

Phylogeny and ultrastructure of *Myxobolus rangeli* n. sp. (Myxozoa, Bivalvulida), a histozoic parasite in Siluriformes fish from the Amazon region

Filogenia e ultraestrutura de *Myxobolus rangeli* n. sp. (Myxozoa, Bivalvulida), um parasita histozóico em peixes Siluriformes da região amazônica

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How to cite: Silva MF, Silva DT, Giese E, Furtado AP, Matos P, Lima AM, et al. Phylogeny and ultrastructure of *Myxobolus rangeli* n. sp. (Myxozoa, Bivalvulida), a histozoic parasite in Siluriformes fish from the Amazon region. *Braz J Vet Parasitol* 2023; 32(2): e005923. <https://doi.org/10.1590/S1984-29612023034>

Abstract

A new species of *Myxobolus* parasitizing the arterial bulb and cardiac musculature of the freshwater fish *Pimelodus ornatus* Kner, 1858, from the Arari river in the municipality of Cachoeira do Arari, island of Marajó, Pará, Brazil, was described. In the present study, the observed prevalence of myxozoan parasites in the heart tissue of the hosts was 20% (6/30). The myxozoans observed had mature biconvex spores, slightly rounded, an anterior end with two pyriform polar capsules and a posterior end with very evident sporoplasm, measuring 8 ± 0.2 μm length. The spore width was 5.8 ± 0.4 μm , with a thickness of 3.4 ± 0.2 μm . The length of the polar capsules was 3.6 ± 0.3 μm and the width was 1.2 ± 0.2 μm , with 6 to 7 turns of the polar filament. The divergences observed, regarding the morphometric and genetic structure of SSU rDNA, in relation to other Myxobolidae already described in the literature, confirm the description of the new species *Myxobolus rangeli* n. sp.

Keywords: Myxozoa, freshwater fish, *Pimelodus ornatus*, Marajó island, Brazil.

Resumo

Descrição de uma nova espécie de *Myxobolus* que parasita o bulbo arterial e a musculatura cardíaca do peixe de água doce *Pimelodus ornatus* Kner, 1858, do rio Arari, no município de Cachoeira do Arari, ilha de Marajó, Pará, Brasil. No presente estudo, a prevalência observada de parasitas mixozoários no tecido cardíaco dos hospedeiros foi de 20% (6/30). Os mixozoários observados apresentavam esporos maduros biconvexos, levemente arredondados, extremidade anterior com duas cápsulas polares piriformes e extremidade posterior com esporoplasma bem evidente, medindo $8 \pm 0,2$ μm de comprimento. A largura do esporo foi de $5,8 \pm 0,4$ μm , com espessura de $3,4 \pm 0,2$ μm . O comprimento das cápsulas polares foi de $3,6 \pm 0,3$ μm e a largura foi de $1,2 \pm 0,2$ μm , com 6 a 7 voltas do filamento polar. As divergências observadas, quanto à estrutura morfológica e genética de SSU rDNA, em relação a outros Myxobolidae já descritos na literatura, confirmam a descrição da nova espécie *Myxobolus rangeli* n. sp.

Palavras-chave: Myxozoa, peixe de água doce, *Pimelodus ornatus*, Ilha de Marajó, Brasil.

Received March 29, 2023. Accepted May 8, 2023.

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Introduction

Myxozoans make up a group of extremely diverse metazoan parasites that develop endoparasitic interactions with aquatic organisms, especially fish (Lom & Dyková, 2006). Despite all the accumulated knowledge about the diversity of this group (Eiras et al., 2014; Abdel-Ghaffar et al., 2017; Okamura et al., 2018), the interactions and responses of the hosts to these organisms have only been partially revealed (Okamura et al., 2015; Naldoni et al., 2020).

Representatives of the genus *Myxobolus* Bütschli, 1882, are among the most frequently reported myxozoans that establish infectious relationships in freshwater fish. They are considered to be an important group of pathogens that can cause environmental and economic damage (Eiras et al., 2014; Molnár & Eszterbauer, 2015; Sindeaux-Neto et al., 2021). In the Amazon region, recent studies have described occurrences of *Myxobolus* sp. and *Myxobolus marajoensis* in the intestinal muscle of Siluriformes fish on the island of Marajó, Pará, Brazil (Abrunhosa et al., 2016, 2017).

