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ORIGINAL PAPER

Morphological and Molecular Characterization of *Myxobolus planilizae* n. sp. (Cnidaria; Myxosporea; Myxobolidae) Infecting the Largescale Mullet *Planiliza macrolepis* (Smith, 1846) Collected From Cochin Backwaters, India

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Abstract

Purpose *Myxobolus planilizae* n. sp. is described from the intestinal muscles of the largescale mullet *Planiliza macrolepis* from Cochin backwaters, Kerala, India.

Methods Host fishes inhabiting Cochin backwaters were collected using Chinese nets/gill nets. The morphometry and morphological studies were carried out using Nomarski differential interference contrast (DIC) optics, followed by molecular and phylogenetic analyses of the small subunit ribosomal DNA gene (SSU rDNA).

Results Plasmodia small, pale white, and infect the muscles of the intestine; measured 0.13–0.22 (0.17) × 0.09–0.14 (0.13) mm. Mature myxospores pyriform in valvular view, and biconvex in sutural and apical views with a short anterior extension, and measured 7.45–8.75 (8.40) × 6.04–6.86 (6.25) µm. Shell valves with sutural ornamentations. Polar capsules two, equal, pyriform, measured 3.96–4.54 (4.45) × 2.22–2.94 (2.52) µm. Polar filament arranged in five coils, measured 24.41–34.44 (28.52) µm when extruded. In morphological and morphometric analysis, the present species exhibit remarkable variations from other species of the genus *Myxobolus*. In molecular analysis, the present species revealed the highest identity of 91.85% and divergence of 9.95% with related species, underlining its molecular uniqueness. In phylogenetic analysis, species of *Myxobolus* infecting mullets appeared as a separate clade and the present species was positioned distinctly with a high bootstrap value.

Conclusions Based on morphology, morphometry, and molecular and phylogenetic analyses, along with tissue/host specificities and geographic location, the present parasite is treated as new and is reported here as *M. planilizae* n. sp.

Keywords Myxoxporean · Myxospore · Plasmodia · Histopathology · Phylogeny · 18S rDNA

Introduction

Myxozoans are a diverse group of metazoans having a complex life cycle, comprising both vertebrate (fish) and invertebrate (annelid) hosts [1, 2]. Multicellular characteristics of the myxospore and molecular information elevated the position of myxozoans from protozoans to metazoans [3]. The striking structural and functional similarities of polar capsules with the nematocysts found in Cnidarians and the presence of Cnidarian-specific minicollagen genes paved the way for the inclusion of myxosporeans in the phylum Cnidaria [4–6]. Presently, myxosporeans represent approximately 20% of the cnidarians [7].

Among the 64 genera of myxosporeans, the genus *Myxobolus* Bütschli, 1882 is the largest with 979 taxa [8–10]. Members of the genus *Myxobolus* are generally histozoic in nature, forming intracellular or intercellular plasmodia. Bivalvular myxospores are ellipsoidal to ovoid or subcircular in valvular view and biconvex in sutural view, with smooth or ornamented shell valves and two pyriform or oval, equal or unequal polar capsules and binucleate sporoplasm, often with an iodinophilous vacuole [11]. Limited and overlapping taxonomic characters together with a large number of species often create difficulties in the taxonomic classification

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of this genus, leading to erroneous species identifications. Many of the myxosporeans exhibit strong host and site/tissue specificities and, hence, these features are also considered along with the morphology and morphometry of myxospores for taxonomic identification [12–16]. The use of molecular tools can reveal the subtle differences between morphologically similar or cryptic species and has been successfully used in resolving the taxonomic ambiguities in many myxosporeans [17].

