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ORIGINAL PAPER

Sphaeromyxa cornuti n. sp., a New Species of Myxosporean Infecting the Gallbladder of the Moorish Idol, *Zanclus cornutus* (Linnaeus, 1758) from Lakshadweep Waters

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Abstract

Purpose The present study describes a new species of myxosporean, *Sphaeromyxa cornuti* n. sp. infecting the gallbladder of the Moorish idol, *Zanclus cornutus* (Linnaeus 1758) collected from Lakshadweep waters of the Arabian Sea.

Methods Fish were collected using traps and cages. The morphology of mature spores recovered from the gallbladder of *Z. cornutus* was studied under Nomarski Differential Interference Contrast (DIC) optics. The molecular and phylogenetic analyses were based on SSU rDNA.

Results *Sphaeromyxa cornuti* n. sp. is characterized by arcuate myxospores with tapering extremities and round ends in valvular, and slightly sigmoid in sutural views (19.2–24.7 µm × 4.1–5.7 µm). The two polar capsules are unequally elongate-ovoid in shape and positioned at opposite ends of the spore (6.2–9.7 µm × 1.7–2.6 µm). Each encloses an irregularly folded, ribbon-like polar tubule, which is oriented parallel to polar capsule axis. In molecular and phylogenetic analyses, the present myxosporean revealed significant differences with related forms and clustered together with *S. hellandi* within the 'incurvata' group of the *Sphaeromyxa* clade with high nodal support.

Conclusions Morphological, morphometric, molecular and phylogenetic differences between our material and previously described species of *Sphaeromyxa,* along with host and geographic variations indicate that the present myxosporean is unique and the name *Sphaeromyxa cornuti* n. sp. is proposed. This forms the first report of a myxosporean parasite-infecting *Z. cornutus.*

Keywords *Sphaeromyxa cornuti* n. sp. · Myxospores · Moorish idol · Gallbladder · Molecular phylogeny

Introduction

The genus *Sphaeromyxa* Thélohan 1892 is an interesting assemblage of myxosporean parasites exclusively infecting the hepatic biliary system of marine fishes [1–3]. Members of the genus *Sphaeromyxa* are characterised by slightly curved or arcuate myxospores with tapering or truncated ends, a straight or curved suture connecting the spore extremities and polar capsules positioned at the opposite ends of the spore. Presence of loosely or irregularly coiled, ribbon-like polar tubules is considered a prominent feature of this genus [1, 2, 4–6]. Sphaeromixids are believed to be evolved from a fresh water *Myxidium* ancestor with a change in polar tubule morphology and expansion into marine hosts [2]. Phylogenetic studies have revealed the monophyletic nature of *Sphaeromyxa* and strongly support the spore morphology-based classification [7, 8]. Based on the spore morphology, the genus *Sphaeromyxa* was initially divided into two groups—'incurvata' possessing arcuate spores and pyriform polar capsules, and 'balbianii' having straight or slightly curved and fusiform or ovoid spores with ovoid polar capsules [9]. Later, a third group, 'limocapitis' was added, which is defined by having fusiform spores with pointed ends and considered to be a distinct lineage within the genus *Sphaeromyxa* [8]. Currently, 54 nominal species of sphaeromyxids have been described [3, 6, 10, 11]. Eight species of *Sphaeromyxa—S. pultai* Tripathi 1953, *S. theraponi* Tripathi 1953, *S. dighae* Sarkar and Majumdar 1983, *S. hareni* Sarkar, 1984, *S. ganapatii* Kalavati and Vaidehi

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1991, *S. opisthopterae*, Sarkar 1999, *S. chacundae* Sarkar 2004 and *S. diacanthusa* Sarkar 2004 have been reported from Indian waters.

The Lakshadweep archipelago with numerous lagoons and submerged reefs is located about 200–300 km off the West Coast of India in the Arabian Sea [12]. Lakshadweep waters have a rich reef fish fauna, with some 300 of the 600 known species considered as ornamental in nature [13]. Previous studies from Australian waters have suggested a high diversity and prevalence of myxosporean infections in reef dwelling fishes [14–20]. However, the parasite profile of the reef dwelling fishes from Lakshadweep waters is less known and the available information on myxosporean parasites infecting them is restricted to the description of two species, *Ceratomyxa lecosternoni* and *C. collarae* [21]. *Zanclus cornutus* (Linnaeus 1758) popularly known as Moorish idol is a marine ornamental fish inhabiting the reefs of the Indo-Pacific and Eastern-Pacific oceans. The present study provides the morphological and molecular description of a new species of *Sphaeromyxa* infecting the gall bladder of *Z. cornutus* collected from Lakshadweep waters.