Also in the state of Pará, Capodifoglio et al. (2019, 2020a, b) described *Myxobolus matosi*, *Myxobolus longissimus*, *Myxobolus colossomatis*, *Myxobolus arapiuns* and *Myxobolus pirapitingae*, parasitizing Characiformes hosts in the Tapajós river basin; and Sindeaux-Neto et al. (2021) published the description of the species *Myxobolus freitasi*, a parasite of the central nervous system of Gymnotiformes fish in the Peixe-Boi river basin, in the eastern portion of the Brazilian Amazon region.

The genus *Pimelodus* La Cépède, 1803, is the most species-rich group of fish in the family Pimelodidae in the Neotropical region (Garavello & Shibatta, 2007). These are small feral catfish commonly known as “mandi”. Myxozoan infections of the genus *Myxobolus* in hosts of this genus of Siluriformes were described by Cellere et al. (2002), in a study carried out in the Paraná river basin, Brazil. The present study deals with redescription and it used aspects of the ultrastructure and molecular biology of new species of *Myxobolus* that were described by Matos et al. (2014).

Materials and Methods

Thirty fish of the species *Pimelodus ornatus* Kner, 1858, from the Arari river, were examined. They were caught in an area adjacent to the municipality of Cachoeira do Arari (01° 00'S; 48° 57' W) on the island of Marajó, in the state of Pará (Brazil). They were bought alive from artisanal fishermen, transported and kept in aquariums for up to 72 hours at the Carlos Azevedo Research Laboratory at the Federal Rural University of the Amazon (UFRA). They were then euthanized by means of anesthesia using tricaine methanesulfonate (MS222 Sandoz) at a concentration of 50 mg/L, in accordance with the procedures approved by the ethics committee for animal experimentation. Following this, necropsy was performed to search for myxosporean infections, through dissection.

During the necropsy, the organs were examined under a stereomicroscope and tissue cysts were collected and analyzed by means of optical microscopy. The presence of parasites was verified.

Tissue fragments were subjected to the transmission electron microscopy (TEM) technique. Specimens were prepared by fixing them in 5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) for 12 h at 4 °C, followed by washing in the same buffer for 12 h at 4 °C and post-fixation in 2% osmium tetroxide buffered with 0.2 M sodium cacodylate for 3 h. The material was dehydrated in an ascending series of ethanol concentrations, followed by propylene oxide, before incorporation into Epon resin. Semi-thin sections were stained with methylene blue, and ultra-thin sections with double contrast with uranyl acetate and lead citrate. These were observed under a JEOL 100CXII transmission electron microscope, which was operated at 60 kV in the Laboratory of Structural and Functional Biology of the Institute of Biological Sciences, at UFPA (LBEF/ICB/UFPA).

For scanning electron microscopy (SEM), fragments of parasitized tissues and free spores were fixed in 5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) for 12 h at 4 °C, followed by washing in the same buffer for 12 h at 4 °C and post-fixation in 2% OsO₄ buffered with 0.2 M sodium cacodylate for 3 h. The material was then dehydrated in an ascending series of ethanol concentrations, freeze-dried to a critical point, coated with gold and examined under a TESCAN Vega 3 LMU tabletop electron microscope at the Ultrastructure Laboratory of the UFRA Institute of Animal Health and Production (LU/ISPA/UFRA).

For molecular and phylogenetic analyses, myxosporid cysts were removed and fixed in 80% ethanol. DNA was extracted using the PureLink® Genomic DNA mini-kit (Invitrogen, USA), following the protocol provided by the manufacturer. The DNA samples were quantified in a Biodrop Duo spectrophotometer (Biodrop) and subjected to the polymerase chain reaction (PCR) technique in order to obtain the partial sequence of the small subunit ribosomal DNA (SSU rDNA), using primers that have been recommended in the literature. The ERIB1/ERIB110 primer sets (Barta et al., 1997) were used in the first round of amplification, followed by the MC3/MC5 primers (Molnár et al.,

2002), nested from the first round, and the ACT3f/ACT3r primers (Hallett & Diamant, 2001), semi-nested in the first pair with ERIB1 and ERIB10.