Mullets belong to the family Mugilidae, which includes 26 genera and 76 species and enjoy a cosmopolitan distribution [18]. They are euryhaline and act as intermediate hosts for many species of myxosporeans [17]. Mullets are considered a delicacy, have high market value, and are cultured widely in many parts of the world [19]. The largescale mullet, *Planiliza macrolepis* (Smith, 1846) inhabits the tropical and subtropical coastal waters, and feeds on small benthic polychaetes, organic matter, detritus, etc., making them vulnerable to parasitic infections, especially myxosporeans. The present study describes a new species of *Myxobolus* infecting the intestine of *P. macrolepis* collected from Cochin backwaters, Kerala, India.

Material and Methods

Sampling

One hundred and eighty-seven samples of *P. macrolepis* measuring 9.2–19.5 cm in length and 18–90 g in weight were collected from Cochin backwaters from October 2018 to September 2021 using Chinese nets/gill nets. Fishes were examined carefully under a Nikon SMZ 1000 stereo zoom microscope (Nikon, Japan) for the presence of external myxosporean parasites. Following standard necropsy procedures, the fish were dissected and their internal organs separated and examined thoroughly for the presence of myxosporeans using a Nikon Eclipse 80i microscope (Nikon, Japan). Spores were preserved in 95% ethanol for molecular analysis. Permanent slides were prepared using air-dried, ethanol-fixed smears stained with Giemsa. The present work was conducted following all regulatory guidelines (CPCSEA Guidelines on Regulation of Scientific Experiments on Animals, 2007).

Myxospore Morphology and Morphometry

Fresh myxospores were studied in detail using a Nomarski differential interference contrast (DIC) objective at × 100 magnification and photomicrographs were taken using a Nikon DS-Fi1c camera (Nikon, Japan). Measurements of myxospores ($n = 30$) were taken using Nikon Elements BR software, following the guidelines proposed by Lom and Arthur [20]. Line drawings were made with the help of Nikon Y-IDT drawing tube (Nikon, Japan). All measurements are given in micrometers (µm) as range, followed by mean ± standard deviation in parentheses.

Histology

Infected tissues were preserved overnight in 10% neutral buffered formalin (NBF), washed, dehydrated in ascending grades of ethanol, cleared with xylene, and embedded in paraffin wax. Sections (5-µm thick) were taken using a rotary microtome (Leica, Germany), stained with hematoxylin and eosin [21], and mounted in DPX, and photomicrographs were taken at different magnifications.

Molecular Analysis

Genomic DNA was extracted from ethanol-fixed myxospores using a Multi-Sample DNA Purification Kit (HiPurA™), following the recommended protocols. Amplification of a small fragment of small subunit ribosomal DNA gene (SSU rDNA) was carried out by semi-nested polymerase chain reaction (PCR) in a Proflex PCR system (Applied Biosystems, USA). PCR was conducted in 30 µl total reaction volumes consisting of 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 template DNA. Eukaryotic primers ERIB1 and ERIB10 [22] were used for the first round of amplification with an initial denaturation at 95 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min and final elongation at 72 °C for 10 min. A second round of reaction was performed with the PCR product yielded from the first reaction using the primers ERIB1-ACT1R [22, 23] (initial denaturation at 95 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, 50 °C for 2 min and 72 °C for 3 min and final elongation at 72 °C for 10 min) and Myxgen4F-ERIB10 primers [22, 24] (initial denaturation at 95 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 56 °C for 1 min and 72 °C for 90 s and final elongation at 72 °C for 5 min). Gel electrophoresis of the final PCR product was carried out using 1.5% agarose gel in Tris–EDTA buffer and stained with ethidium bromide. The positive amplicons were excised, purified, and Sanger sequenced through a commercial company.

