of the 18S rDNA gene of myxosporidians deposited in GenBank and was generated using the neighbor-joining method. The tree was oriented by using the 18S rDNA sequence of *Buddenbrockia plumatella* as the outgroup. Numbers above the branches are bootstrap confidence levels. GenBank accession numbers are given after the species name

deposited in GenBank under accession number JX456461.

Taxonomic summary of *Ortholinea saudii* sp. nov. (Myxosporaea: Ortholineidae)

Type host: *Siganus rivulatus* Forsskål & Niebuhr, 1775

Location in the host: Kidney (lumen of the renal tubules and rarely in the glomeruli)

Type locality: Jeddah Red Sea coast, Saudi Arabia (21° 31' N, 39° 13' E)

Type specimens: A partial sequence of the 18S rDNA gene was deposited in GenBank under accession number JX456461.

Prevalence of infection: 5% (2/40)

Etymology: The specific epithet '*saudii*' was derived from the locality of the host, i.e. Saudi Arabia

Spore shape and structure: Subspherical in the frontal view, slightly wider than long

Shape of polar capsules: Spherical

Polar filament: With 3 coils perpendicular to the longitudinal axis of the polar capsules

Sporoplasm: Binucleated

DISCUSSION

To date, 14 species of *Ortholinea* have been described (Table 1). *O. australis* can easily be distinguished from *O. saudii* sp. nov. by having more rounded spores and oval polar capsules compared to the present myxospores which are characterized by rounded, triangular-shaped, and spherical polar capsules. In addition, *O. australis* exhibits different numbers of valvular ridges (5 to 9 vs. 10 to 14). *O. gobiusi* also differs from *O. saudii* in having pointed posterior end myxospores in addition to markedly smaller polar capsules, while *O. undulans* has pyriform capsules and undulated sutures in contrast to the spherical capsules and the straight sutures exhibited by *O. saudii*. In the same way, *O. fluviatilis* can be distinguished from *O. saudii* by having ellipsoidal myxospores with undulated sutures. Also, *O. striateculus* has larger and pyriform polar capsules, while *O. divergens* has subspherical, pyriform capsules, and the large hazelnut-shaped and irregular ellipsoidal myxospores of *O. irregularis* can be easily differentiated from the rounded triangular myxospores of *O. saudii*. On the other hand, spores of *O. polymorpha* have smooth surfaces and elongated polar capsules

compared with the ridged myxospore valves and spherical polar capsules exhibited by *O. saudii*. *O. orientalis*, *O. antipae*, and *O. gadusiae* have thinner spores with smaller polar capsules. Also, *O. orientalis* and *O. antipae* have spore ridges with triangular intercapsular processes. Furthermore, the combination of different hosts and geographic locations separate all compared species from *O. saudii* (Table 1). *O. saudii* clearly does not match the characters of any of the species discussed above, and thus it is suggested as a new species.

With the exception of *O. fluviatilis*, thus far no ultrastructural study of any *Ortholinea* species has been reported. Therefore, we had difficulties in comparing some characters in relation to our results. However, the ultrastructure of the myxospore morphogenesis followed the usual pattern valid for most myxosporean genera (Ali et al. 2007, Abdel-Baki 2011, Azevedo et al. 2011).

Regarding the host habitat, *Ortholinea* was believed to be restricted to marine fishes only. *O. fluviatilis* and *O. africanus* (Lom & Dyková 1995) are the only 2 exceptions described from freshwater fishes. Thus, it can be concluded that the genus *Ortholinea* shares freshwater as well as marine fish hosts.

Conserved genes are the best choice for phylogenetic analysis. One of these conserved genes is the 18S rDNA, which is widely used for revealing phylogenetic relationships among taxa (Avisé 2004). Although the universal eukaryotic primers failed to amplify the 18S rDNA gene from the tested samples, 831 bp were successfully amplified using the myxosporean-specific 18S rDNA primers as nested primers. These nested primers amplify partial 18S rDNA sequences (800–950 nt) from myxosporean species (Fiala 2006). Sequence analysis of the amplified products displayed varying percentages of identity to different myxosporean species. The overall similarity, including gaps, of the sequenced samples to other myxosporean sequences was 86 to 90%, as mentioned in the 'Results', whereas the overall similarity, including the aligned position, was 60.1 to 69.7%. *Ortholinea* sp. (AL-2006) was the closest sequence to the isolated sample sequence and showed 90 and 69.7% overall similarity including gaps or aligned position, respectively. This percent of homology is enough to relate this parasite to the genus *Ortholinea*. However, it failed to show a high percent of homology to any species of the genus *Ortholinea* deposited in GenBank. Results of the phylogenetic tree also grouped the new parasite with *Ortholinea* sp. (AL-2006) in 1 clade