The amplifications were performed in a final reaction volume of 25 μ l, containing 1 x ReddyMix PCR Master mixture (Thermo Scientific, USA), 75 mM Tris-HCl (pH 8.8), 20 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each nucleotide triphosphate (Thermo Scientific, USA), 10 pmol of each primer, 1.25 U of Taq DNA polymerase (Thermo Scientific, USA) and the DNA model (10-50 ng/ μ l). The reaction protocol for the ERIB1/ERIB10 primers consisted of an initial extension at 95°C for 5 minutes, followed by 35 cycles of 95°C for 60 seconds, 56°C for 60 seconds and 72°C for 120 seconds, with a final extension step of 72°C for 10 min. For the other reactions, the reaction protocol was 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, annealing temperatures of 56°C (nested PCR) or 58°C (semi-nested PCR) for 30 seconds and 72°C for 60 seconds, with a final extension step of 72°C for 10 min.

Subsequently, 3 μ l of the PCR mixture was electrophoresed on 1% agarose gel with 1X Tris-borate-EDTA (TBE), stained with SYBR® Safe (Invitrogen, USA) and viewed under blue light. The PCR products were purified by means of DNA GFX™ PCR and use of a gel strip purification kit (GE Healthcare, UK), in accordance with the manufacturer's instructions. The sequencing reactions were conducted using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA), following the manufacturer's instructions, in an ABI 3100 genetic analyzer (Applied Biosystems, USA).

The sequences obtained through this procedure were aligned in the BioEdit software (Hall, 2007) and any ambiguous bases were clarified using the respective chromatograms. Sequences of the SSU rDNA gene of myxozoan species that had previously been deposited in NCBI GenBank (Sayers et al., 2022) were aligned in Clustal X 1.8 (Thompson et al., 1997). Similarity scores greater than 80% in the Basic Local Alignment Search Tool (BLAST) were used as a criterion for selecting GenBank strings for inclusion in the analysis. The jModelTest software, version 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008) was used to identify the best nucleotide replacement model for the data set.

Bayesian inferences were implemented in MrBayes, version 3.1.2 (Ronquist & Huelsenbeck, 2003). The Markov chain Monte Carlo method was used to search for two simultaneous executions of four chains of 10,000,000 generations, in which each 500th tree was sampled. A consensus tree was generated by means of the TreeAnnotator v1.8.4 tool, with a burn-in of 10%, and this was edited and plotted in FigTree v.1.4.3 (Rambaut et al., 2018). The reliability of the phylogenetic findings was verified by means of likelihood mapping analysis, in Tree-Puzzle 5.2 (Schmidt et al., 2002). Genetic distances were calculated in PAUP* 4.0b1 (Swofford, 2002), using the standard p parameter for the SSU rDNA gene.

Illustrative drawings were made from photomicrographs that had been obtained with the aid of a camera coupled to a computer.

Results

The macroscopic analysis showed the presence of whitish cysts, located in the cardiac musculature and in the region of the bulbus arteriosus of six specimens of *P. ornatus*, containing pyriform spores. The spore valves were symmetrical, with two equal polar capsules and a binucleated sporoblast (Figure 1).

Electron microscopy was performed on mature spores present in the bulbus arteriosus and in the heart muscle (Figure 2), and the presence of two identical conical valves was shown (Figures. 3A and 3C). The two polar capsules (PCs) were equal in size, pyriform and elongated, and they converged at the apex of the spore, with a polar filament (PF) presenting 6-7 turns (Figure 3B).