Phylogenetic Analysis

Partial SSU rDNA sequences were assembled from two overlapping fragments, edited using BioEdit V7.2.5 [25], and the resulting 1884 bp-long sequence was analyzed with a NCBI BLAST search and deposited in GenBank (Accession number: OM690617). Phylogenetic analysis was conducted using partial 18S SSU rDNA sequences of 24 species

of *Myxobolus* downloaded from GenBank (selected based on the identity scores) along with the sequence of *Buddenbrockia plumatellae* (Accession number: KJ731698) as outgroup. Sequences were aligned using Clustal W module in MEGA 7 [26]. General Time Reversible (GTR + I + G) model appeared to be the best-fit model for phylogenetic analysis in MEGA7. Genetic distance analysis was carried out using the *p*-distance model. The maximum likelihood (ML) tree was constructed with the rapid bootstrap method using 1000 ML bootstrap replicates. Bayesian inference (BI) was carried out in MrBayes, version 3.2.7 [27], and Markov chain Monte Carlo (MCMC) search was performed for 20,00,000 generations using default parameters, with every 100th tree being sampled. The first 25% of trees were discarded as burn-in. The resulting ML and BI trees were visualized, edited, and annotated in FigTree v.1.4.3 [28] and Adobe Photoshop (Adobe Systems Inc. San Jose, California, USA), respectively.

Results

Of the 187 *P. macrolepis* screened, 6 (3.2%) were infected with plasmodia of a novel species of *Myxobolus*. The plasmodia were observed infecting the muscular layer, just below the outer surface of the intestine and four to six plasmodia were recovered from each fish. Plasmodia appeared pale white, circular, and measured 0.13–0.22 (0.17 ± 0.02) mm in length and 0.09–0.14 (0.13 ± 0.01) mm in width (Fig. 1a).

Morphology of Myxospores

Mature myxospores were bivalvular, pyriform in valvular view, and biconvex in sutural and apical views, with a short, well-defined anterior extension and measured 7.45–8.75 (8.40 ± 0.24) µm in length and 6.04–6.86 (6.25 ± 0.33) µm ($n = 30$) in width (Fig. 1b–e; Fig. 2a and b). Sutural ridge

