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Morphological, ultrastructural, and phylogenetic analysis of two novel *Myxobolus* species (Cnidaria: Myxosporea) parasitizing bryconid fish from São Francisco River, Brazil

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ABSTRACT

Twelve *Myxobolus* species have been previously described to parasitize Bryconidae fish in South America. Here, we describe two novel myxosporean species that parasitize economically important Bryconidae from the São Francisco River basin in Brazil. Myxospores morphometry, morphology, small-subunit ribosomal DNA - ssrDNA sequences, and other biological traits were used in the taxonomic analysis. Phylogenetic analysis was performed to assess the position of the new *Myxobolus* species among the closest *Myxobolus/Henneguya*. *Myxobolus iecoris* n. sp. was found infecting the liver of *Salminus franciscanus* (dourado). Myxospores were oval with the anterior region aculiform in frontal view and biconvex in lateral view and measured 11.4–14.2 (12.8 ± 0.8) μm long, 7.7–9.9 (8.7 ± 0.6) μm wide, 6.5–7.5 (6.9 ± 0.4) μm thick. Two pyriform and equal-sized polar capsules measuring 4.9–7.4 (5.9 ± 0.5) μm long and 2.3–3.5 (3.0 ± 0.2) μm wide contained polar tubules with 8–9 turns. *Myxobolus lienis* n. sp. was found infecting the spleen of *Brycon orthotaenia* (matrinxã). Myxospores were round to oval in frontal view and biconvex in lateral view and measured 10.3–13.8 (12 ± 0.6) μm long, 6.8–9.3 (8.3 ± 0.5) μm wide, and 6.9–7.0 (7.0 ± 0.6) μm thick. Two oval and equal-sized polar capsules measured 3.9–5.8 (4.6 ± 0.5) μm long and 2.0–3.5 (2.8 ± 0.3) μm wide contained polar tubules with 5–6 turns. Ultrastructural analysis revealed asynchronous sporogenesis with germinative cells and young sporogonic stages in the periphery of the plasmodia. A connective tissue capsule was observed surrounding *Myxobolus lienis* n. sp., but it was absent for *Myxobolus iecoris* n. sp. Maximum likelihood and Bayesian inferences showed the two novel species clustering in a well-supported subclade composed by *Myxobolus* spp. of bryconids. *Myxobolus iecoris* n. sp. appeared as a sister species of *M. aureus* and *Myxobolus lienis* n. sp. as sister to *M. umidus*.

1. Introduction

Myxozoans are a diverse group of obligatory endoparasites comprising over 2500 species [1,2,13]. They are cnidarians and have a complex life cycle that typically alternates between an annelid and a fish; however, bryozoans, amphibians, reptiles, birds, and mammals can also be exploited as hosts [2]. Some species are highly pathogenic in commercially important fish and cause severe damage to both wild and farm fish populations [3–5].

Myxobolus Bütschli 1882 (Myxosporea: Myxobolidae) is the most speciose genus in the subphylum Myxozoa, and of the 850 species described worldwide, twelve have been reported parasiting South American bryconids [6–11]. Although the description of new myxosporean species in South America has been increasing over the years, given the high diversity of fish species (all them potential hosts), the diversity of these parasites in this geographic region is likely still underestimated [12,13].

In this paper, we described two novel *Myxobolus* species of Bryconidae fish by combining myxospore morphology through light and electron microscopy and phylogentic analysis using small-subunit ribossomal sequences (ssrDNA). We found the novel species infecting two characiforms of Bryconidae that were caught in the São Francisco

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Table 1
Prevalence of myxosporean infection in *S. franciscanus* and *B. orthotaenia* in the São Francisco River in several periods between July 2010 and November 2013.

Fish species	Period	No. of fish examined	No. of fish infected	Prevalence
Salminus franciscanus	July 2010	2	2	100%
	July 2011	7	3	42.8%
	December 2011	17	11	64.7%
	December 2012	12	3	25%
	November 2013	4	2	50%
Brycon orthotaenia	July 2010	5	3	60%
	July 2011	5	3	60%
	December 2011	12	7	58.3%
	December 2012	14	10	71.4%
	November 2013	5	4	80%

River basin in Brazil: *Salminus franciscanus* Lima and Britski, 2007, popularly known as "dourado," and *Brycon orthotaenia* Günther, 1864, popularly known as "matrinxã". These fish are endemic to the São Francisco River basin and have great importance to commercial fishing [14,15]. In addition, we used the ssrDNA sequence data from the novel species to explore their phylogenetic relationships.