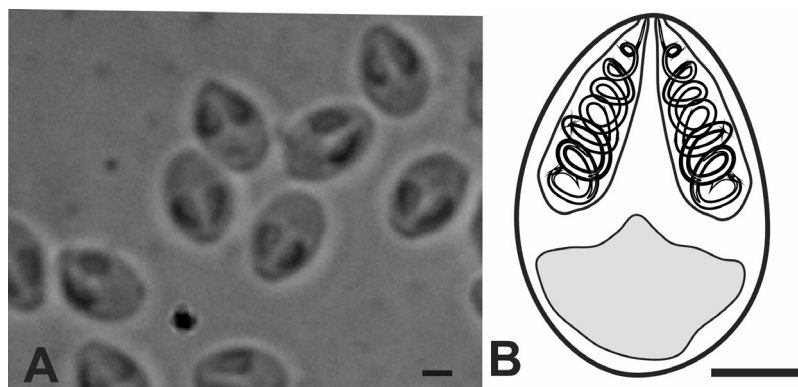


Figure 1. A: Photomicrography of the fresh spores. B: Diagram of spore of *Myxobolus rangeli* n. sp., in valvular view. Scale bar: 2 μ m.

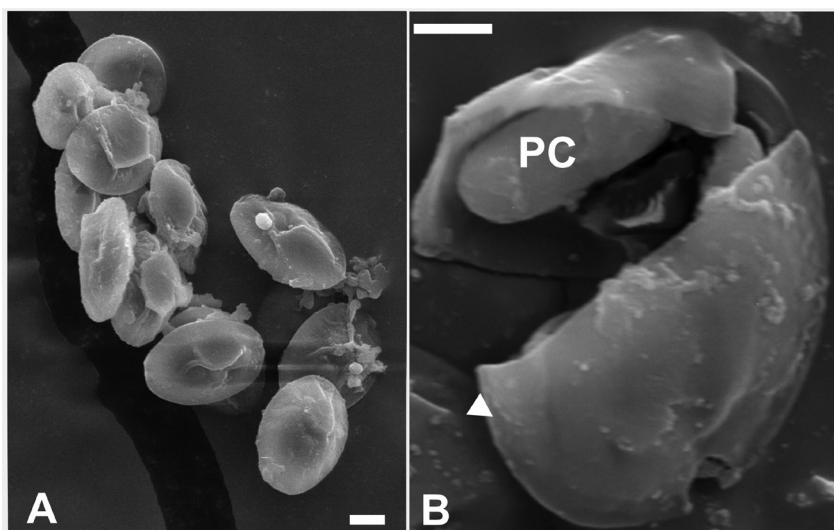


Figure 2. Scanning electron microscope (SEM) image of *M.rangeli* n. sp. A: View of myxospores after cyst rupture. B: View of the polar capsule (PC) and the valve wall (white arrow). Scale bar: 2 µm.

