Morphology and phylogeny of two new species of *Sphaeromyxa Thélohan*, 1892 (Cnidaria: Myxozoa) from marine fish (Clinidae and Trachichthyidae)

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SUMMARY

Our survey of marine fish from South Africa and Indonesia revealed the presence of two new myxosporean species of the genus *Sphaeromyxa* for which we provide morphological and sequence data. *Sphaeromyxa clini* n. sp. detected in three *Clinus* spp. and *Muraenoclinus dorsalis* from South Africa is morphologically similar to *Sphaeromyxa noblei* previously described from *Heteroclinus whiteleggii* from Australia and to several other sphaeromyxids with arcuate spores and rounded ends. This similarity is reflected by phylogenetic positioning of *S. clini* n. sp. which clusters within the '*incurvata*' group of the *Sphaeromyxa* clade. It differs from morphologically similar species by spore and polar capsule dimensions, host specificity and geographic distribution. *Sphaeromyxa limocapitis* n. sp., described from *Gephyroberyx darwinii* from Java, is morphologically similar to sphaeromyxids with straight spores and to marine *Myxidium* species with spindle-shaped spores but differs from them by spore and polar capsule dimensions, host specificity and geographic distribution. *S. limocapitis* n. sp. represents a separate lineage of the *Sphaeromyxa* clade and appears to be a missing link in the evolution of sphaeromyxids.

Key words: Myxosporea, Clinidae, *Gephyroberyx*, ultrastructure, evolution, *Sphaeromyxa* clade, SSU rDNA, new species.

INTRODUCTION

The marine genus *Sphaeromyxa* Thélohan, 1892 (Myxozoa: Myxosporea) comprises almost 50 nominal species, all of which parasitize the hepatic biliary systems of their fish hosts typically occupying the gall bladder (Lom and Dyková, 2006; Karlsbakk *et al*. 2013; Kristmundsson and Freeman, 2013; Whipps and Font, 2013). A number of sphaeromyxids are known to have low host specificity and a wide geographic distribution (Lom, 2004; Kristmundsson and Freeman, 2013).

Among myxosporeans, species of the genus *Sphaeromyxa* are unique by a number of morphological and evolutionary features. The polar filaments are not spirally coiled as in most myxosporeans, but instead folded over several times upon themselves. The filaments are also typically broad at their base gradually tapering towards the distal ends (Lom and Dyková, 2006). The ribbon-like character of these polar filaments was the main criterion used to establish the suborder Sphaeromyxina Lom et Noble, 1984 and was found to represent a single synapomorphic trait of sphaeromyxids in the myxosporean evolution (Fiala and Bartošová, 2010). However, in the light of recent molecular findings the suborder Sphaeromyxina was suppressed and the monotypic family Sphaeromyxidae Lom et Noble, 1984 was transferred to the suborder Variisporina Lom et Noble, 1984 (Kristmundsson and Freeman, 2013). The specific ultrastructural features of genus *Sphaeromyxa* are marked surface projections of their large flat plasmodia and an endoplasm containing a number of extremely large vacuoles and lobocytes (Lom, 1969, 2004).

Sphaeromyxa is one of a few myxozoan genera where the classification by spore morphology is consistent with estimates of phylogeny (i.e. it is monophyletic). Marine sphaeromyxids unusually cluster within the 'hepatic biliary group' which besides *Sphaeromyxa* spp. mostly includes species that infect the gall bladders of freshwater hosts but with some from marine and exceptionally from terrestrial hosts (Fiala, 2006; Kristmundsson and Freeman, 2013). *Sphaeromyxa* species further divide into two morphologically distinct groups, (i) the '*balbianii*' group with straight or slightly curved fusiform or ovoid spores, usually truncate spore ends and ovoid polar capsules and (ii) the

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'*incurvata*' group with arcuate spores, twisted valves, usually rounded spore ends and pyriform polar capsules (Laird, 1953; Karlsbakk *et al*. 2013). This morphology-based grouping was supported by recent phylogenetic analyses of molecular data where a monophyletic *Sphaeromyxa* clade groups the members with zig-zag folded polar filament further clustering within the '*balbianii*' or '*incurvata*' subclade (Karlsbakk *et al*. 2013; Kristmundsson and Freeman, 2013; Whipps and Font, 2013). Some authors even proposed the establishment of two different genera for members of the two groups (Karlsbakk *et al*. 2013).

Five *Sphaeromyxa* species have been recorded in intertidal blennies. *Sphaeromyxa tripterygii* Laird, 1953 was described from *Forsterygion varium* (Forster) and *Bellapiscis medius* (Günther) (Perciformes: Tripterygiidae) off New Zealand (Laird, 1953). *Sphaeromyxa incurvata* Doflein, 1898 was found in *Blennius ocellaris* L. (Perciformes: Blenniidae) from the Mediterranean (Doflein, 1898; Kudo, 1919). *Sphaeromyxa* species from clinid blennies (Perciformes: Clinidae) were recorded (i) from the Pacific Ocean off California, USA, i.e. *Sphaeromyxa gibbonsia* Noble, 1938 from *Gibbonsia elegans* (Cooper) and *Gibbonsia metzi* Hubbs, (ii) along the Australian New South Wales coast, i.e. *Sphaeromyxa noblei* Lom, 2004 from *Heteroclinus whiteleggii* (Ogilby) and (iii) from the south coast of Africa, i.e. an undescribed *Sphaeromyxa* species from *Pavoclinus graminis* (Gilchrist & Thompson) (Noble, 1939; Lom, 2004; Reed *et al*. 2009). To our knowledge, five myxosporean species have so far been reported from the beryciform fishes (Beryciformes) (Gaevskaya and Kovaleva, 1980; Kovaleva and Gaevskaya, 1981, 1988); however, no *Sphaeromyxa* species have previously been recorded in any representatives of this fish order.

The present work examines intertidal blennies from the genera *Clinus* and *Muraenoclinus* from South African coast as well as the deep sea fish *Gephyroberyx darwinii* (Johnson) from Java (Indonesia) for the presence of myxosporean parasites from the genus *Sphaeromyxa*.

MATERIALS AND METHODS

Sample collection, study area and time schedule

In total, 140 fish of the family Clinidae (Perciformes) belonging to *Clinus acuminatus* (Bloch & Schneider), *Clinus brevicristatus* Gilchrist et Thompson, *Clinus cottoides* Valenciennes, *Clinus superciliosus* (L.), *Clinus venustris* Gilchrist et Thompson, and *Muraenoclinus dorsalis* (Bleeker) were collected using small hand nets from intertidal rock pools from several localities in South Africa in March–April 2008, November 2009 and October–November 2012 (Fig. 1). One individual of *G. darwinii* was purchased at the fish market in Pelabuhan Ratu, Java, Indonesia (6°59′02″N, 106°32′37″E) in March 2008.

A DNA sample of Sphaeromyxa longa Dunkerley, 1921 (isolate M0313; Table 1) from *Trisopterus minutus* (L.) was available from a collection in the Laboratory of Fish Protistology, BC ASCR where it had been archived from a previous sampling trip conducted to the North Sea off Scotland in June 2003 (Fiala, 2006).

Processing of samples

Gall bladders and livers of fishes were examined for the presence of myxosporean infections by light microscopy (LM) on Leica DM750 and Olympus BX51 microscope. Plasmodia and spore morphology were documented with Leica DM750 and Olympus DP70 digital camera using differential interference contrast. The spores ($n = 20$) were measured according to the guidelines of Lom and Arthur (1989) using ImageJ v.1_44p software (Wayne Rasband, http://imagej.nih.gov/ij/). Samples were stored in 400 μL of TNES urea buffer (10 mM Tris-HCl with pH 8, 125 mM NaCl, 10 mM EDTA, 0·5% SDS and 4 M urea) or in 90% ethanol for subsequent DNA extraction.