Materials and Methods

Sample Collection and Morphological Analysis

Six specimens of *Z. cornutus* ranging from 7.5 cm to 10.1 cm in length and 16.6 g to 28.5 g in weight, were captured using cages/traps from Lakshadweep waters (10° 52.030′ N; 72° 12.289′ E). The collected fish were frozen, brought to the laboratory and examined for the presence of external myxosporean infections under a Nikon SMZ 1000 stereo zoom microscope (Nikon, Japan). The fish were dissected and internal organs/tissues and contents of gallbladder/urinary bladder were screened under a Nikon ECLIPSE 80i microscope (Nikon, Japan) for the presence of myxosporeans. For molecular analysis, infected bile was fixed in 95% ethanol. A portion of the infected sample was used for preparing permanent smears stained with Giemsa. Morphology of fresh myxospores was studied in detail under Nomarski Differential Interference Contrast (DIC) optics at 100 × magnification. Photomicrographs were taken using a Nikon DS Fi1C camera and myxospores were measured ($n = 30$) using Nikon-Elements BR software, following Lom and Arthur [22]. All measurements are stated in micrometers (µm) and expressed as mean ± SD followed by range in parenthesis. Myxospores were identified based on their morphology and morphometry with the help of appropriate key [23]. Line drawings of spores were prepared with the help of a Nikon Y-IDT drawing tube (Nikon, Japan) and digital images.

Molecular Analysis

Total genomic DNA of the parasite was extracted from the gallbladder content of one of the infected fish using HipurA™ multisample DNA purification kit (Himedia, India) in accordance with the manufacturer's protocol. The 18S small subunit ribosomal DNA (SSU rDNA) gene was amplified by nested PCR using two sets of primers, ERIB1—ERIB10 [24] and Myxospec F—18R [25, 26] in a Proflex PCR system (Applied Biosystems, USA). Both reactions were performed in 30 µl reactions containing 15 µl Dream Taq Green PCR master mix 2X (Thermo scientific, USA), 12 µl nuclease free water, 0.6 µl of each primer and 1.8 µl of DNA sample. First set of reactions was carried out using the primers ERIB1 and ERIB10 and the PCR cycling conditions were as follows: an initial denaturation at 94 ℃ for 5 min, followed by 35 cycles at 94 ℃ for 1 min, 56 ℃ for 1 min, 72 ℃ for 2 min and a final elongation of 72 ℃ for 10 min. To obtain a more specific product, a second round of amplification was performed using the product from the first reaction as template using the primers, Myxospec F and 18R, with the following PCR conditions: an initial denaturation at 95 ℃ for 4 min, followed by 35 cycles at 94 ℃ for 45 s, 58 ℃ for 45 s, 72 ℃ for 2 min and a final elongation of 72 ℃ for 10 min. The final PCR product obtained was visualized on a 1.5% Agarose Gel stained with Ethidium bromide and the bands were visualized under a LED transilluminator. The amplified PCR product was sequenced through a commercial firm (Agrigenome Labs Pvt. Ltd, Kochi, India).

Phylogenetic Analysis

Contigs of both the forward and reverse sequences were assembled in Bioedit 7.2.5 [27] and the resulting 1532 bp sequence was deposited in NCBI GenBank (Accession number: MZ997334). Standard BLAST search was performed to find similar sequences in NCBI GenBank database. Genetic distance between similar species of *Sphaeromyxa* was calculated using MEGA X [28]. To understand the phylogenetic relationship with other closely related species, both Bayesian Inference (BI) and Maximum Likelihood (ML) trees were constructed. Phylogenetic trees were constructed using sequences from 18 species of *Sphaeromyxa* selected from BLAST results along with the sequence of *Choromyxum leydigi* (Accession number: AY604199) as out group. The sequences were aligned in MEGA X with Clustal W programme [29]. The general time reversible model (GTR + I + G) determined using MEGA X was employed for tree construction. BI analysis was carried out in MrBayes, v.3.2.7

[30]. Markov Chain Monte Carlo (MCMC) analysis was simultaneously run for 1.5 million generations, with every 100th tree being sampled. The first 25% samples were discarded as burn-in. ML analysis was performed in MEGA X and nodal support was determined based on 1000 bootstrap replicates. The resulting ML and BI trees were depicted in FigTree v.1.4.3 [31], edited and annotated using Adobe Photoshop (Adobe Systems Inc. San Jose, CA).