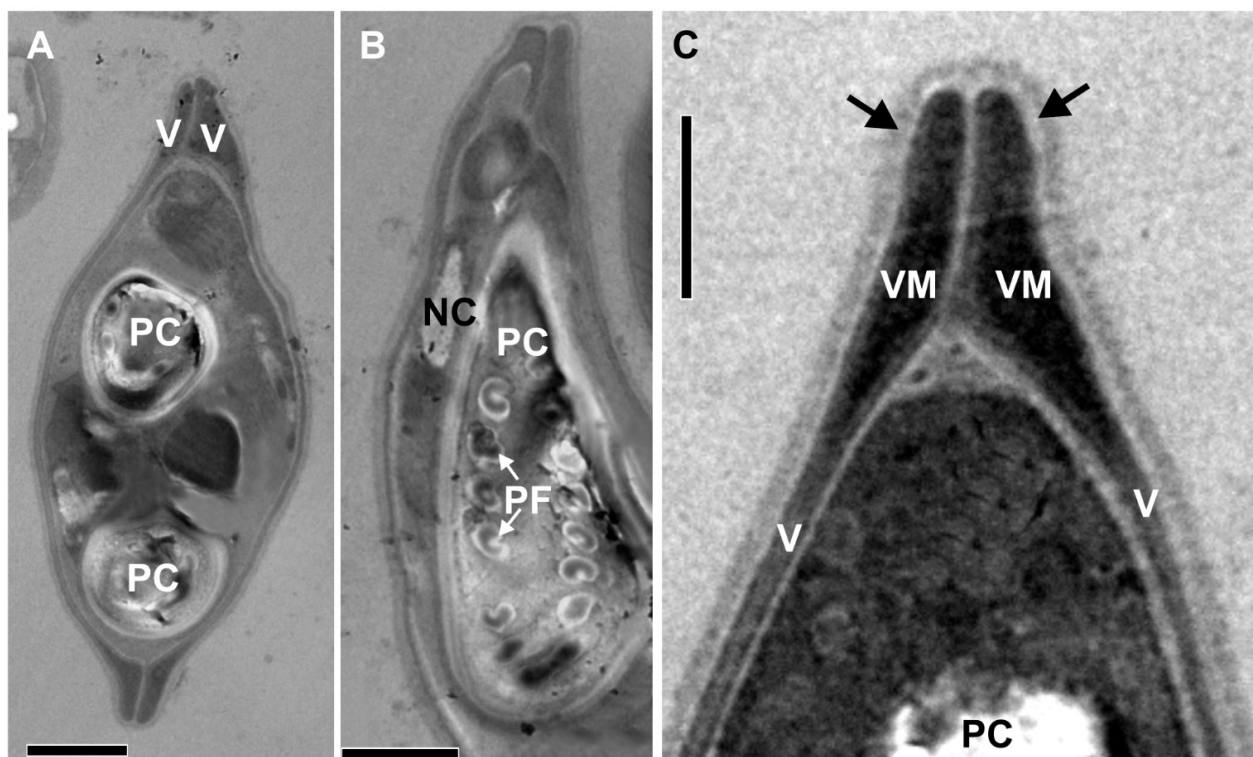


Figure 3. Electron micrographs of myxospores of *Myxobolus rangeli* n. sp., a parasite of the heart of *Pimelodus ornatus*. A: mature developmental stage of myxospores, with polar capsule (PC) and valves (V);scale bar: 2µm. B: longitudinal section of the myxospore, showing nucleus of capsulogenic cell (NC) and polar capsule (PC) with polar filaments (PF) (white arrow);scale bar: 2 µm. C: Detail of region of junction of the valves (v), showing valve-forming material (vm); PC – polar capsule;scale bar: 1µm.

Taxonomic Summary

- Phylum: Cnidaria Verrill, 1865.
- Unranked subphylum: Myxozoa Grassé, 1970.
- Class: Myxosporidia Bütschli, 1881.
- Order: Bivalvulida Shulman, 1959.

Family: Myxobolidae Thélohan, 1892.

Genus: *Myxobolus* Bütschli, 1882

Species: *Myxobolus rangeli* n. sp.

Host type: *Pimelodus ornatus* Kner, 1858

Infection site: heart (bulbus arteriosus / cardiac muscle)

Description of cysts: The cysts were found to have an oval shape, showing a whitish color and averaging 286 µm (213-408) in length and 175 µm (122-245) in width.

Mature spores: The spores were 8.0 ± 0.2 µm long (n = 25), 5.8 ± 0.4 µm wide (n = 25) and 3.4 ± 0.2 µm thick (n = 15). The mature spores of *Myxobolus rangeli* n. sp. were relatively small compared with other representatives of the genus *Myxobolus*, and were described as parasitizing fish in Brazil. They were morphometrically closer to *Myxobolus flavus* Carriero et al. 2013 and *M. brycon* (Table 1).

Table 1. Morphometry of myxobolids of the genus *Myxobolus* parasites of fish from Brazil.