For histological examination, approximately 8 mm^3 of liver tissue of *M. dorsalis* (Mouille Point) was fixed for 24 h in Davidson fixative and stored in 70% ethanol. Samples were routinely dehydrated and embedded into paraffin. Sections were stained by haematoxylin and eosin (H&E) and Giemsa. For examination of fine structure of spores and plasmodia by transmission electron microscopy (TEM), 6 mm^3 of liver tissue from *C. acuminatus* (Mouille Point) was fixed in cacodylate buffered 3% glutaraldehyde at 4 °C, rinsed in 0·1 M cacodylate buffer and postfixed in 1% osmium tetroxide. After graded acetone dehydration series, the samples were embedded in Spurr's resin. Semithin sections (1–2 μM) were stained with toluidine blue and used for additional histological observation. Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate and examined in a JEOL JEM 1010 electron microscope operating at 80 kV. Images were collected with Megaview II soft paging system using analySIS software.

Host species identification

Most of the fish species were identified using morphological features (Froese and Pauly, 2014). The morphology-based identification of clinid fish hosts, that all have very similar morphology, was verified by sequencing the taxonomically informative genes cytochrome c oxidase I (COI) or cytochrome b (*cytb*) using previously described primers (Folmer *et al*. 1994; Boore and Brown, 2000).

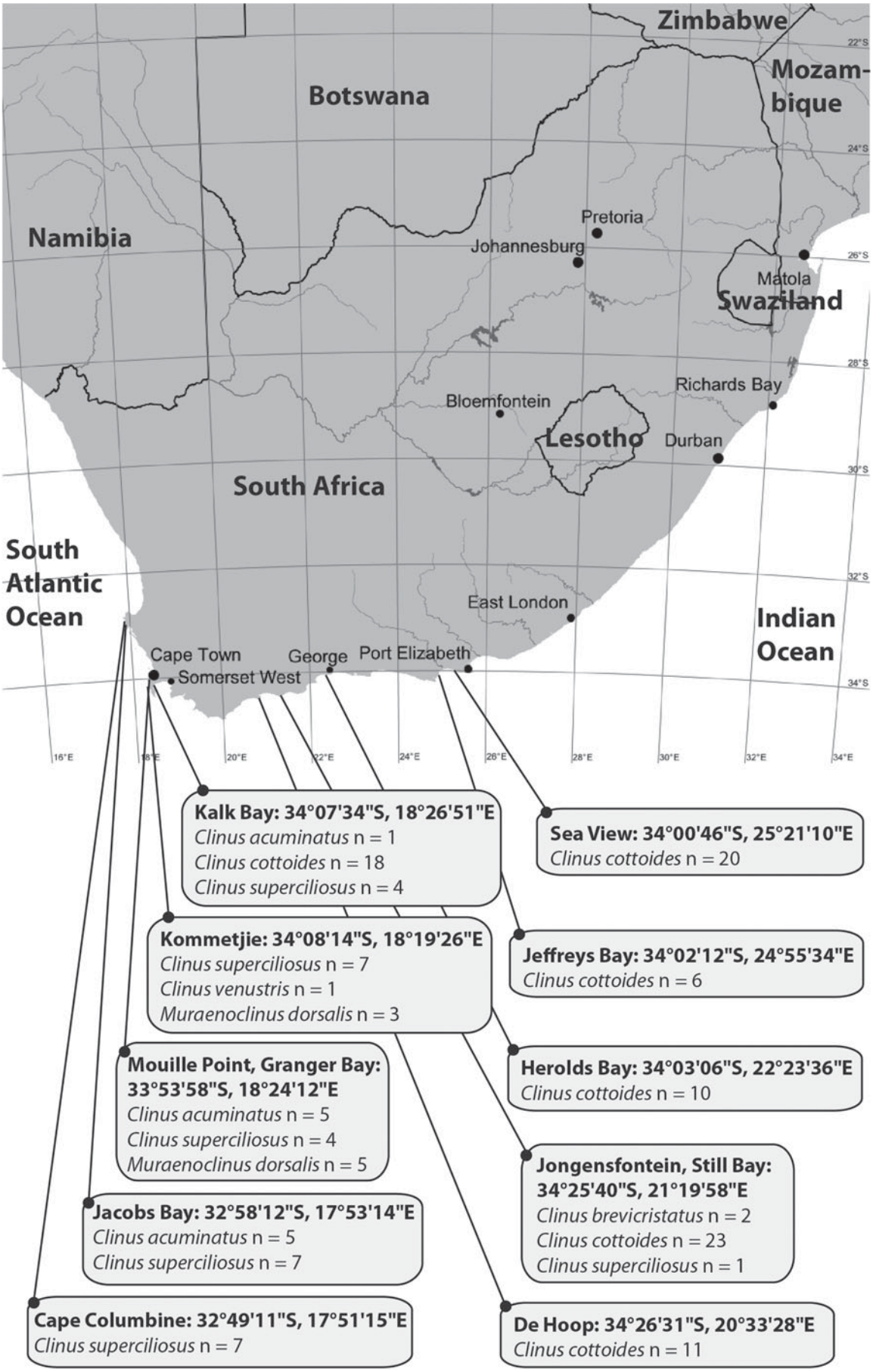

Fig. 1. Map of South Africa showing collection localities and including information on host species and numbers of samples.

Table 1. New sequence data on *Sphaeromyxa* species from this study

Myxosporean species	Fish host	Locality	Host organ	*cytb*/COI of host	DNA sample	SSU rDNA of *Sphaeromyxa*
Sphaeromyxa clini n. sp.	*Clinus cottoides*	Kalk Bay, SA	GB	KM201345[a]	813	KM201336
	Clinus acuminatus	Mouille Point, SA	L	KM201346	1512	KM201337
	Clinus superciliosus	Cape Columbine, SA	GB	–	1460	KM201338
	Muraenoclinus dorsalis	Mouille Point, SA	GB	KM201347	1456	KM201339
	Muraenoclinus dorsalis	Jacobs Bay, SA	L	–	1542	KM201340
	Muraenoclinus dorsalis	Kommetjie, SA	L	–	931	KM201341
	Muraenoclinus dorsalis	Granger Bay, SA	GB	KM201348	933	KM201342
Sphaeromyxa limocapitis n. sp.	*Gephyroberyx darwinii*	Pelabuhan Ratu, Java, Indonesia	GB	–	754	KM201343
Sphaeromyxa longa	*Trisopterus minutus*	North Sea	GB	–	313	KM201344

[a]*cytb* sequence, the rest of sequences in the row are host COI sequences; SA, South Africa; GB, gall bladder; L, biliary ducts in liver.

DNA extraction, PCR amplification, cloning and sequencing

Total DNA was extracted using a standard phenol–chloroform protocol, after an overnight digestion with proteinase K (50 μg mL^{-1}; Serva, Germany) at 55 °C. DNA was re-suspended in 50–100 μL^{-1} of DNAse-free water and left to dissolve overnight at 4 °C.

Almost complete SSU rDNA sequences of a *Sphaeromyxa* species from *C. cottoides* (Kalk Bay) as well as a *Sphaeromyxa* species from *Gephyroberyx darwinii* were obtained using universal eukaryotic ERIB1–ERIB10 primers in the first PCR. Two overlapping fragments of the parasite SSU rDNA were amplified in nested PCR by a combination of ERIB1–ACT1r and Myxgen4F–ERIB10 for a *Sphaeromyxa* species from *C. cottoides* or MyxGP2F–ERIB10 for a *Sphaeromyxa* species from *G. darwinii* (Barta *et al*. 1997; Kent *et al*. 1998; Hallett and Diamant, 2001; Diamant *et al*. 2004). The composition of PCR reaction mixture was identical as for the Taq-Purple DNA polymerase protocol described by Bartošová *et al*. (2013). Following PCR cycling conditions were used for the first and nested PCR, respectively: 95 °C 3 min, (95 °C 1 min, 48 °C 1 min, 72 °C 1 min 30 s) 30×, 72 °C 10 min or 95 °C 3 min, (95 °C 1 min, 50 °C 1 min, 72 °C 1 min) 30×, 72 °C 10 min.