Results

Morphology of Myxospores

Mature myxospores were observed freely floating in the bile. Myxospores were arcuate with tapering extremities and round ends in valvular view (Figs. 1a; 2a), and slightly sigmoid in sutural view (Figs. 1b; 2b). Delicate longitudinal striations were present on the spore surface. Though the striations were difficult to observe in fresh preparations, they were visible in Giemsa-stained spores (Fig. 1d). Sutural ridge absent, suture line less prominent, sigmoid in

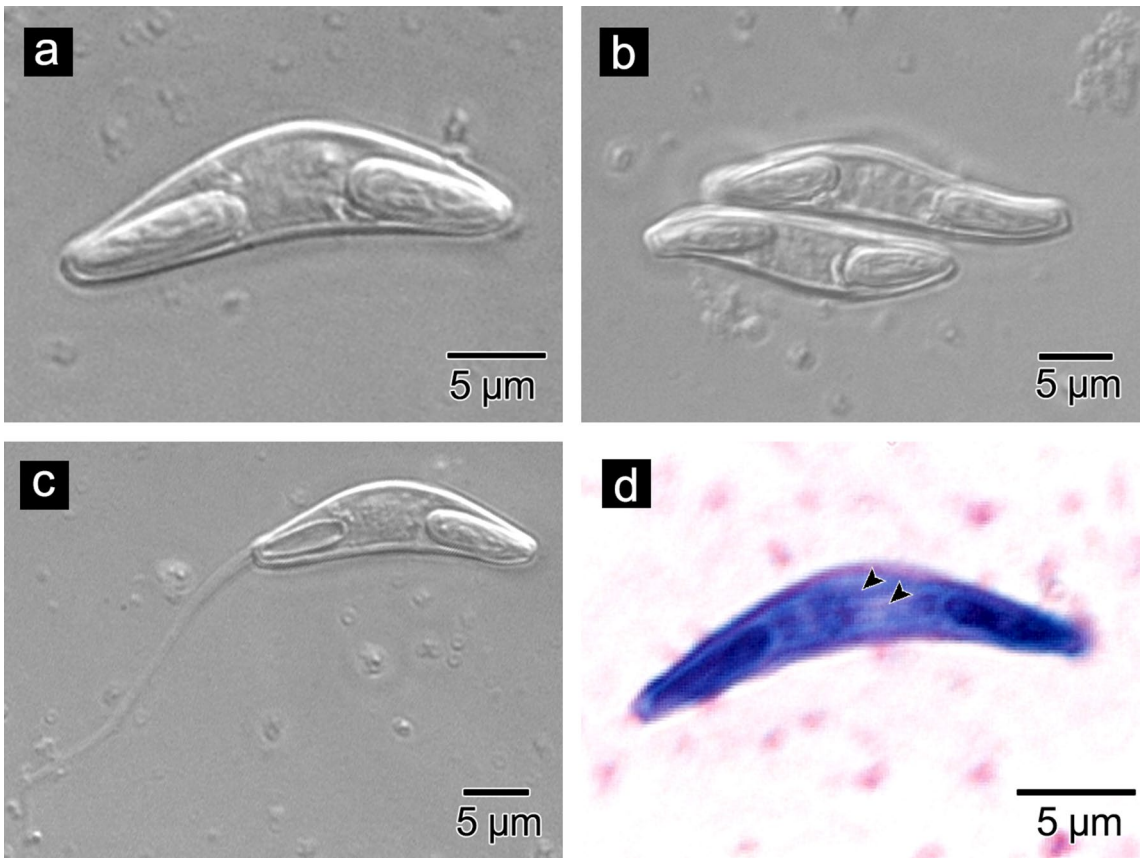

Fig. 1 Microphotographs of myxospores of *Sphaeromyxa cornuti* n. sp. **a** arcuate spore in valvular view; **b** sigmoid spore in sutural view; **c** spore with extruded polar capsule showing ribbon-like polar tubule; **d** Geimsa stained spores with surface striations indicated by arrowheads