Species	Spore Length	Spore width	Polar capsule length	Polar capsule width	Polar filament coils	References
<i>Myxobolus rangeli</i> n. sp.	8 ± 0.2	5.8 ± 0.4	3.6 ± 0.3	1.2 ± 0.2	6–7	Present study
<i>M. aureus</i>	12.6 ± 0.5	8.3 ± 0.3	5.7 ± 0.3	2.9 ± 0.2	7–8	Carriero et al. (2013)
<i>M. brycon</i>	6.9 ± 0.6 (6.5–7.2)	4.2 ± 0.5 (3.9–4.8)	2.5 ± 0.7 (1.9–2.8)	1.9 ± 0.6 (1.7–2.5)	8–9	Azevedo et al. (2011)
<i>M. colossomatis</i>	11.8 (11.4–12.2)	6.9 (6.6–7.2)	6.0 (5.8–6.6)	6.0 (5.8–6.6)	7–8	Molnár & Békési (1993)
<i>M. cordeiroi</i>	11.1 ± 0.2	7.3 ± 0.1	5.4 ± 0.3	1.4 0.1	-	Adriano et al. (2009)
<i>M. cuneus</i>	10.0 ± 0.6	5.1 ± 0.3	5.7 ± 0.3	1.7 ± 0.2	8–9	Adriano et al. (2006)
<i>M. flavus</i>	9.2 ± 0.2	6.5 ± 0.3	4.5 ± 0.2	1.6 ± 0.1	4–5	Carriero et al. (2013)
<i>M. heckelii</i>	12.7 (12.2–13.1)	6.6 (6.3–6.9)	2.9 (2.7–3.3)	1.7 (1.4–2.0)	4–5	Azevedo et al. (2009)
<i>M. maculatus</i>	21.0 (9.7–23.0)	8.9 (7.9–9.5)	12.7 (11.8–13.8)	3.2 (3.0–3.6)	14–15	Casal et al. (2002)
<i>M. metynnis</i>	12.9–13.5	7.5–8.3	5.0–5.5	5.0–5.5	8–9	Casal et al. (2006)
<i>M. myleus</i>	19.3 ± 0.5 (19–20)	8.3 ± 0.5 (7.5–9)	13.2 ± 0.4 (12.5–13.5)	3.0 ± 0.3 (2.5–3.5)	19–21	Azevedo et al. (2012)
<i>M. niger</i>	11.3 ± 0.4	6.8 ± 0.2	5.0 ± 0.3	2.0 ± 0.1	6–7	Mathews et al. (2016)
<i>M. piraputangae</i>	10.1 ± 0.5	8.7 ± 0.5	5.2 ± 0.4	3.0 ± 0.3	4–5	Carriero et al. (2013)
<i>M. umidus</i>	13.5 ± 0.7	7.8 ± 0.4	5.1 ± 0.4	2.7 ± 0.3	4–5	Carriero et al. (2013)

Locality type: Arari River, in the municipality of Cachoeira do Arari, Marajó Island, Pará, Brazil.

Prevalence: 6 out of 30 hosts examined (20%).

Type sample: Slides containing spores were obtained from the layer of the arterial bulb/cardiac muscle; these samples were deposited in the International Collection of Protozoan Samples of the National Institute of Amazonian Research (INPA), in Manaus, Amazonas, Brazil (catalog number: INPA 72).

Etymology: The species *Myxobolus rangeli* n. sp. is named in honor of Prof. Dr. Nello de Moura Rangel, who was an eminent professor and researcher in the fields of histology and embryology at the Federal University of Minas Gerais, Brazil (in memoriam).

Histopathology: The infection was characterized macroscopically by hypertrophy of the bulbus arteriosus and cardiac muscle that exhibited normal staining. Microscopic analysis of the bulbus arteriosus and cardiac muscle revealed infection by *Myxobolus rangeli* n. sp., which was organized in the form of cysts on the pericardium, and with free spores in the myocardium. In association with areas of myocardial infection by free spores, there was degeneration and multifocal necrosis of muscle fibers, and discrete accumulation of mixed inflammatory infiltrate. It was concluded that the fish presented myocarditis secondary to infection by *Myxobolus rangeli* n. sp.

Representative sequence: the SSU rDNA sequence of *M. rangeli* n. sp. has been deposited at GenBank under the accession number MT990755.

A partial sequence of 1,805 bp corresponding to the SSU rDNA was applied through sequencing spores of *Myxobolus rangeli* n. sp. found in the heart of *P. ornatus*.