Approximately 500 bp of the SSU rRNA gene of additional *Sphaeromyxa* samples from other clinid fish (Table 1) were amplified by a combination of primers newly designed in this study, i.e. SphmyxSSU520F (5′-AGGTCGTTCCCAMAAGGATTC-3′)–SphmyxSSU1140R (5′-ATCACCCGAAGACAGCAACC-3′). The composition of PCR reaction mixture was identical as for the TITANIUM Taq DNA polymerase protocol described by Bartošová *et al*. (2013). Following PCR cycling conditions were used: 95 °C 3 min, (94 °C 40 s, 57 °C 40 s, 68 °C 50 s) 35×, 68 °C 8 min.

The missing 3′end of SSU rRNA gene sequence of *S. longa* was amplified from the identical DNA sample (isolate M0313) for which partial SSU and almost complete LSU rDNA data exist in GenBank (NCBI: DQ377691, FJ417072). For this purpose, Myxgen4F–ERIB10 primers were used. The composition of PCR reaction mixture was identical as for the TITANIUM Taq DNA polymerase protocol described by Bartošová *et al*. (2013). Following PCR cycling conditions were used: 95 °C 3 min, (95 °C 50 s, 55 °C 50 s, 68 °C 1 min 40 s) 35×, 68 °C 10 min. The product, which was weakly amplified most probably due to low DNA concentration of parasite in the sample, was re-amplified using identical primers and PCR cycling parameters as in previous PCR.

PCR products were purified using a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd, USA) and sequenced directly. As a mixed sequence was detected for *S. longa* in the chromatogram of the direct sequencing product, the amplified fragments were cloned into the pDrive Vector with a PCR Cloning Kit (Qiagen, Germany) and transformed into TOP10 chemically competent *Escherichia coli* cells (Life Technologies, Czech Republic). Plasmid DNA was isolated using a High Pure Plasmid Isolation Kit (Roche Applied Science, Germany) and two colonies were sequenced on ABI PRISM 3130 × 1 automatic sequencer (Applied Biosystems, Czech Republic) in the sequencing facility of the Faculty of Science and BC ASCR.

Alignments and phylogenetic analyses

The SSU rDNA sequences were aligned in MAFFT v6·864b (Katoh *et al*. 2002) using the L-INS-i multiple alignment method, with a default gap opening penalty (–op = 1·53) and gap extension penalty (–ep = 0·0). The alignment was manually edited in BioEdit v7·0·5·2 (Hall, 1999) and the ambiguous sections were removed manually.

The SSU rDNA-based alignment included newly obtained *Sphaeromyxa* sequences as well data from all *Sphaeromyxa* species available in GenBank and 12 non-sphaeromyxid members of the hepatic biliary group sensu Kristmundsson and Freeman (2013). *Chloromyxum cristatum* (NCBI: AY604198; under the synonym *Chloromyxum cyprini* in GenBank) and *Chloromyxum fluviatile* (NCBI: GU471264) were used as outgroups.

Maximum parsimony (MP) analysis was performed in PAUP* v4.b10 (Swofford, 2003), using a heuristic search with random taxa addition, the ACCTRAN option, TBR swapping algorithm, all characters treated as unordered, a Ts/Tv ratio of 1:2, and gaps treated as missing data. Maximum likelihood (ML) analysis was performed in RAxML v7·0·3 (Stamatakis, 2006) using the GTR + Γ model. Bootstraps were based on 1000 replicates for both analyses. Bayesian inference (BI) analysis was performed in PhyloBayes v3·3.f. (Lartillot *et al*. 2009) using the CAT-GTR model of evolution. Two intertwined chains were run in parallel for a minimum of 500 cycles. Then, every 100 cycles, the bipartition frequencies and the summary variables were automatically done and the run stopped once all the discrepancies were lower or equal to 0·3 and all effective sizes were larger than 50. Burnin period was equal to one fifth of the total length of the chain.

RESULTS

Four out of the six clinid fish species examined, i.e. *C. acuminatus, C. cottoides, C. superciliosus* and *M. dorsalis* were found to be infected with a *Sphaeromyxa* which we describe as a new species using light microscopical, ultrastructural, histological and molecular data. The morphology of a *Sphaeromyxa* found in the gall bladder of *G. darwinii* also did not match any existing nominal *Sphaeromyxa* species and is described as new species of *Sphaeromyxa* in this study.

We obtained a novel *cytb* sequence of *C. cottoides* (NCBI: KM201345), the host of a *Sphaeromyxa* species from South Africa, which showed 81% sequence similarity with *Abudefduf sparoides* (Perciformes: Pomacentridae; NCBI: JQ707166; *cytb* of clinids missing in GenBank). We also obtained three COI sequences of *C. acuminatus* (NCBI: KM201346) and *M. dorsalis* (NCBI: KM201347, KM201348), additional hosts of the abovementioned *Sphaeromyxa* species, which showed 100% and 98% sequence similarity with their nucleotide GenBank reference data (NCBI: JF493211, JF493914, respectively) and 100 % sequence similarity with their amino acid GenBank reference data (NCBI: AEB16730, AEB17430), respectively (Table 1).

Species descriptions.

Phylum: Cnidaria Hatschek, 1888
Unranked subphylum: Myxozoa Grassé, 1970
Class: Myxosporea Bütschli, 1881
Order: Bivalvulida Schulman, 1959
Suborder: Variisporina Lom et Noble, 1984
Family: Sphaeromyxidae Lom et Noble, 1984
Genus: Sphaeromyxa Thélohan, 1892

***Sphaeromyxa clini* n. sp.**

Type host: *Clinus acuminatus*, sad klipfish.

Other hosts: *Clinus cottoides*, bluntnose klipfish; *C. superciliosus*, super klipfish; *M. dorsalis*, nosestripe klipfish (Perciformes: Clinidae).

Type locality: South Africa: Mouille Point (Fig. 1).

Other localities: South Africa: Cape Columbine; Jacobs Bay; Kalk Bay; Kommetjie (Fig. 1).

Description of sporogonic stages: Flat polysporic plasmodia with irregular margins, pansporoblasts producing one or predominantly two spores (Fig. 2A and B). Ectoplasm composed of a compact 2·5–3·5 μm thick outer layer and a finely granular 4·5–7·5 μm thick inner layer. Endoplasm vacuolated and loosely connected (Fig. 2B). Plasmodia with small projections on their surface (Fig. 3D and E; more detailed description in ultrastructure section).

Description of myxospores (measurements from *C. acuminatus, C. superciliosus* and *M. dorsalis*): Spores arcuate in frontal view tapering towards the ends (Fig. 2A–E) and slightly sigmoid in sutural view (Fig. 2D and E). Spore length 17·4–20·6 μM (mean ± S.D. = 18·8 ± 0·9), spore width 4·5–6·1 μM (5·3 ± 0·4), spore thickness 4·0–6·0 μM (5·0 ± 0·7). Sutural line relatively indistinct, sigmoid (Fig. 2E). Two elongate oval polar capsules (PCs) with ribbon-like polar filament irregularly folded twice to three times inside each PC (Fig. 2A and C–E). PC length 5·3–6·6 μM (5·9 ± 0·3), PC width 2·1–3·1 μM (2·5 ± 0·2). Single binucleated sporoplasm occupying the space between PCs (Fig. 2D and E). Spore ridges not observed by LM. Mature spores were observed from March to April and from October to November.

Localization of sporogonic stages: Coelozoic, gall bladder, liver–bile ducts (Table 1).

Prevalence: 9% of all fish individuals (n = 140); detailed data: *C. acuminatus*: Mouille Point 60 % (n = 5); *C. cottoides*: Kalk Bay 6% (n = 18); *C. superciliosus*: Cape Columbine 14% (n = 7), Jacobs Bay 29% (n = 7); *M. dorsalis*: Mouille Point 100% (n = 5), Kommetjie 33% (n = 3); the rest of fish examined negative.