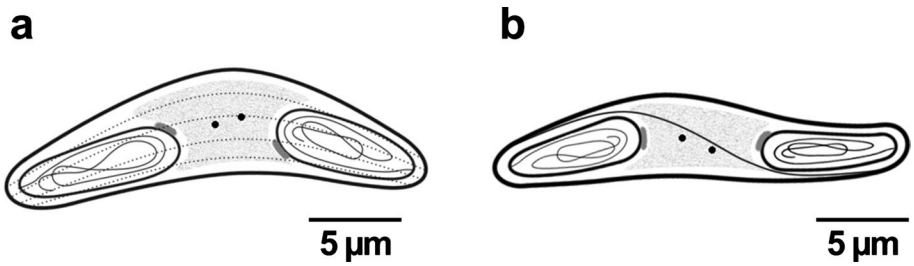

Fig. 2 Diagramatic representation of mature myxospore of *Sphaeromyxa cornuti* n. sp. **a** Valvular view; **b** sutural view

shape, and almost connecting the spore extremities (Fig. 2b). Myxospores measured 22.0 ± 1.3 (19.2–24.7) µm in length and 4.7 ± 0.5 (4.1–5.7) µm in thickness ($n = 30$) (Table 1). Polar capsules two, unequal and elongate-ovoid in shape, and were positioned at the opposite ends of the spore. One of the polar capsules appeared slightly longer and narrower than the other (Figs. 1a–d; 2a and b). Polar capsules measured 7.7 ± 0.8 (6.2–9.7) µm in length and 2.4 ± 0.2 (1.7–2.6) µm in width ($n = 30$) (Table 1). A capsulogenic cell nucleus was observed adjacent to each of the polar capsules. Polar tubule ribbon-like, irregularly folded, more or less parallel to the longitudinal axis of the polar capsule. The number of polar tubule coils could not be established due to their irregular folding pattern. When extruded, the filament appeared flat and broad at its base and gradually tapered towards its tip (Fig. 1c). Sporoplasm binucleate, occupied most of the extra-capsular space (Figs. 1a–c; 2a and b). Vegetative or developmental stages were not observed.

Taxonomy

Phylum: Cnidaria
Unranked subphylum: Myxozoa
Class: Myxosporea Bütschli, 1881
Order: Bivalvulidae Shulman, 1959
Family: Sphaeromyxidae Lom and Noble, 1984
Genus: *Sphaeromyxa* Thélohan, 1892

Species: *Sphaeromyxa cornuti* n. sp
Type host: *Zanclus cornutus* (Linnaeus, 1758) (Family: Zanclidae)
Type locality: Lakshadweep islands, Arabian Sea
Site/organ: Gallbladder
Prevalence: Two of six *Z. cornutus* were infected (33%)
Etymology: The species name of the parasite refers to the specific name of the host
Type material: Permanent, Giemsa-stained slide of the voucher specimen was deposited with the Marine Biodiversity Museum, Central Marine Fisheries Research Institute, India (Accession number: CG.3.2.1.16). The SSU rDNA gene sequence (1532 bp) was submitted to NCBI GenBank database (Accession number: MZ997334); ZooBank species registration no: D50C34FE-6DA2-4EF2-B97B-7A8DF4CCF920

Molecular and Phylogenetic Analysis

The partial SSU rDNA of the present species was sequenced and the 1532 bp long sequence was submitted to GenBank (Accession number: MZ997334). In BLASTn analysis, the present species revealed the highest percentage of pairwise sequence identity of 96.4% with *Sphaeromyxa hellandi* from *Mellanogrammus aeglefinus* (DQ377693), followed by 96.2% with *S. hellandi* from *Helicolenus dactylopterus* (DQ377701; Table 2). In genetic divergence study, the

Table 1 Showing comparative descriptions of *Sphaeromyxa cornuti* n. sp. with eight morphologically similar species