In the phylogenetic tree generated by means of Bayesian inferences, two main groups were formed (Figure 4), which both presented high nodal support (posterior probability). The clade formed by parasites of freshwater fish was divided into two subclades: the first comprised hosts of the orders Perciformes and Characiformes; while the second was formed by parasites of the genera *Henneguya* and *Myxobolus*, which are parasites of hosts of the order Siluriformes. *Myxobolus rangeli* n. sp. was present in the subclade of parasites of siluriform fish in South America, composed of a subclade of hosts of the family Pimelodidae, which presented high nodal support (posterior probability).

Among the parasites of this clade, *Myxobolusrangeli* n. sp. is phylogenetically close to *M.flavus* and *Myxobolus tapajosi* Zatti et al. 2018, which parasitize, respectively, *Pseudoplatystoma corruscans* Spix and Agassiz 1829 and *Brachyplatystoma rousseauxii* Castelnau, 1855, which are both Siluriformes living in the Amazon Basin, in Brazil. *Myxobolus flavus*, a branchial branch parasite, behaves like a sister species of *Myxobolus rangeli* n. sp.

Likelihood analysis, which generated 10,000 combinations of quartets, further confirmed the topology of the phylogenetic tree (Figure 4B). The alignment of the sequence contained 92.9% of the phylogeny within the tree. Only 5% of the quartets were partially resolved, while 2.1% were unresolved.

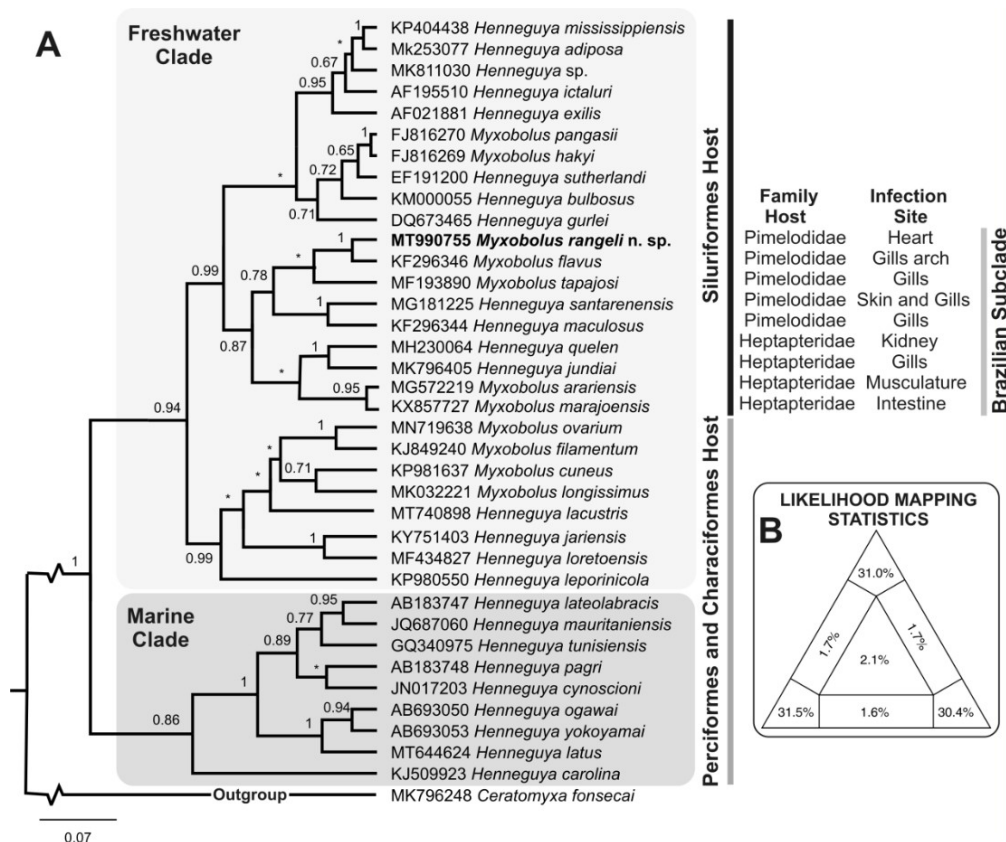


Figure 4. A: Results from Bayesian inference analysis on the partial sequence of the SSU rDNA gene of *Myxobolus rangeli* n. sp., combined with those of the 35 most relevant myxozoan species of the family Myxobolidae that have been registered in the NCBI GenBank, with a correspondence of over 80%;the numbers indicate Bayesian posterior probabilities. B: Likelihood mapping of the partial sequence of the SSU rDNA gene.