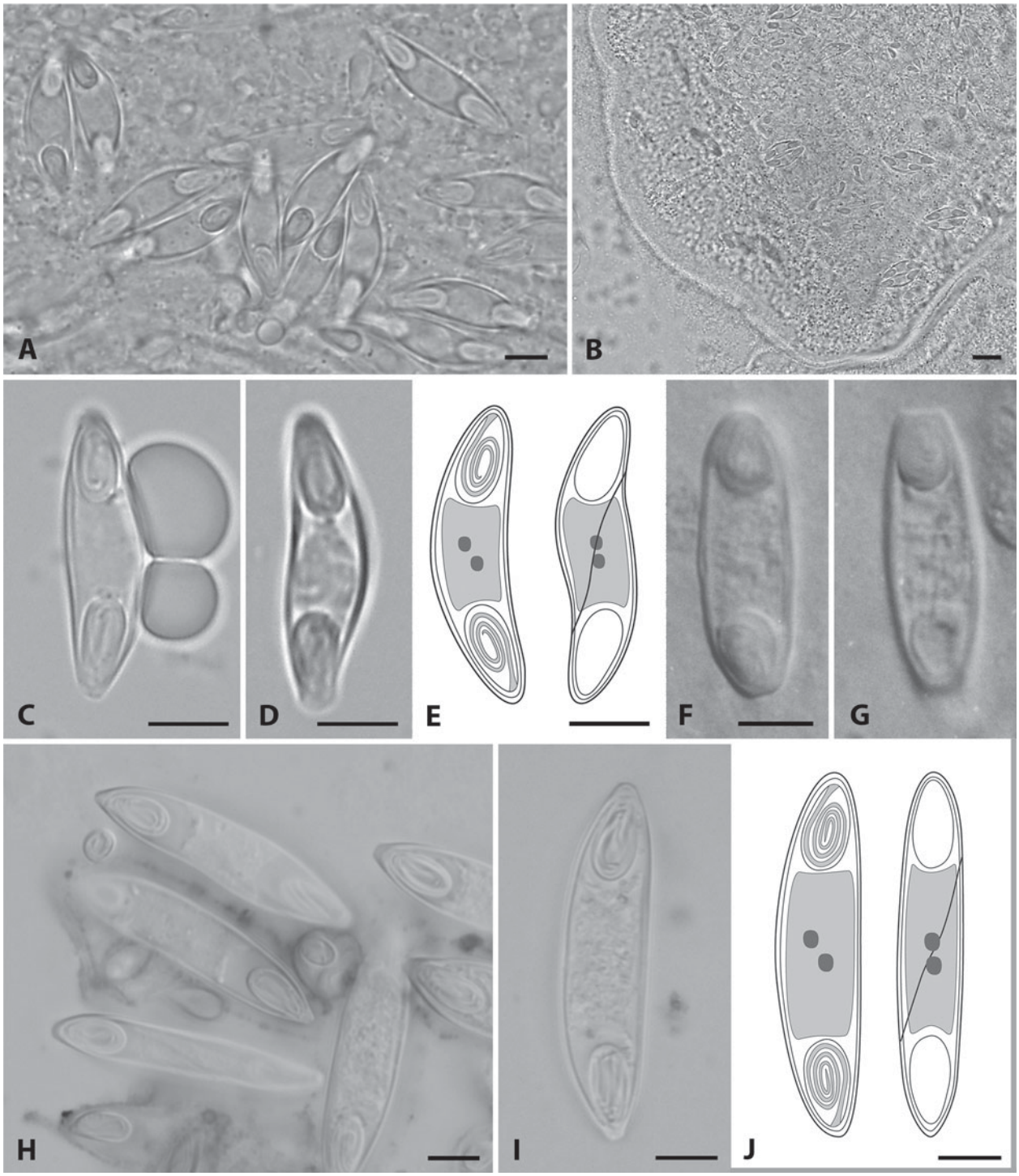

Fig. 2. Light microscopy pictures and line drawings of *Sphaeromyxa* spores and plasmodia as seen in Nomarski differential interference contrast. (A–E) *Sphaeromyxa clini* n. sp. stages from (A–C) *M. dorsalis* and (D) *C. acuminatus*. (A) Plasmodium with spores in pansporoblasts; (B) Plasmodium with focus on the ectoplasmic and endoplasmic layers; (C) Myxospore in frontal view; (D) Myxospore in sutural view; (E) Myxospore in frontal (left) and sutural view (right). Myxospores of *Sphaeromyxa longa* in (F) frontal and (G) sutural view. *Sphaeromyxa limocapitis* n. sp. myxospores in (H, I) frontal and (H) sutural view. (J) Myxospore in frontal (left) and sutural view (right). Scale bar 5 μM.

Pathology: In histology sections, the biliary ducts completely occluded by plasmodia causing dilatation (Fig. 3A and C).

Materials deposited: DNA samples (Table 1) stored in −80 °C, paraffin block No. 363 and block in resin nr. AK005 stored in the Laboratory of Fish Protistology, Institute of Parasitology, BC ASCR.

Molecular data: Seven SSU rDNA sequences (NCBI: KM201336–KM201342; Table 1) with 100% sequence identity in the overlapping region. Intragenomic variability: one polymorphic site