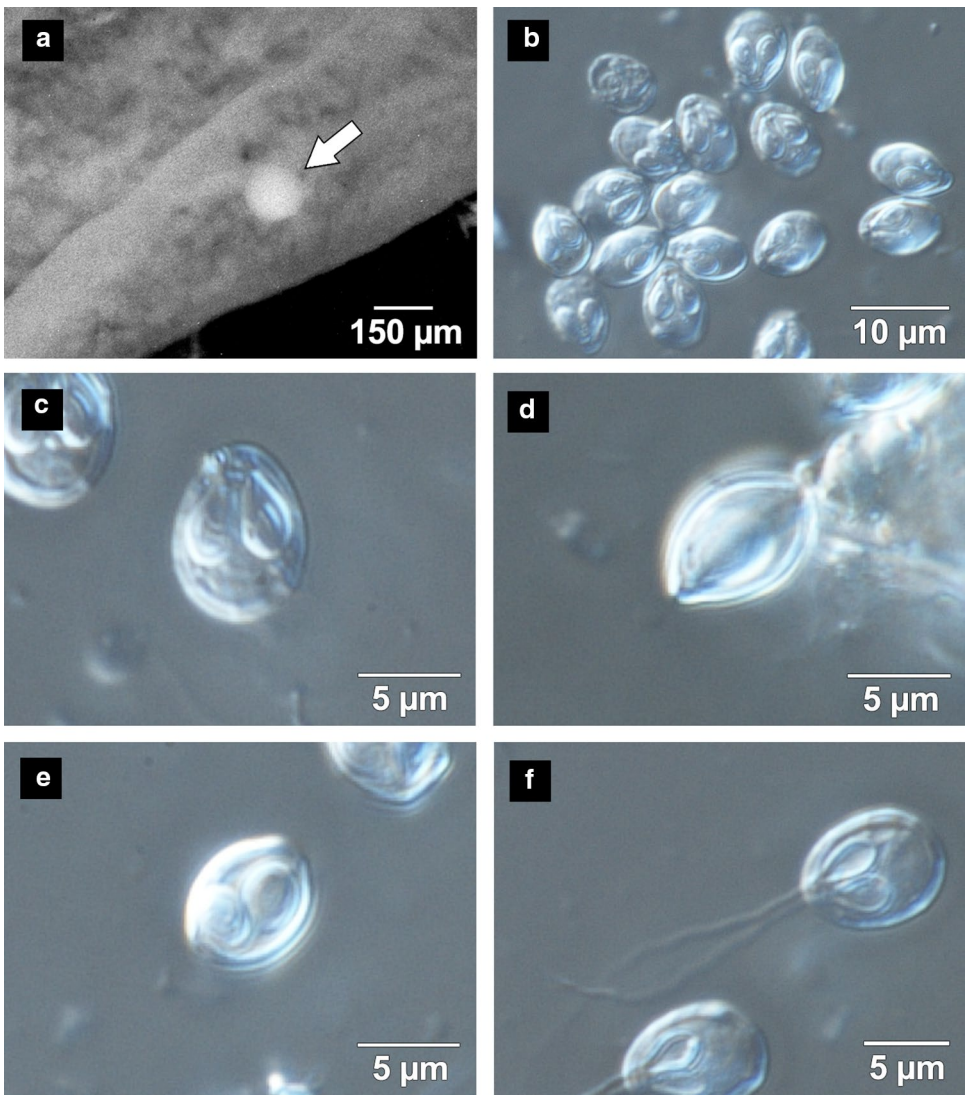

Fig. 1 *M. planilizae* n. sp. **a** plasmodium attached to the intestinal muscles; **b** myxospores; **c** myxospore—frontal view; **d** myxospore—sutural view; **e** myxospore—apical view; **f** myxospores showing extruded polar filaments

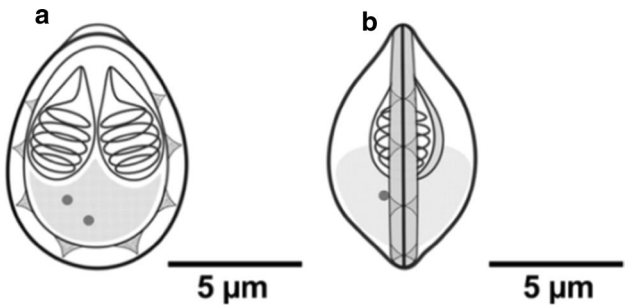

Fig. 2 Line drawings of *M. planilizae* n. sp.: **a** frontal view; **b** sutural view

presented with eight sutural folds, which were restricted to the posterior three-fourths of the shell valves (Fig. 1c; Fig. 2a and b). Spores harbored two, equal, and pyriform polar capsules with pointed anterior ends, measuring 3.96–4.54 (4.45 ± 0.38) µm in length and 2.22—2.94 (2.52 ± 0.24) µm ($n = 30$) in width. Polar filament, arranged in five coils, measured 24.41–34.44 (28.52 ± 3.38) µm ($n = 6$) when extruded. Sporoplasm with two capsulogenic nuclei filled the entire extracapsular space (Fig. 2a and b).

Taxonomy

Phylum: Cnidaria.

Unranked Subphylum: Myxozoa Grassé, 1970.

Class: Myxosporea Bütschli, 1881.

Order: Bivalvulidae Shulman, 1959.

Family: Myxobolidae Thelohan, 1892.

Genus: *Myxobolus* Bütschli, 1882.

Species: *Myxobolus planilizae* n. sp.

Type host: *Planiliza macrolepis* (Smith, 1846).

Type locality: Cochin backwaters, Southwest coast of India (9.9312 ^{0} N, 76.2673 ^{0}E).

Site/Organ: Intestinal muscles.

Prevalence: 6 of 187 *P. macrolepis* were infected (3.2%).

Etymology: named after the generic name of the host species.

Type material: Giemsa-stained slides deposited in the parasite collections of the Marine Biodiversity Museum, Central Marine Fisheries Research Institute, India (Accession number: CD. 1. 1. 1. 2) ,and partial sequences of 18S SSU rDNA gene (1884 bp) deposited in NCBI GenBank (Accession number: OM690617). Zoo Bank species registration no: urn:lsid:zoobank.org:act: 55676E06-0100–4467-9017-B49529671388.