Table 1. Comparative descriptive measurements (means, with ranges in parentheses; μm) of *Ortholinea saudii* sp. nov. and morphologically similar species. nd: no data

Species	Host	Site of infection	Locality	Spore		Polar capsule		References
				Length	Thickness	Length	Width	
<i>O. divergens</i>	<i>Hippoglossoides platessoides</i>	Urinary bladder	North Atlantic	9.2	9.4	2	2.4	Shulman (1966)
<i>O. polymorpha</i>	<i>Opsanus tau</i>	Urinary bladder	US Atlantic coast	6.5–10	nd	4.4	2.4	Lom & Dyková (1992)
<i>O. orientalis</i>	<i>Clupea</i> spp.	Kidney, urinary and gall bladders	Northern Pacific	7.8	6	2.5	2.1	Shulman & Shulman-Albova (1953)
<i>O. irregularis</i>	<i>Drepanopsetta platessooides</i>	Urinary bladder	North Sea	10.6 (8–11)	7.1 (6.0–9)	2.2	2.2	Kabata (1962)
<i>O. gobiusi</i>	<i>Gobius ophicephalus</i>	Urinary bladder	Black Sea	8.8	8.4	1.9	1.9	Lom & Dyková (1992)
<i>O. undulans</i>	<i>Peltothamphus novaezelandiae</i> and <i>Caulopsetta scapha</i>	Urinary bladder, ureter	New Zealand	8.3 (7–10)	7.4 (6–9)	2.9 (2–4)	2.2 (2–3)	Meglitsch (1970)
<i>O. alata</i>	<i>Chaetodon rainfordi</i>	Kidney tubules	Australia	12.6	9.6	4.6	4.6	Kent & Moser (1990)
<i>O. australis</i>	<i>Acanthopagrus australis</i> and <i>Rhabdosargus sarba</i>	Hepatic duct	Australia	8.7 (7.8–10.4)	8 (7.3–9.5)	3.7 (2.8–4.4)	2.9 (3.3–3.2)	Lom et al. (1992)
<i>O. striatoculus</i>	<i>Leptatherina presbyteroides</i>	Ureters	Australia	10.1	10	3.5	2.9	Su & White (1994)
<i>O. fluviatilis</i>	<i>Tetraodon fluviatilis</i>	Renal tubules	South-east Asia	8.3 (7.9–8.4)	7.8 (7.3–8.0)	3.1 (2.8–3.3)	3.1 (2.8–3.3)	Lom & Dyková (1995)
<i>O. gadusiae</i>	<i>Gadusia chapra</i>	Urinary bladder	Bay of Bengal, India	10.8 (9.0–11.7)	8.0 (7.2–9.0)	3 (2.3–3.2)	nd	Sarkar (1999)
<i>O. basma</i>	<i>Clinus agilis</i>	Urinary bladder	South Africa	13.5 (12–15)	12.3 (11.8–13)	4.3 (4–4.8)	3.5 (3–4.3)	Ali (2000)
<i>O. antipae</i>	<i>Alosa caspia</i>	Urinary bladder	Black Sea	6.8–7.5	5.0–5.4	1.8–2.5	nd	Moshu & Trombitsky (2006)
<i>O. africanus</i>	<i>Oreochromis niloticus</i>	Urinary bladder	Egypt	7.71 (6.93–8.47)	7.67 (6.93–8.47)	2.87 (2.31–3.85)	2.87 (2.31–3.85)	Abdel-Ghaffar et al. (2008a)
<i>Ortholinea saudii</i> sp. nov.	<i>Siganus rivulatus</i>	Kidney	Saudi Arabia	10 (9–11)	12 (11–13)	4.5 (4.0–5.0)	4.5 (4.0–5.0)	Present study

away from the other *Ortholinea* species used in constructing this tree.

Based on the spore morphology and the molecular data, which differ from the known *Ortholinea* species, we propose to establish the present species as a new one, with the name *Ortholinea saudii* sp. nov.

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