Species	SL	ST	PCL	PCW	Host	Type locality	Source
S. reinhardti	21.2–23.3	3.7–5.0	–	–	*Engraulis mordax*	Pacific Ocean, California	Jameson [35]
S. curvula	19.0–22.0	4.0–6.0	7.0–9.0	2.0–3.0	*Pachymetopon blochii*	False Bay, South Africa	Fantham [36]
S. elegini	17.0–20.0	6.0	5.0–6.0		*Eleginus gracilis*	Andreeva Bay, Sea of Japan, Russia	Dogiel [37]
S. hareni	27.5 (23.3–28.9)	5.1 (4.7–5.6)	9.3 (8.9–10.3)	4.3 (3.0–5.1)	*Plicofollis platystomus*	Digha, Bay of Bengal, India	Sarkar [38]
S. noblei	20.0 (18.5–21.5)	5.0 (4.8–5.2)	5.9 (5–6.5)	2.6 (2.5–2.7)	*Heteroclinus whiteleggii*	Arrawara, New South Wales, Australia	Lom [39]
S. kenti	18.5 (17.5–19.8)	4.4 (3.8–5.2)	7.9 (6.9–8.6)	2.3 (2–2.6)	*Gobiosoma bosc*	Louisiana, USA	Whipps and Font [7]
S. clini	18.8 (17.4–20.6)	5.0 (4.0–6.0)	5.9 (5.3–6.6)	2.5 (2.1–3.1)	*Clinus acuminatus*	Mouille Point, South Africa	Bartošová-Sojková *et al.* [8]
S. xiamenensis	21.1(19.0–23.0)	4.3 (3.0–5.0)	8.4 (6.5–9.8)	2.8 (1.8–3.2)	*Siganus fuscescens*	Xiamen, China	Chen *et al.* [10]
S. cornuti n. sp.	22.0 ± 1.3 (19.2–26.4)	4.7 ± 0.5 (4.1–5.7)	7.8 ± 0.9 (6.2–9.7)	2.3 ± 0.2 (1.7–2.6)	*Zanclus cornutus*	Lakshadweep, Arabian sea, India	Present study

The measurements are given in µm

SL Spore length, *ST* spore thickness, *PCL* polar capsule length, *PCW* polar capsule width

Table 2 Showing the percentage pairwise sequence identity obtained from BLAST (upper right diagonal) and nucleotide divergence from MEGA X (lower left diagonal) of *Sphaeromyxa cornuti* n. sp. with nine related species

	1	2	3	4	5	6	7	8	9	10
1 *Sphaeromyxa cornuti* n. sp. (MZ997334)		96.42	96.16	93.33	93.08	92.25	90.88	90.72	89.43	89.17
2 *Sphaeromyxa hellandi* from *Mellanogrammus aeglefinus* (DQ377693)	3.25		99.70	93.58	93.16	92.32	91.29	90.46	89.26	88.48
3 *Sphaeromyxa hellandi* from *Helicolenus dactylopterus* (DQ377701)	3.52	0.40		93.38	92.96	92.10	91.09	90.25	89.01	88.27
4 *Sphaeromyxa clini* (KM201336)	7.12	6.91	7.18		92.73	93.34	91.03	90.67	89.65	88.49
5 *Sphaeromyxa xiamenensis* (MK335952)	6.72	7.25	7.52	7.40		92.08	91.32	90.16	94.22	88.40
6 *Sphaeromyxa theraponi* (MK335953)	8.14	8.06	8.33	5.95	7.59		90.18	89.58	89.12	88.58
7 *Sphaeromyxa lycodi* (KC524734)	10.08	9.79	10.06	9.58	9.06	10.18		94.20	88.48	89.83
8 *Sphaeromyxa kenti* (JX443489)	10.08	10.19	10.47	9.65	10.00	11.12	6.03		88.14	89.94
9 *Sphaeromyxa* sp.(MT840081)	9.42	9.94	10.21	9.74	4.05	9.87	10.70	11.70		86.19
10 *Sphaeromyxa artedielli* (KF135220)	11.72	11.08	11.35	11.34	11.57	12.16	10.01	10.07	13.79	

present species revealed the least divergence of 3.2% with *S. hellandi* from *M. aeglefinus* followed by 3.5% with *S. hellandi* from *H. dactylopterus* (Table 2). In phylogenetic analysis, topologies of both ML and BI trees were identical. In both analyses, trees were divided into two main lineages. The first lineage includes the 'incurvata' and 'limocapitis' groups while the second lineage contains species belonging to 'balbianii' group. The present species clustered with *S. hellandi* within the 'incurvata' group with high bootstrap and BI values (100/1) (Fig. 3).