Molecular and Phylogenetic Analysis

The partial SSU rDNA sequence obtained from the present species was 1884 bp long and deposited in NCBI GenBank (Accession number: OM690617). BLASTn results showed the highest identity of 91.85% with *M. muscularis* (MK203075). This was followed by 90.96% identity with *M. adiposus* (MK203076), 90.53% with *M. cerveirensis* (MK203079), and 90.49% with *M. peritonaeum* (MK203080). Genetic distance analysis using the *p*-distance model revealed a divergence of 9.95% with *M. muscularis*, the nearest sequence in BLASTn analysis.

In phylogenetic analysis, trees constructed using both ML and BI methods were identical, with the species of *Myxobolus* forming a separate clade (clade A). The present species occupied a distinct position with high bootstrap support along with other species of *Myxobolus* recovered from mullets (Fig. 3). Species of *Henneguya* along with *M. filamentum* appeared as a second clade (Clade B).

Histopathology

With histopathology, the plasmodium was observed within the muscular layer, just below the outer surface of the intestine (Fig. 4a). The muscular layer surrounding the plasmodium appeared compressed, probably due to the pressure exerted by the plasmodium. Mild hemocytic infiltration was observed in the surrounding tissues (Fig. 4a and b). The central region of the plasmodium was filled with mature myxospores, while immature spores and developing stages were observed near the periphery.

Remarks

Among the known species of *Myxobolus* possessing elongate, oval spores with sutural ornamentations and equal polar capsules, the present species resembles *M. phylloides* Schulman, 1962, *M. episquamalis* Egusa *et al.* 1990, *M. macrolepi* Padma Dorothy & Kalavati 1992, *M. sclerii* Kaur *et al.* 2010, *M. chittalii* Kaur *et al.* 2011, *M. basuhaldari* Szekely *et al.* 2015, *M. szentendrensis* Cech *et al.* 2015, *M. arariensis* Abrunhosa *et al.* 2018, *M. arcasii* Rocha *et al.* 2019, *M. cerveirensis* Rocha *et al.* 2019, *M. labrosus* Rocha *et al.* 2019 and *M. ovarium* Naldoni *et al.* 2020 in its morphology. A comparison of the present myxosporean with the above 11 species of *Myxobolus* is provided in Table 1.