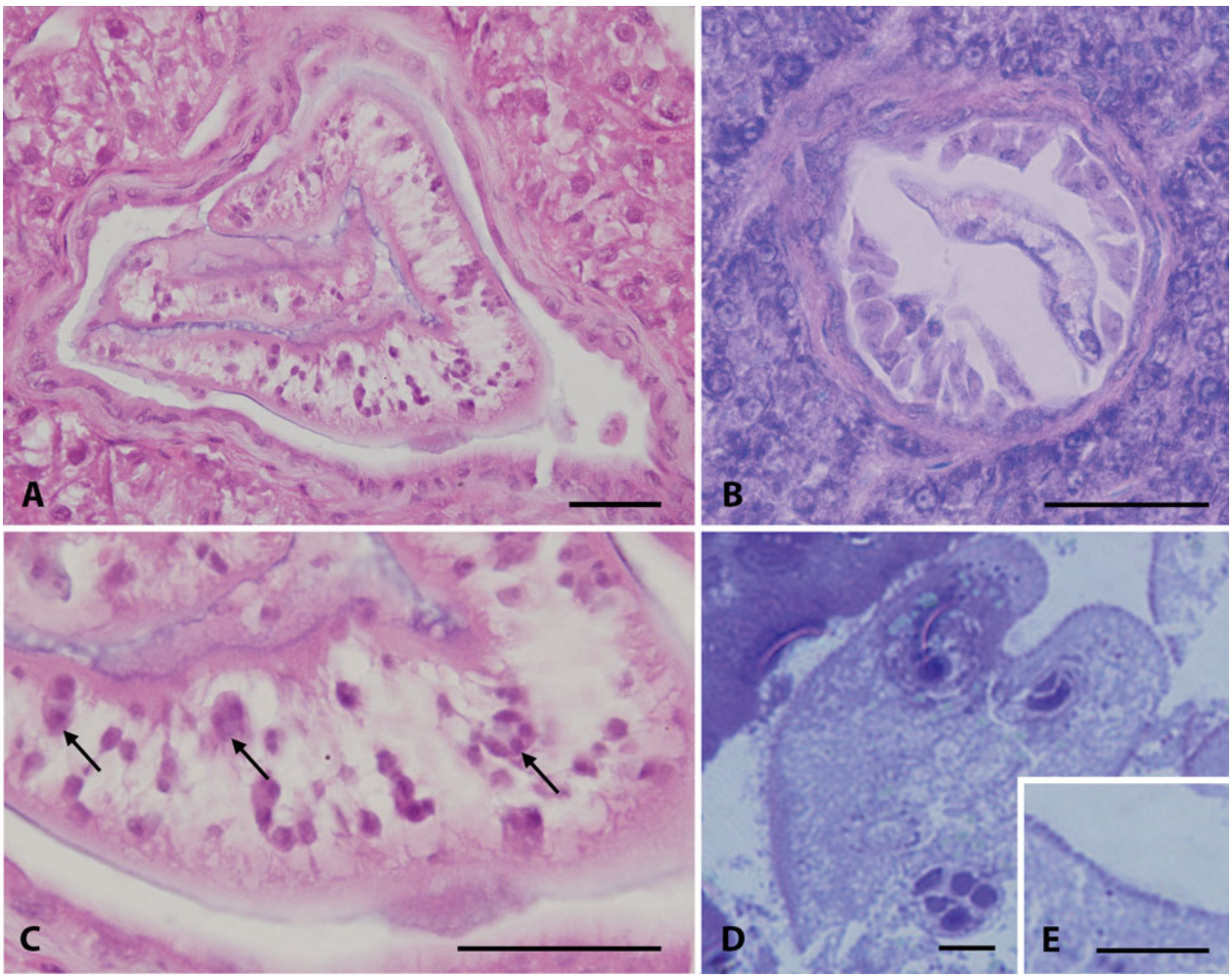

Fig. 3. Histological and semi-thin sections of *Sphaeromyxa clini* n. sp. in the bile ducts of (A–C) *M. dorsalis* and (D–E) *C. acuminatus*. (A) H&E stained section of infected bile duct of *M. dorsalis* completely occluded with the sporogonic plasmodium of *S. clini* n. sp.; (B) Giemsa stained bile duct of *M. dorsalis* co-infected by the sporogonic stages of *S. clini* n. sp. (centrally) and *Ceratomyxa* sp. (peripherally); (C) Picture detail of the plasmodium with sporogonic stages of *S. clini* n. sp. (arrows); (D) Sporogonic plasmodium of *S. clini* n. sp. in the bile duct of *C. acuminatus* (E) with the detail on plasmodial surface with small projections covered with mucus layer. Scale bars 20 μM (A–C) and 5 μM (D–E).

(A/G) at position 895 (related to the almost complete sequence NCBI: KM201336). The almost complete sequence of *S. clini* n. sp. (NCBI: KM201336) was used for the phylogenetic analyses. Clusters within the '*incurvata*' group of the *Sphaeromyxa* clade.

Ultrastructure: Surface of the plasmodium extends in fine projections ranging about 130–350 nm (Fig. 4A, B and D–F), covered with a double cell membrane (Fig. 4E). Cell membrane frequently invaginated to form small (approximately 100 nm) pinocytotic vesicles which were observed also deeper in the ectoplasm (Fig. 4B and C). Network of mucus up to 450 nm thick layer covering the plasmodial projections (Fig. 4A, B, D, and F). Approximately 1 μM thick ectoplasmic layer (Fig. 4B and F) containing small pinocytotic vesicles (50–80 nm). Endoplasm additionally filled with electron-dense granules of variable size (0·5–1·0 μM), lamellar structures, mitochondria and sporoblasts (Fig. 4F). Two secondary cells in a primary cell observed as the earliest developmental stages of sporogony (Fig. 4F and G). Pansporoblasts harbouring a single developing spore (Fig 4I). During capsulogenesis, PC primordium filled with granular substance and attached to the transversely striated external tube (Fig. 4H). Apex of the maturing polar capsule covered with an electron-dense cap (stopper-like structure) in the preexisting channel for polar filament discharge; three folds of the polar filament observed in the longitudinal section of PC (Fig. 4I). Polar filament with dark lumen and bright surface (Fig. 4I). Shell valves of the maturing spore having much darker cytoplasm than the cytoplasm of surrounding sporoblast. Spore most probably possessing ridges – these could not be clearly distinguished in our material due to the absence of mature spores in the examined plasmodium. Desmosome-like junction of the suture discernible between valve cells (Fig. 4I).

Etymology: *clini* refers to the name 'Clinidae' of fish family where both of its fish host genera belong.

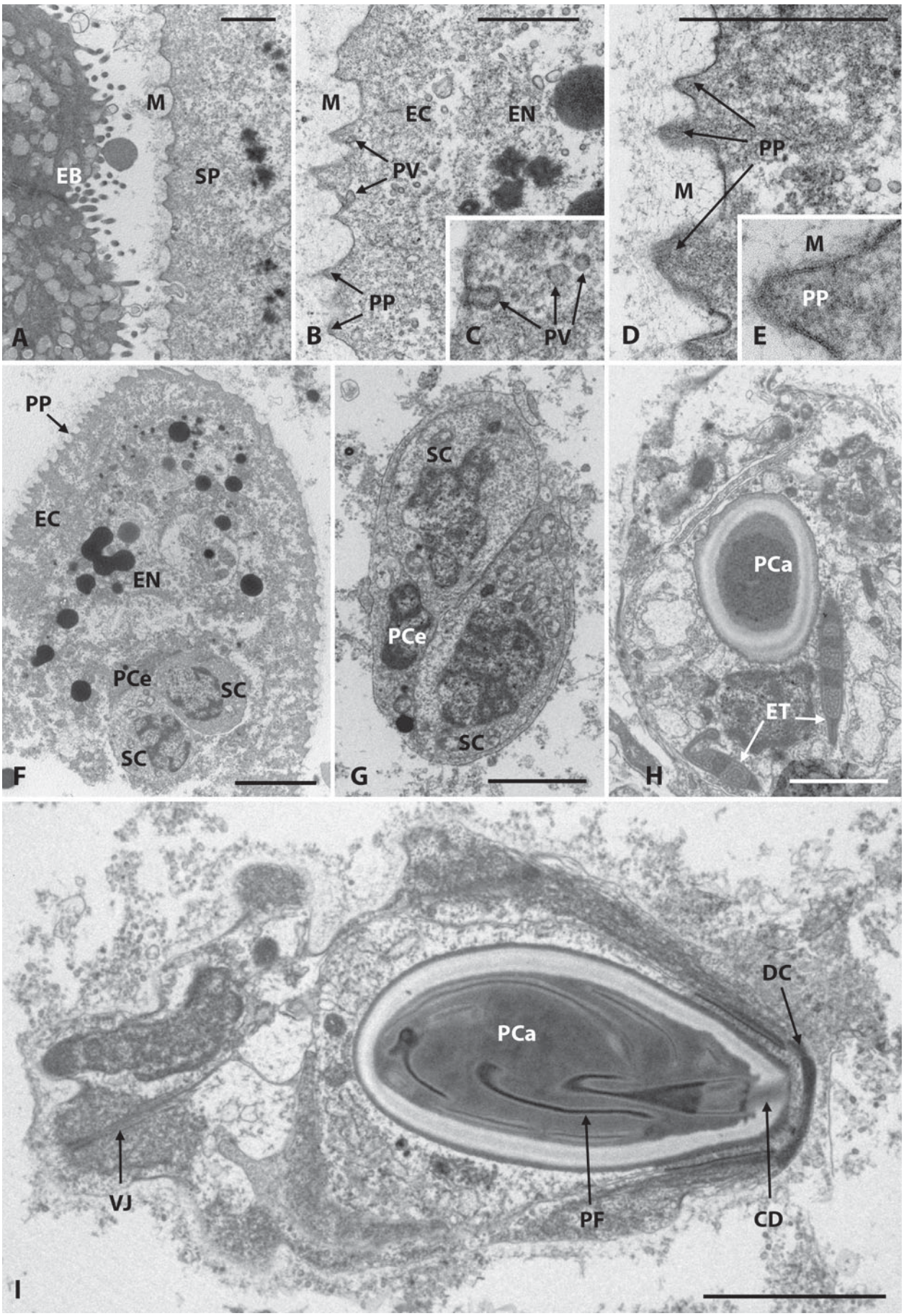

Fig. 4. Ultrastructure of *Sphaeromyxa clini* n. sp. infection in the bile ducts of *C. acuminatus*. (A) Contact zone of the plasmodium with host bile duct endothelium; (B) Plasmodium containing pinocytotic vesicles and dense granules with (C)

Differential diagnosis: South African *S. clini* n. sp. is most similar to *S. noblei* described from a different clinid fish *H. whiteleggii* from Australia (Lom, 2004). Besides host species and locality, they differ by a number of morphological features, i.e. spore shape (less arcuate in *S. clini* n. sp.) and mean length (18·8 μM in *S. noblei vs* 20 μM in *S. clini* n. sp.), thickness and structure of mucus layer on the plasmodial surface (thicker and ramified in *S. noblei vs* thinner and less branched in *S. clini* n. sp.), and thickness of plasmodial ectoplasm (15 μM in *S. noblei vs* 1 μM and 7–11 μM in *S. clini* n. sp. as observed by TEM and LM, respectively) (Table 2). The comparison of the thickness of ectoplasm layer has to be taken with caution, as it may vary with plasmodial size and development, i.e. the small unsporulated plasmodia from the liver ducts may not be readily comparable with large plasmodia from the gall bladder. Moreover, as deduced from the TEM figures in Lom (2004), the thickness of ectoplasm of *S. noblei* appears to be 5 μM rather than 15 μM as referred in the text. *Sphaeromyxa clini* n. sp. has several additional characteristics which most probably remained unnoticed or are absent in *S. noblei*; more specifically wider tissue specificity (bile ducts additionally to gall bladder), double-layer plasmodial membrane and formation of pinocytotic vesicles.