Remarks Based on the morphology and morphometry of the myxospores, the present species exhibits close similarities with *Sphaeromyxa reinhardti* Jameson, 1929, *Sphaeromyxa curvula* Fantham, 1930, *Sphaeromyxa elegini* Dogiel, 1948, *Sphaeromyxa hareni* Sarkar, 1984 *Sphaeromyxa noblei* Lom, 2004, *Sphaeromyxa kenti* Whipps and Font, 2013, *Sphaeromyxa clini* Bartošová-Sojková *et al*. 2015 and *Sphaeromyxa xiamenensis* Chen *et al*. 2020. A comparison of the above eight species with the present myxosporean is provided in Table 1. *S. reinhardti* differs from the present myxosporean in having thin, straight to slightly bent myxospores with blunt and truncated ends. Further, spores of *S. reinhardti* possessed ovoid polar capsules and a small, centrally located sporoplasm. *S. curvula* can be differentiated from the present parasite in having curved myxospores with blunt extremities, and broader polar capsules. Shorter and broader spores with blunt ends and presence of smaller polar capsules distinguish *S. elegini* from the present species. *S. hareni* differs from the present species in having large, slightly curved spores without striations and large, oval polar capsules. Shorter myxospores with 6–9 striations and shorter and broader polar capsules differentiate *S. noblei* from the present species. Smaller, elongate myxospores with blunt extremities and 5–9 striations, longitudinal suture and a central sporoplasm differentiates *S. kenti* from the present species. Shorter myxospores, short, oval and broad polar capsules, and absence of surface striations separates *S. clini* from the present species. Unlike the present species, *S. xiamenensis* has comparatively smaller spores devoid of striations, possessed blunt ends and larger polar capsules. Except *S. kenti,* all the above compared forms have equal polar capsules. In addition, the present species differs from all the above compared species in its fish host and geographic location. Hence, considering the above-mentioned differences with the previously described species of *Sphaeromyxa,* the present myxosporean is treated as unique and the name *Sphaeromyxa cornuti* n. sp. is proposed.