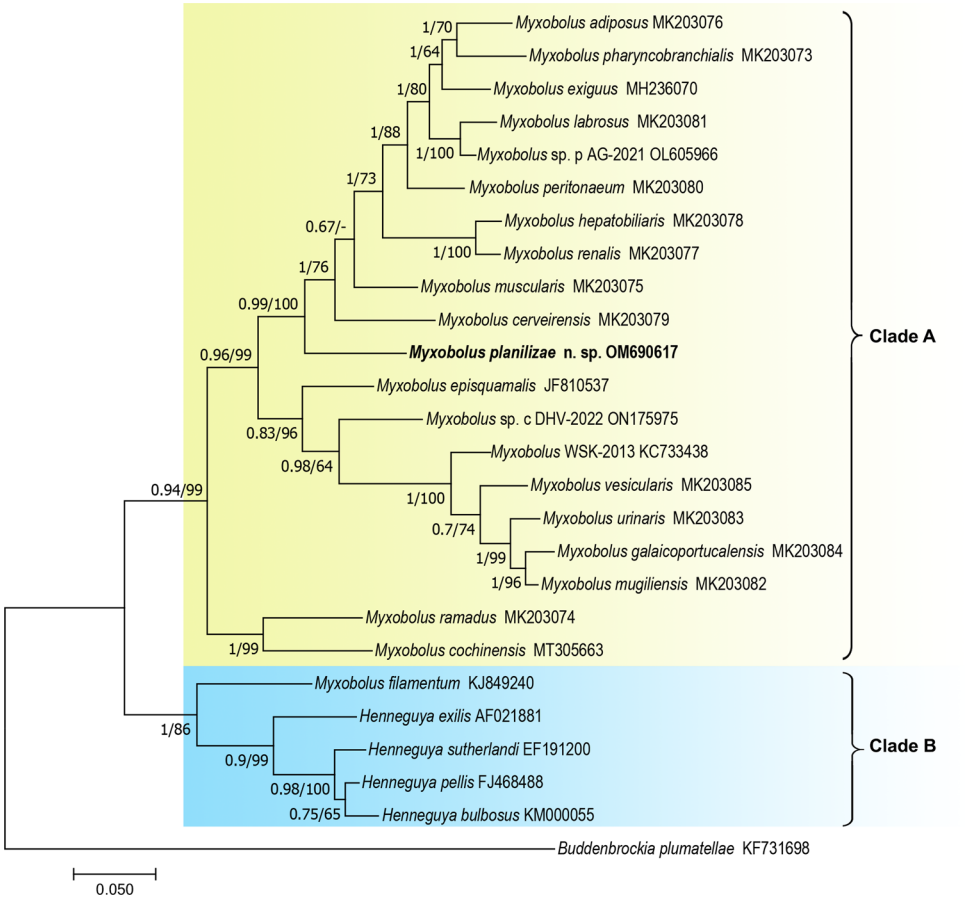

Fig. 3 Phylogenetic tree showing the position of *M. planilizae* n. sp. (in bold) with other related species of myxosporeans downloaded from GenBank. Posterior probability and bootstrap for BI and ML are represented at nodes, and species names are followed by their accession number. Dashes at nodes indicate the bootstrap value (ML) < 50

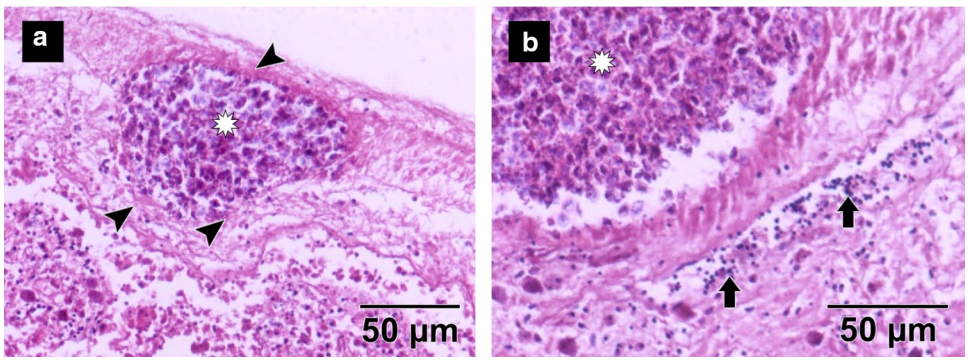

Fig. 4 Histology of the intestine infected with the plasmodium of *M. planilizae* n. sp.: **a** plasmodium in the intestinal wall (*) compressed surrounding muscles (arrowheads); **b** hemocytic infiltration around the plasmodium (arrows)

Spores of *M. phylloides, M. szentendrensis, M. arariensis, M. arcasii* and *M. labrosus* are larger than the present species, while those of *M. macrolepi* and *M. basuhaldari* are smaller, distinguishing the present species. *M. macrolepi* also differs in having three to four polar filament coils. *M. ovarium* differs from the present species in having longer spores, longer and narrower polar capsules, and eight to nine polar filament coils. Though both the species infect the intestine, the presence of narrower spores and polar capsules and a well-defined anterior extension differentiate the present species from *M. cerveirensis. M. episquamalis* can be distinguished from the present species in having broader polar capsules and in its site of infection. *M. sclerii* infects the eyeball and has narrower spores, and longer and narrower polar capsules when compared to the present species, while *M. chittalii* differs in having longer spores. Among the compared species, *M. macrolepi* and *M. cerveirensis* infect the intestine of their hosts, while the remaining species infect various other organs. The present species also differs from all other compared species except *M. macrolepi, M. sclerii, M. chittalii* and *M. basuhaldari* in its geographical location. The marked differences in morphology and morphometry,