Sphaeromyxa clini n. sp. differs considerably from *Sphaeromyxa* sp. from the South African clinid *Pavoclinus graminis* by more arcuate longer spores with longer PCs (Reed *et al*. 2009). Spores of another species from intertidal blennies, *S. triptherygii*, are comparable in their length with *S. clini* n. sp.; however, they are relatively slender with truncate ends and narrower PCs (Laird, 1953). Other two *Sphaeromyxa* species from the tidal blennies, *S. gibbonsia* and *S. incurvata*, have longer spores and larger ovoid or pyriform PCs than *S. clini* n. sp. (Doflein, 1898; Kudo, 1919; Noble 1939) (Table 2).

The phylogenetically most closely related species, *Sphaeromyxa hellandi*, resembles to *S. clini* n. sp. in spore shape but differs by longer spores and longer PCs, host specificity and geographic distribution (Kalavati and MacKenzie, 1999). Other species of the '*incurvata*' clade, *Sphaeromyxa lycodi* Kristmundsson et Freeman, 2013 and *Sphaeromyxa kenti* Whipps et Font, 2013, differ from *S. clini* n. sp. by possessing more arcuate spores, longer PCs, host species spectrum and geographic distribution. *Sphaeromyxa kenti* has similar spore dimensions as *S. clini* n. sp. whereas spores of *S. lycodi* are larger (Kristmundsson and Freeman, 2013; Whipps and Font, 2013). Other arcuate species, *Sphaeromyxa arcuata* Fantham, 1930, *Sphaeromyxa elegini* Dogiel, 1948, *Sphaeromyxa exneri* Awerinzew, 1913, *Sphaeromyxa curvula* Fantham, 1930, *S. incurvata* Doflein, 1898, *Sphaeromyxa maiyai* Morrison et Pratt, 1973, and *Sphaeromyxa sabrazesi* Laveran et Mesnil, 1900 differ from *S. clini* n. sp. by their geographic distribution, host species, spore size and shape of PCs (Doflein, 1898; Kudo, 1919; Fantham, 1930; Shulman, 1966; Morrison and Pratt, 1973; Aseeva, 2002). Moreover, some of them possess specific features atypical for *Sphaeromyxa* species, i.e. disporic plasmodia in *S. elegini* and absence of mucus on the plasmodial membrane in *S. maiyai* (Shulman, 1966; Morrison and Pratt, 1973; Aseeva 2002) (Table 2).

Other remarks: Co-infections of *S. clini* n. sp. and *Ceratomyxa* sp. sporogonic stages observed in the infected tubules (Fig. 3B).

Sphaeromyxa limocapitis n. sp.

Type host: *G. darwinii*, Darwin's slimehead (Beryciformes: Trachichthyidae).

Type locality: Fish market at Pelabuhan Ratu, Java, Indonesia (6°59′02″N, 106°32′37″E).

Description of sporogonic stages: Not observed.

Description of myxospores: Spores straight to slightly curved in frontal view (straight on one side and slightly convex on another side) tapering towards the pointed ends (Fig. 2H–J); spore straight in sutural view (Fig. 2H and J). Spore length 26·7–30·1 μM (27·7 ± 1·3), spore width 5·6–6·7 μM (6·1 ± 0·4), spore thickness 4·1–5·7 μM (5·1 ± 0·7). Sutural line slightly sigmoid (Fig. 2J). Two elongate oval PCs with ribbon-like polar filament irregularly folded four times inside each PC (Fig. 2H–J). PC length 6·0–7·8 μM (6·8 ± 0·6), PC width 2·7–3·5 μM (3·2 ± 0·2). Single binucleated sporoplasm occupying the space between PCs (Fig. 2I and J). Spore ridges not observed.

detail of pinocytotic vesicles in the membrane and outer layer of the endoplasm; (D) Projections and mucus layer on the surface of plasmodium with (E) detail of double cell membrane of plasmodium; (F) Plasmodium with projections and mucus layer on its surface; dense granules and early sporoblasts present in the endoplasm; (G) Early sporogonic stage with two secondary cells in the primary cell; (H) Immature spore with polar capsule primordium filled with granular substance and transversely striated external tube; (I) Monosporic pansporoblast containing maturing spore with polar capsule covered with an electron-dense cap and filled with polar filament folded three times and connected to the discharge canal; valve cells connected by desmosome cell junction. Abbreviations: DC, electron-dense cap; CD, channel for polar filament discharge; EN, endoplasm; EC, ectoplasm; ET, external tube; EB, endothelium of bile duct; M, mucus layer; PCa, polar capsule; PCe, primary cell; PF, polar filament; PP, plasmodial projections; PV, pinocytotic vesicle; SC, secondary cell; SP, *Sphaeromyxa* plasmodium; VJ, valve cell junction. Scale bar 1 μM (A, B, D) and 2 μM (F–I).

Table 2. Comparison of *Sphaeromyxa clini* n. sp. with morphologically similar members of the '*incurvata*' group

Sphaeromyxa species	Host species	Distribution	Plasmodium	Spore shape and surface	Spore dimensions (L × W × T in μM)	PCs shape	PCs dimensions (L × W in μM)	Reference
Sphaeromyxa clini n. sp.	*Clinus acuminatus, C. cottoides, C. superciliosus, Muraenoclinus dorsalis*	South Africa	Flat with irregular margins, polysporic	Arcuate, bluntly rounded ends, fine striation	17·4–20·6 × 4·5–6·1 × 4·0–6·0	Elongate oval	5·3–6·6 × 2·1–3·1	This study
Sphaeromyxa arcuata	*Macroplus nasutus, Bathygobius soporator, Argyrozona argyrozona*	Atlantic Ocean off Namibia	–	Arcuate, bluntly rounded ends	21·3–23·3 × 3·7–5 × NG	Pyriform	7–10 × 1·5–2	(Fantham, 1930)
Sphaeromyxa curvula	*Helicolenus dactylopterus, Pachymetopon blochii*	Atlantic Ocean off Namibia	–	Arcuate, bluntly rounded ends	19–22 × 4–6 × NG	Pyriform	7–9 × 2–3	(Fantham, 1930)
Sphaeromyxa elegini	*Eleginus gracilis*	Japan Sea, Bering Sea	Round, disporic	Arcuate, bluntly rounded to truncate ends, fine striation	15–23·5 × 4–6·5 × NG	Pyriform	5–7·6 × NG	(Shulman, 1966; Aseeva, 2002)
Sphaeromyxa exneri	*Thrysanophrys japonicas, Sarritor leptorhynchus*	Indian Ocean (Mosambique Channel), Japan Sea	–	Arcuate, slightly tapering ends	75–80 × 18–20 × NG	Pyriform	30–35 × NG	(Kudo, 1919)
Sphaeromyxa gibbonsia	*Gibbonsia elegans, G. metzi*	Pacific Ocean	Leaf-like, polysporic	Slightly curved, elongate, rounded ends	27–5·2 × NG	Ovoid	10 × 4	(Noble, 1939)
Sphaeromyxa hellandi	*Brosme brosme, Macroramphosus scolopax, Mellanogrammus aeglefinus, Merlangius merlangus, Molva molva, Pholis gunnellus*	Northeast Atlantic, Barents Sea	Disc-like, polysporic	Slightly curved, bluntly rounded ends, smooth valves	22·5–30·0 × 4·5–7·5 × NG	Long pyriform	8·5–12·5 × 2·5–3·5	(Kalavati and MacKenzie, 1999)
Sphaeromyxa incurvata	*Blennius ocellaris, Pegusa lascaris*	Mediterranean Sea, Black Sea	Thin hollow ball, polysporic	Arcuate, bluntly rounded ends, smooth valves	30–35 × 8 × NG	Pyriform	12–15 × 4–5	(Doflein, 1898; Kudo, 1919)
Sphaeromyxa kenti	*Gobiosoma bosc*	Lousiana, USA	Disc-like, polysporic	Arcuate, bluntly rounded ends, fine striation	17·5–19·8 × 3·8–5·2 × NG	Elongate elliptical	PC1: 6·9–8·6 × 2·0–2·6; PC2: 5·8–7·5 × 2·0–2·6	(Whipps and Font, 2013)
Sphaeromyxa lycodi	*Lycodes pallidus, L. reticulatus, L. seminudus, L. eudipleurostictus, L. gracilis*	Iceland	Long slender, polysporic	Arcuate, bluntly rounded ends, fine striation	19·6–25·3 × 4·6–6·9 × 4·5–6·2	Pyriform	5·8–9·8 × 2·5–4·5	(Kristmundsson and Freeman, 2013)
Sphaeromyxa maiyai	*Microgadus proximus*	Off Newport, Oregon	Disc-like, polysporic	Arcuate, bluntly rounded to truncate ends, fine striation	23–30 × 5–7 × NG	Pyriform	6·0–8·5 × 3·0–3·6	(Morrison and Pratt, 1973)
Sphaeromyxa noblei	*Heteroclinus whiteleggii*	South Wales (Australia)	Leaf-like, polysporic	Arcuate, bluntly rounded ends, fine striation	18·5–21·5 × 5·2–6·0 × 4·8–5·2	Elongate oval	5·0–6·5 × 2·5–2·7	(Lom, 2004)