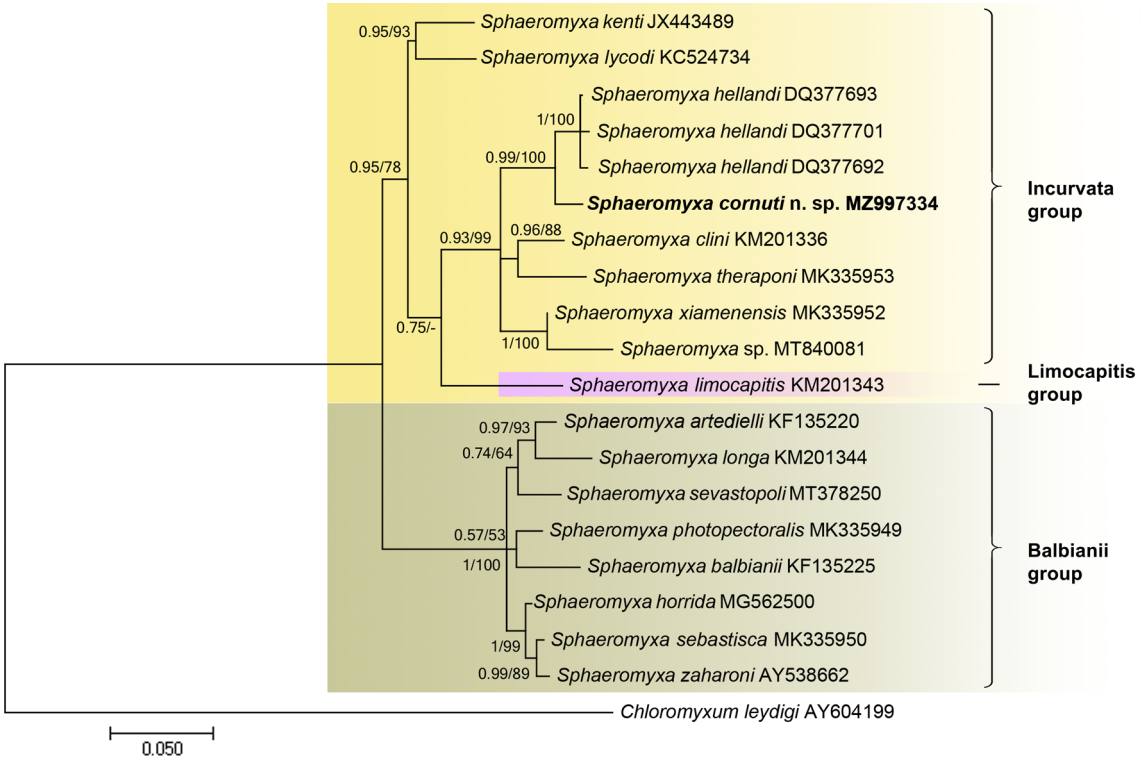

Fig. 3 Phylogenetic tree based on SSU rDNA dataset indicating the position of *Sphaeromyxa cornuti* n. sp. (represented in bold italics). Numbers at nodes represent Bayesian posterior probability and bootstrap values (BI/ML). Dashes at nodes indicate nodal support (ML) < 50. GenBank accession numbers follow each taxon

Discussion

The present study describes a new species of myxosporean, *Sphaeromyxa cornuti* n. sp. infecting the gallbladder of *Z. cornutus.* Arcuate or slightly curved myxospores with elongate-ovoid polar capsules place the present myxosporean in the 'incurvata' group of the genus *Sphaeromyxa* [4, 9]. The present species differs significantly from the previously reported species of *Sphaeromyxa* in terms of morphology, morphometry, host and geographic location (Table 1). Limited morphological features of myxospores and their overlapping nature often create difficulties in the traditional, spore-based species identification in myxosporeans. Hence a comprehensive approach incorporating a combination of morphology, morphometry, molecular phylogeny, host/site specificity and geographical location is preferred.

In molecular analysis, *S. cornuti* n. sp. exhibits highest molecular similarity of 96.4% with *S. hellandi* from *M. aeglefinus*. While in divergence study, the present species exhibits least divergence of 3.2% with *S. hellandi* from *M. aeglefinus* (Table 2). However, in morphology and morphometry, *S. hellandi* differs significantly from the present species in having larger spores and large, ellipsoidal polar capsules, and in its host and geographical distribution. A genetic divergence of 1- 1.3 percent has been considered by many authors for species delineation in myxosporeans [17, 18, 20]. However, the divergence value may vary in different groups, and intraspecific divergence varying from 0.1 to 30% has been reported for the genus *Myxobolus* [25, 32–34].

Phylogenetic analyses of SSU rDNA gene of myxosporeans reveal the presence of paraphyletic and polyphyletic taxa indicating that spore morphology cannot be directly correlated with phylogenetic relationships in myxosporeans [25]. *Spheromyxa* represents one of the few monophyletic groups of myxozoan taxa as its myxospore morphology based grouping is consistent with phylogenic divisions [2, 8]. However, of the 54 nominal species of *Sphaeromyxa,* SSU rDNA sequence data is available for only 15 species. Hence, availability of molecular information for more species will provide a conclusive picture on the monophyly of this genus.

In the present phylogenetic analyses, genus *Spheromyxa* appears as a monophyletic group which is further subdivided into two lineages, supporting the earlier myxospore-based classification [3, 8, 9, 11, 34]. The first lineage hosts the 'incurvata' and 'limocapitis' groups while the second harbours the 'balbianii' group. In the present study, *S. cornuti* n. sp. is nested within the 'incurvata' group alongside *S. hellandi* as sister with high bootstrap and BI values (100/0.1). The phylogenetic positioning of *S. limocapitis* has been

inconsistent, in one of the earlier studies it appeared as a separate lineage, as sister to 'incurvata' and 'balbianii' groups [8]. In some other studies, *S. limocapitis* clusters within the 'balbianii' group [11], or appears as sister to 'incurvata' [3] or clusters within the 'incurvata' group [34] as in the present case. Hence, many authors prefer to consider *S. limocapitis* as an unstable lineage which switches between 'balbianii' and 'incurvata' groups and may be considered as a missing link in the evolution of sphaeromyxids [8].

Based on the morphological, morphometric, molecular and phylogenetic differences with the previously described species of *Sphaeromyxa,* coupled with host and geographic differences, the present myxosporean is treated as novel and the name *Sphaeromyxa cornuti* n. sp. is proposed. The present study forms the first report of a myxosporean parasite-infecting *Z. cornutus.*

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Declarations

Conflict of Interest The authors declare that they have no conflicts of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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