2. Material and methods

2.1. Sampling and morphological analysis

Adult specimens of *S. franciscanus* and *B. orthotaenia* were caught using a seine net or fishing hook in the São Francisco River in the municipality of Pirapora, Minas Gerais State, Brazil between July of 2010 to November of 2013 (Table 1). Fish sampling and access to the genetic data were authorized by the Brazilian Ministry of the Environment (SISBIO n° 27,964–1 and SISGEN A33CB83, respectively). After capture, fish were killed by an overdose of benzocaine solution (70 mgl^{-}1), measured, necropsied, then all organs were inspected for the presence of myxozoans. The research methodology was approved by the Ethics Committee on Animal Use, of the University of Campinas (CEUA/UNICAMP n° 2334–1) in accordance with Brazilian laws (Federal Law N^{o}. 11.794, dated of 8 October 2008). The plasmodia were fixed in formalin (10%) for morphologic characterization [16] and in 100% ethanol for ssrDNA sequencing, then in 2.5% glutaraldehyde with 0.1 M cacodylate buffer (pH 7.4) for electron microscopy. Half of each fixed plasmodium was mounted between a slide and coverslip and observed under light microscopy. Myxospores from at least two plasmodia related to the same myxosporean species were photographed and measured using differential interference contrast (DIC) and a computer equipped with Axivision 4.1 image capture software coupled to an Axioplan 2 Zeiss Microscope. The guidelines of Lom and Arthur [16] were followed. Note that here, we use the term "polar tubule" instead of "polar filament" [17]. The dimensions of formalin-fixed myxospores are shown as a range in μm, followed by the mean ± standard deviation in parentheses, and number of specimens measured in brackets. Smears containing free myxospores were air-dried onto glass slides, stained with Giemsa, mounted in a low-viscosity mounting medium (Cytoseal™), and deposited in the Museum of Zoology "Adão José Cardoso" of the University of Campinas, São Paulo State, Brazil.

2.2. Ultrastructural analysis

For transmission electron microscopy (TEM), plasmodia were fixed in 2.5% glutaraldehyde for at least 12 h, washed in a glucose-saline solution for 2 h and then post-fixed in OsO_4. All steps were performed at 4 °C. After dehydration that used increasing concentrations of an acetone series, the material was embedded in EMbed 812 resin (Electron Microscopy Sciences, Hatfield, USA). Ultrathin sections, double stained with uranyl acetate and lead citrate, were examined in a LEO 906 electron microscope at 60 kV (at the University of Campinas - UNICAMP, São Paulo, Brazil). Two plasmodia of each *Myxobolus* species taken from the two fish specimens to be examined were observed using TEM to ensure the presence of the observed plasmodial structures.

2.3. DNA extraction, amplification and sequencing

Total DNA was extracted from plasmodia fixed in 100% ethanol using the DNeasy® Blood & Tissue Kit (animal tissue protocol) (QIAGEN Inc., California, USA, following the manufacturer's instructions). The product was quantified in a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) at 260 nm.

Partial ssrDNA was amplified using a two-round polymerase chain reaction (PCR). In the first round, DNA was amplified with the universal primer pair ERIB1 (ACCTGGTTGATCCTGCCAG; [18]) and ERIB10 (CTTCCGCAGGTTCACCTACGG; [18]). In the second round, the primer combinations were ERIB1 with ACT1r (AATTTCACCTCTCGCTGCCA; [19]), and Myxgen4F (GTGCCTTGAATAAATCAGAG [20]) with ERIB10, which amplified two overlapping fragments of approximately 1000 bp and 1200 bp, respectively. PCR was performed in 25 μl reaction volumes that comprised: 10–50 ng of extracted DNA, 1 × Taq DNA polymerase buffer, 0.2 mmol of dNTP, 1.5 mmol of $MgCl_2$, 0.2 pmol of each primer, 0.25 μl (1.25 U) of Taq DNA polymerase (all reagents from Invitrogen, Thermo Fisher Scientific, Massachusetts, USA), and ultrapure water. PCR cycling was performed on Matercycler® nexus (Eppendorff, Hamburg, Germany) with an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 60 s and annealing at 62 °C for the new species of *Myxobolus* infecting *S. franciscanus*, or at 58 °C for the new species of *Myxobolus* infecting *B. orthotaenia* for 60 s, and finally extension at 72 °C for 90 s, followed by a terminal extension at 72 °C for 5 min. Amplicons were electrophoresed in 1.5% agarose gel in a TAE buffer (Tris 40 mM, Acetic Acid 20 mM, EDTA 1 mM) stained with SYBRsafe® (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) alongside a 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) at 90 V for 30 min. These were then analyzed on a blue light LED transilluminator (Kasvi, Paraná, Brazil). The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN Inc., California, USA), according to the manufacturer's instructions. Sequencing was carried out with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™) in a 3500 DNA sequencing analyser (Applied Biosystems, California, USA) at Helixxa Company (Paulinia City, São Paulo State, Brazil), using the same primers used for amplification plus the additional primers MC5 (CCTGAGAAACGGCTACCACATCCA; [21] and MC3 (GATTAGCCTGACAGATC ACTCCACGA; [21]).

2.4. Sequencing assembly and phylogenetic analysis

Primary DNA sequence data was verified using visual reference to the corresponding ABI chromatograms, and sequences were then assembled into a single contig in BioEdit [22]. Basic Local Alignment Search Tool (BLAST) searches were performed against the NCBI nucleotide database to determine sequence similarity to known myxozoans for phylogenetic analysis [23].