Table 2. (*Cont.*)

Sphaeromyxa species	Host species	Distribution	Plasmodium	Spore shape and surface	Spore dimensions (L × W × T in μM)	PCs shape	PCs dimensions (L × W in μM)	Reference
Sphaeromyxa sabrazesi	*Hippocampus brevirostris*, *H. guttulatus*, *Syngnathus acus*, *Motella tricirrata*	Mediterranean Sea, Atlantic Ocean	Disc-like, polysporic	Arcuate, truncate ends, fine striation	22–28 × 34·3 × NG	Pyriform	8–10 × 2–3	(Kudo, 1919)
Sphaeromyxa sp.	*Pavoclinus graminis*	South Africa	Leaf-like, polysporic	Arcuate, bluntly rounded ends, fine striation	26·5–27·5 × 5·0–5·2 × NG	Elongate ovoid	7·0–9·0 × 2·0–3·0	(Reed *et al*. 2009)
Sphaeromyxa tripterygii	*Forsterygion varium*, *Bellapiscis medius*	Off New Zealand	Leaf-like, folded upon itself into a hollow ball	Arcuate, truncate ends	17·2–21·1 × 3·4–3·8 × NG	Pyriform	4·6–5·1 × 1·4	(Laird, 1953)

NG, spore thickness not given in the literature.

Localization of sporogonic stages: Coelozoic, gall bladder.

Prevalence: 100% ($n = 1$).

Materials deposited: DNA samples (Table 1) stored in −80 °C in the Laboratory of Fish Protistology, Institute of Parasitology, BC ASCR.

Molecular data: SSU rDNA sequence (NCBI: KM201343; Table 1). Clusters in a separate lineage of the *Sphaeromyxa* clade.

Etymology: *limocapitis* refers to the English name 'slimehead' of the family Trachichthyidae where *G. darwinii* belongs.

Differential diagnosis: *Sphaeromyxa limocapitis* n. sp. described from the Central Indo-Pacific and possessing straight/slightly curved spores with pointed ends has an uncommon spore shape rather similar to certain marine *Myxidium* spp. than to most of sphaeromyxids. From the ten *Sphaeromyxa* spp. reported from the Western Indo-Pacific (Bay of Bengal) and Central Indo-Pacific (the coast of Borneo Island), only *Sphaeromyxa bengalensis* Sarkar, 2010, *Sphaeromyxa diacanthusa* Sarkar, 2004 and *Sphaeromyxa lomi* Moser et Noble, 1977 have similar fusiform spores as *S. limocapitis* n. sp. However, they differ from the new sphaeromyxid by host species, locality and by having (i) smaller striated spores with bluntly rounded ends (*S. diacanthusa*) or rounded/truncated spore ends (*S. lomi*) or (ii) spores with moderately truncated ends and spirally coiled polar filament hence with overall morphology similar to *Myxidium* morphotype (*S. bengalensis*) (Sarkar 2004; Sarkar, 2010 and others reviewed in Sarkar, 1999 and Lom, 2004). *Sphaeromyxa magna* Zhukov, 1964 and *Sphaeromyxa reinhardti* Jameson, 1929 having similar morphology as *S. limocapitis* n. sp. but described from different marine realm (i.e. Arctic and Temperate Northern Pacific, respectively), differ from the new species by smaller spores with truncated ends, host specificity and distribution (Jameson 1929; Shulman 1966).

Phylogenetic analyses

The strongly supported *Sphaeromyxa* clade (node A in Fig. 5A) clustering within the freshwater myxosporean lineage split into (i) the '*incurvata*' group, (ii) the '*balbianii*' group and (iii) *S. limocapitis* n. sp. lineage. *Sphaeromyxa clini* n. sp. clustered with high nodal support sister to the clade composed of *S. hellandi* sequences within the '*incurvata*' group. *Sphaeromyxa longa* (Fig. 2F and G), for which additional 765 bp at its 3′end were newly sequenced in this study (NCBI: KM201344), clustered with strong nodal support with *Sphaeromyxa artedielli* Karlsbakk, Einen et Bartošová, 2013 within the '*balbianii*' group (Fig. 5A). The new species *S. limocapitis* n. sp. created a separate lineage which was (i) positioned at the base of the *Sphaeromyxa* clade in the ML analysis (Fig. 5A and B left), (ii)