Table 1 Comparative myxospore dimensions (μm) of the present myxosporean with morphologically similar species

Species	Myxospore measurements					Site of infection	Host	Geographic region	Reference
	SL	SW	PCL	PCW	NC				
M. phylloides	9.0–10.0	7.0–7.5	5.7–6.5	2.8–3.5	–	Mesentery and kidney	*Hypophthalmichthys molitrix*	Iraq	Schulman [29]
M. episquamalis	7.5–9.5 (8.6)	6.0–7.5 (6.8)	3.8–5.0 (4.4)	2.0–3.0 (2.3)		Scales	*Mugil cephalus*	Japan	Egusa [30]
M. sclerii	7.9–9.5 (8.7 ± 1.13)	4.3–5.7 (5.0 ± 0.9)	4.0–5.4 (4.7 ± 0.98)	1.0–2.6 (1.8 ± 1.31)	4–5	Eyeball	*Catla catla*	India	Kaur [31]
M. chittalii	8.8–9.2 (9.0)	5.88–6.48 (6.18)	4.0–5.0 (4.5)	2.0–2.8 (2.4)	4–5	Gill lamellae	*Puntius sophore*	India	Kaur [32]
M. basuhaldari	6.8–7.8 (7.3 ± 0.31)	5.1–6.2 (5.5 ± 0.35)	2.9–3.7 (3.2 ± 0.31)	2.0–2.2 (2.1 ± 0.08)	5	Gill lamellae	*Labeo rohita*	India	Szekely [33]
M. szentendrensis	8.8–9.6 (9.2)	7.6–8.0 (7.9)	4.8–5.6 (5.3)	2.8–3.2 (3.0)	6	Gill lamellae	*Chondrostoma nausus*	Hungary	Cech [34]
M. arariensis	10.7–12.6 (11.4)	6.4–7.9 (7.2)	3.6–4.3 (4)	1.7–2.2 (1.9)		Skeletal muscle	*Rhamdia quelen*	Brazil	Abrunhosa [35]
M. arcasii	9.3–10.7 (9.7 ± 0.5)	8.0–8.7 (8.1 ± 0.2)	3.3–4.3 (3.9 ± 0.3)	2.7–3.3 (3.0 ± 0.2)	6	Kidneys	*Achondrostoma arcasii*	Portugal	Rocha [17]
M. cerveirensis	7.7–8.7 (8.1 ± 0.2)	6.7–7.3 (6.8 ± 0.2)	4.0–4.7 (4.2 ± 0.2)	2.3–3.0 (2.8 ± 0.2)	4–5	Intestine	*Chelon ramada*	Portugal	Rocha [17]
M. labrosus	9.7–10.3 (10.0)	7.7–8.7 (8.1)	4.0–5.0 (4.5)	2.3–2.7 (2.5)	5–7	Urinary bladder	*Chelon labrosus*	Portugal	Rocha [17]
M. ovarium	9.2–11.0 (9.8)	5.6–6.9 (6.5)	3.9–6.2 (4.7)	1.8–2.4 (2.1)	8–9	Ovary	*Brycon orthotaenia*	Brazil	Naldoni [36]
M. planilizae n. sp.	7.45–8.75 (8.4 ± 0.24)	6.04–6.86 (6.25 ± 0.33)	3.96–4.54 (4.45 ± 0.38)	2.22–2.94 (2.52 ± 0.24)	5	Intestine	*Planiliza macrolepis*	India	Present study

(*SL* spore length, *SW* spore width, *PCL* polar capsule length, *PCW* polar capsule width, *NC* no. of coils)

when compared with the other described species of *Myxobolus*, indicate that the present myxosporean is morphologically unique and is considered a new species, and the name *Myxobolus planilizae* n. sp. is proposed.