For pairwise comparisons, a new alignment was obtained, including only the species of *Myxobolus* that were parasites of Pimelodidae hosts, grouped with *M. rangeli* n. sp. The minimum genetic distance (p) was 4.2%, between *Myxobolus rangeli* n. sp. and *M. flavus* (KF296346), and the distance was 6.4% in relation to *M. tapajosi* (MF193890) (Table 2).

Table 2. Genetic distances (p-distances) between all Myxobolides species in the Pimelodidae fish subclade (Figure 3).

Species	(1)	(2)	(3)	(4)
(1) <i>Myxobolus rangeli</i> n. sp. (MT990755)				
(2) <i>Myxobolus flavus</i> (KF296346)	0.042			
(3) <i>Myxobolus tapajosi</i> (MF193890)	0.064	0.055		
(4) <i>Henneguya santarensis</i> (MG181225)	0.063	0.055	0.059	
(5) <i>Henneguya maculosus</i> (KF296344)	0.067	0.066	0.071	0.049

Discussion

The morphology of the myxospores observed in the cardiac tissue of *Pimelodus ornatus* is consistent with the general characteristics defined for the genus *Myxobolus* Bütschli, 1882 (family Myxobolidae) (Lom & Dyková, 2006). *Myxobolus* infections in the cardiac musculature of different species of fish are rarely recorded in the scientific literature. Examples are the occurrence of *M. bulbocordis* in the heart of *Barbus sharpeyi* in Iran (Masoumian et al., 1996) and *M. muscularis* in the fibers of the heart and skeletal muscle of *Chelon ramada* in Portugal (Rocha et al., 2019).

Polyphyletism is a striking feature in most myxozoan genera (Kent et al., 2001; Azevedo et al., 2021). In addition to the combination of morphological and molecular analyses, factors related to the environment and tropism in relation to the host have been shown to be important in establishing the evolutionary relationships of this group (Fiala, 2006; Rocha et al., 2019). It was seen in the results described here that there were clear indications of grouping in relation to the type of environment, thus forming a well-defined clade of myxozoa that infect freshwater fish.

These results reinforce the hypothesis that host affinity is a strong evolutionary signal for myxobolids (Moreira et al., 2014; Vieira et al., 2018) and that the origin and irradiation of these parasites reflect the evolution of their hosts (Azevedo et al., 2021). The phylogeny presented here reinforces this finding, since the sequences analyzed were grouped according to the order and family of the hosts.

The morphological and morphometric characteristics of the spores and the partial sequence of the SSU rDNA gene obtained in the present study enabled description of *Myxobolus* parasites of the cardiac musculature of specimens of *Pimelodus ornatus* that were native to the island of Marajó, Brazil (Matos et al., 2014). The description of the new species, *Myxobolus rangeli* n. sp. contributes to knowledge of the biodiversity and phylogenetic relationships of myxozoan parasites of freshwater fish in the eastern portion of the Amazon region.

Acknowledgements

This study was supported financially by the Brazilian Coordination Office for Higher-Education Personnel Training (CAPES) and the National Council for Scientific and Technological Development (CNPq). ERM was supported through the CNPq/Universal (441645-2014-3) and CNPq/Productivity in Research (301497/2016-8) programs. IGH was supported through the CNPq/Universal (432209/2018-2) and CAPES/ PROCAD AM (88881.200563/2018-01) programs. We are grateful to the State University of the Tocantina Region of Maranhão (UEMASUL) and the Maranhão State Research Foundation (FAPEMA).

Ethics declaration

The procedures were approved by the UFRA ethics committee for animal experimentation (CEUA 013/2014).

Conflict of interest

The authors have no conflicts of interest.

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