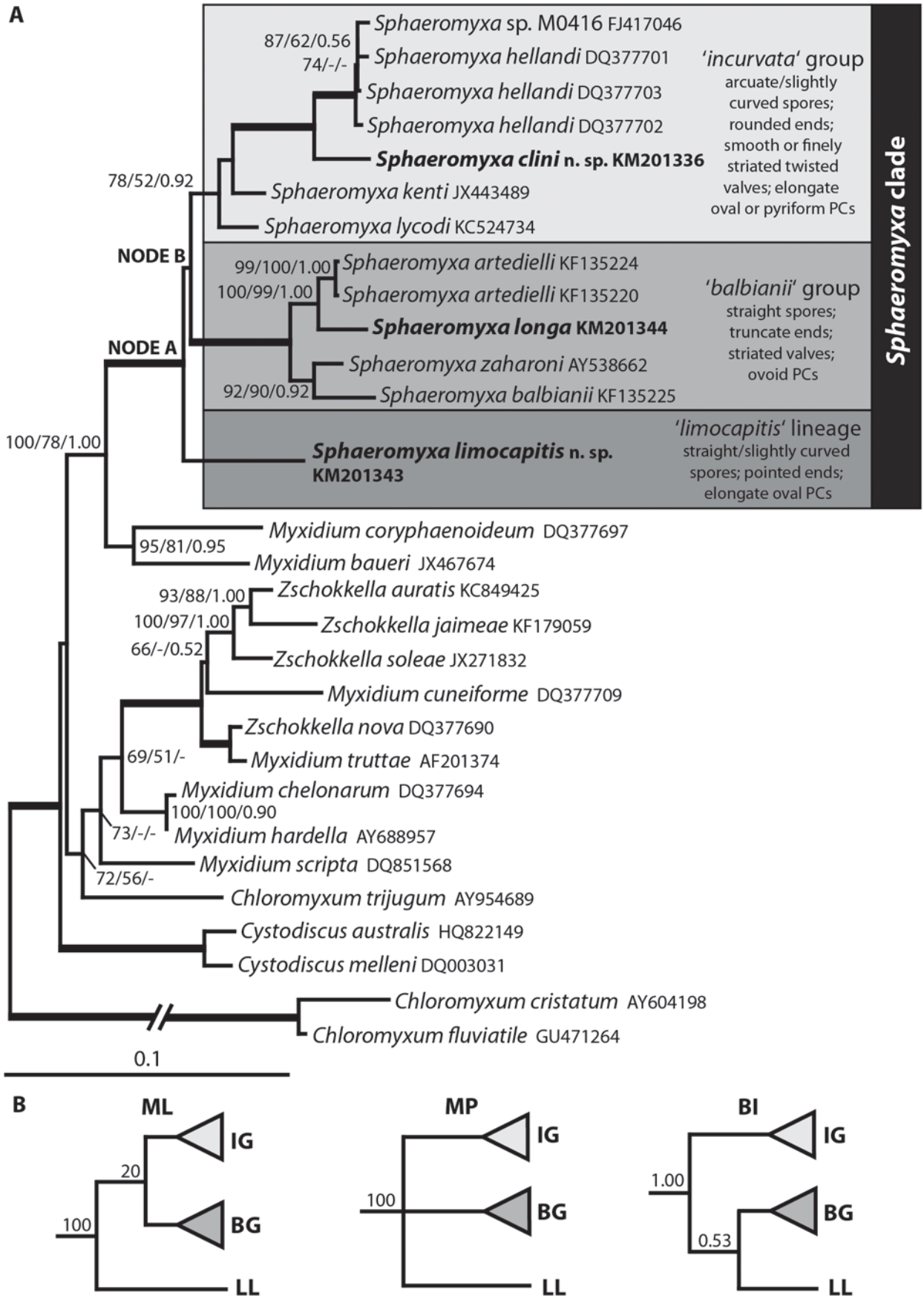

Fig. 5. Phylogeny of myxosporeans focused on the *Sphaeromyxa* clade. (A) Maximum likelihood (ML) tree showing the clustering of *Sphaeromyxa* species with new sequence data within the *Sphaeromyxa* clade. *Chloromyxum fluviatile* and *Chloromyxum cristatum* were selected as outgroups, their common branch shortened to 50% of original length. Newly sequenced taxa in bold. Bold branches lead to a node with a 100% ML/maximum parsimony (MP) bootstrap support and

unresolved within the *Sphaeromyxa* clade in the MP consensus tree (Fig. 5B middle) or (iii) positioned sister to the '*balbianii*' group in the BI analysis (Fig. 5B right).

DISCUSSION

Sphaeromyxa clini n. sp. from South African *C. acuminatus, C. cottoides, C. superciliosus* and *M. dorsalis* (this study) was found to be most similar to *S. noblei* described from *H. whiteleggii* from Australia (Lom, 2004) by overall shape of their spores and plasmodia. However, both species possess several differences in their morphology, host species and geographic distribution. Future sequencing of *S. noblei* from its type host and type locality and its subsequent comparison with the sequence data obtained for *S. clini* n. sp. in this study can help to ascertain if the observed differences are expressed as species-specific features or are due to variability in one species (*S. noblei*). The second option would be reasoned by low host specificity and a wide distribution reported for a number of *Sphaeromyxa* spp. (Lom, 2004) and by evolutionary implications. More specifically, even though the abovementioned clinid fish hosts of *S. clini* n. sp. and *S. noblei* are restricted to different biogeographic realms (temperate Southern Africa *vs* temperate Australasia; Spalding *et al*. 2007), they are closely related phylogenetically (Lin and Hastings, 2013). Clinids have an ancestral distribution in the temperate region and successfully crossed intervening tropical regions during their evolutionary history (Lin and Hastings, 2013). The common ancestor of clinids lived some 20 Ma ago (Lin and Hastings, 2013) when continents had almost the same position as at present, i.e. Australia was placed a bit more south from its current position. Therefore, we assume these clinid fish may possibly share a similar parasite fauna inherited from their ancestor.

Regarding the intraspecies variability, no sequence differences were observed in *S. clini* n. sp. isolates from different fish species and localities even though oceanographic barriers do affect the population structuring of South African intertidal fishes (Von der Heyden *et al*. 2011) along with their parasites, as for example shown for a myxosporean *Ceratomyxa cottoidi* closely associated with its host *C. cottoides* (Reed *et al*. 2007, 2011).

As for the fish host identification of *S. clini* n. sp., the 2% sequence divergence observed between the COI nucleotide sequence of *M. dorsalis* from Moullie Point and GenBank reference data of *M. dorsalis* from Haga Haga and Wooleys Pool (both South Africa) is due to the cryptic species nature of the fish host resulting from phylogeographic breaks (Von der Heyden *et al*. 2011).

The *Sphaeromyxa* species, which are all parasites of marine fish, cluster with marine *Myxidium* species, i.e. *Myxidium coryphaenoidium* Noble, 1966 and *Myxidium baueri* Kovaljova & Gaevskaya, 1982, within the freshwater myxosporean lineage (Fiala, 2006; Kalavati *et al*. 2013). *Sphaeromyxa* species, especially *S. limocapitis* n. sp. forming a separate lineage of the *Sphaeromyxa* clade, share many morphological similarities with phylogenetically closely related *M. coryphaenoidium* and *M. baueri* as well as with other morphologically similar myxidiids without sequence data in GenBank i.e. *Myxidium bajacalifornium* Noble, 1966, *Myxidium melanocetum* Noble, 1966, *Myxidium melanostigmum* Noble, 1966 and *Myxidium noblei* Konovalov, 1966 (Noble, 1966; Zubchenko and Krasin, 1980). Besides similar fusiform/spindle shaped spores (straight with tapering ends) and infecting the gall bladder of marine fish, the abovementioned *Myxidium* spp. possess rough polar filament (Zubchenko and Krasin, 1980) which is heavy at its proximal end and becoming thinner and more tightly coiled at distal end (Noble, 1966). *Sphaeromyxa* species also have a thick polar filament with a broad base, however being zig-zag folded and not spirally coiled as in *Myxidium* species. This morphological, biological and molecular evidence strongly corresponds with the hypothesis that *Sphaeromyxa* species had evolved by a change in the character of polar filament from the spindle-shaped *Myxidium* ancestor, which then returned to the marine environment (Fiala and Bartošová, 2010; Kristmundsson and Freeman, 2013). Our phylogenetic analyses indicate that the sphaeromyxids then diverged into species still possessing ancestral characters (the '*limocapitis*' lineage with straight/slightly curved fusiform spores, pointed spore ends and elongate oval PCs) or expressing evolutionary novelties (the '*incurvata*' group with arcuate or slightly curved spores, rounded spore ends, smooth or finely striated twisted valves and elongate oval or pyriform PCs; the '*balbianii*' group with straight fusiform or ovoid spores, truncate spore ends, striated valves and ovoid PCs). Thus, *S. limocapitis* n. sp. may represent a missing link in the evolution of sphaeromyxids.

1·00 Bayesian inference (BI) posterior probability. Dashes indicate bootstrap values <50 or node not present in the MP or BI tree. PCs, polar capsules; (B) Variable positioning of *S. limocapitis* n. sp. within the *Sphaeromyxa* clade in the ML, MP and BI analyses. Nodal supports (even <50) shown for nodes A and B (defined in Fig. 5A). IG, '*incurvata*' group; BG, '*balbianii*' group; LL, '*limocapitis*' lineage.

Herein, we describe a *Sphaeromyxa* species parasitizing the deep sea trachichthyid fish, *S. limocapitis* n. sp., based on new morphological and sequence data. We also provide morphological and sequence data for a newly described species *S. clini* n. sp. from clinid fishes. Further sequencing of additional *Sphaeromyxa* species, especially of those with unique spore morphologies, may reveal the existence of other phylogenetic clades within this myxosporean group and thus provide more insights into *Sphaeromyxa* radiation. Moreover, future study of the life cycles of *Sphaeromyxa* representatives, for which the invertebrate host remains unknown, may help to clarify why these parasites of marine fish cluster within the clade encompassing parasites which mostly alternate between freshwater fish and oligochaetes.

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