Discussion

The present study describes a new species of myxosporean parasitizing the intestinal muscles of the largescale mullet *P. macrolepis* from Cochin backwaters. Morphological features like bivalvular and elongate-oval myxospores, ornamented shell valves, equal and pyriform polar capsules and binucleate sporoplasm clearly assign the present species to the genus *Myxobolus.* However, 979 existing species along with limited and overlapping taxonomic features make myxospore morphology-based species delineation a daunting task in the genus *Myxobolus*. Though information from molecular and phylogenetic analyses, host/site/organ specificities, and geographical location are also considered in addition to spore morphology and morphometry [16], the absence of molecular information on many of the earlier described species adds to the difficulties.

Morphological and morphometric analyses reveal that the present species exhibit remarkable differences from all the previously described species of the genus *Myxobolus*. Though the present species exhibited 91.85% molecular identity match with *M. muscularis*, closely followed by *M. adiposus, M. cerveirensis*, and *M. peritonaeum,* it differed significantly from all these species in morphology and morphometry. *P*-distance analysis revealed a high divergence value of 9.95% with *M. muscularis.* There is no specific reference point for divergence value in the molecular taxonomy of myxosporeans, and depending on the groups studied, the values may vary widely. Generally, 1–1.3% genetic differences are considered satisfactory for delineating myxosporeans [37–39]. However, values varying from 0.2 to 30% have been reported for the genus *Myxobolus* [40–43]. Rocha [17] reported 0 to more than 4% interspecific variability for *Myxobolus* spp. reported from mullets. Hence, it is always prudent to consider the morphology and morphometry together with divergence values for differentiating myxosporeans. In

the present case, the lower identity and higher divergence values revealed in the molecular analysis clearly establish the molecular uniqueness of the present myxosporean. In phylogenetic analysis, members of the genus *Myxobolus* infecting mullets congregated into a separate clade (Clade A) except for *M. filamentum,* which occupied the *Henneguya* clade (Clade B). *M. planilizae* n. sp. was positioned distinctly within the *Myxobolus* clade with a high bootstrap value, supporting the morphological and morphometric observations.

Among the myxosporeans infecting fishes, histozoic species are generally more harmful while a few are considered fatal [11, 44]. In the present study, histopathology of the infected intestine revealed compression of the surrounding muscular layers, which may be due to the pressure exerted by the plasmodium, and mild hemocytic infiltration. However, cases of heavy infections leading to constriction and narrowing of the gut lumen have been reported earlier [45, 46].

M. planilizae n. sp. showed a low prevalence (3.2%) of infection in *P. macrolepis* and prevalence varying from 4.5% to 78.6% has been reported for *Myxobolus* spp. infections in mugilids [17, 46–49]. Numerous factors including the abundance of susceptible invertebrate communities, the migratory nature of fish hosts, and varied environmental conditions can influence the prevalence of myxosporeans [17, 50].

So far, more than 80 species of myxosporeans have been reported from mullets [17], of which 52 species belong to the genus *Myxobolus* [17, 51–53]. *Myxobolus* spp. has been reported from almost all organs of mugilid fishes including scales, skin, fins, gills, intestine, pancreas, gallbladder, swim bladder, liver, kidney, gonad, spleen, and brain [17, 41, 46, 49, 52, 54–59]. Rocha [17] opined that the high biodiversity of myxozoans observed in mullets could be due to the hyperdiversification of *Myxobolus* spp. in this host species.

Conclusion

Considering the marked differences in morphology and morphometry in tandem with molecular and phylogenetic analysis and differences in the host and geographic locations, the present myxosporean is treated as a new species and named *Myxobolus planilizae* n. sp. Detailed studies on the invertebrate definitive host may shed more light on the life cycle of the parasite.

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Declarations

Conflict of Interest The authors declare that there is no conflict of interest.

Human and Animal Rights All applicable institutional, national, and international guidelines for the care and use of animals were followed in the present study.

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