To explore the phylogenetic relationships of the new species with other myxozoans, we downloaded 51 ssrDNA sequences from the most closely related myxozoans with similarity > 80%, as determined by the BLAST search, and included additional sequences of species of the genus *Myxobolus* and *Henneguya* from South America, which were available from the NCBI database. *Parvicapsula bicornis* Køie, Karlsbakk, and Nylund, 2007 (EF429097) and *Parvicapsula irregularis* Kodadková, Dyková, Tyml, Ditrich, and Fiala 2014, (KF874229) were used as the outgroup. The set of sequences were aligned in BioEdit using ClustalW and default settings [24]. Gaps were treated as missing data. jModelTest 0.1 software [25] was used to determine the best-fit nucleotide substitution model from 82 alternative evolutionary models. Phylogenetic analyses were performed using maximum likelihood (ML) and Bayesian inference (BI). ML was done in the PhyML 3.0 implemented via the web server (http://www.atgc-montpellier.fr/phyml/) [26], with topology assessed by bootstrapping with 1000 replicates, using the GTR + I + G model of evolution. BI was done using MrBayes v.3.0 [27], under same model, with posterior probabilities estimated from 1 million generations with 2 independent runs of 4 simultaneous Markov Chain Monte Carlo (MCMC) algorithms, with every 1000th tree saved. Results were verified with TRACER v1.4.1 [28] to ascertain the length of burn-in (30,000). We visualized trees using Figtree 1.3.1 [29] and prepared figures using Adobe Photoshop (Adobe Systems Inc., California, USA). We determined genetic distances using the p-distance model matrix in MEGA version 7 [30]. Gaps and missing data were deleted.

3. Results

Plasmodia containing myxospores characteristic of *Myxobolus* were observed in the liver of 52.3% (22/42) of *S. franciscanus* and in the spleen of 65.8% (27/41) of *B. orthotaenia* caught in São Francisco River, Minas Gerais State, Brazil (Table 1).

3.1. Taxonomic summary and morphological data

Phylum: Cnidaria Verrill, 1865.
Unranked subphylum Myxozoa Grassé (1970).
Class: Myxosporea Bütschli, 1881.
Order: Bivalvulida Shulman, 1959.
Family: Myxobolidae Thélohan, 1892.
Genus: *Myxobolus* Thélohan, 1892.

Myxobolus iecoris n. sp. (Fig. 1A–D).

Type host: *Salminus franciscanus* Lima and Britski, 2007, Characiformes, Bryconidae.

Prevalence: 52.3% (22/42).

Site of infection: Liver.

Intensity: up to five plasmodia per host.

Type-locality: São Francisco River, municipality of Pirapora, Minas Gerais State, Brazil (coordinates: 17°12′ 06.05"S, 44°50′16.35" W).

Etymology: The specific name refers to the organ where the parasite develops (liver). Latin, "*iecoris*" = from liver.

Type material

A glass slide with stained spores (syntype) was deposited in the collection of the Museum of Zoology "Adão José Cardoso" of University of Campinas (UNICAMP), São Paulo, Brazil (accession number ZUEC MYX 80). The ssrDNA sequence was deposited in GenBank, with accession number MH500002.

Description

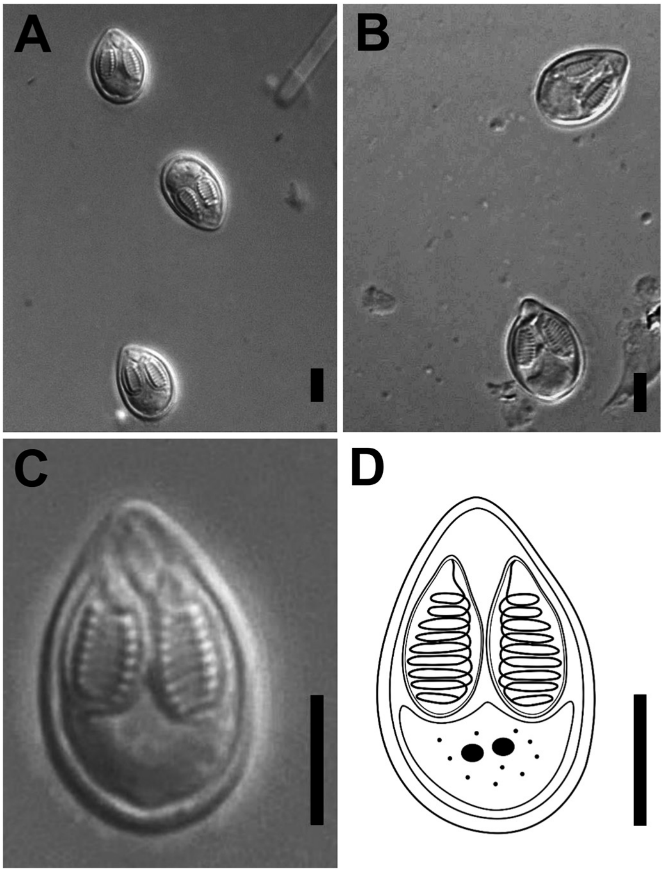

Fig. 1. Myxospores of *Myxobolus iecoris* n. sp., parasite of liver of *Salminus franciscanus*. A–C: photomicrographs using DIC of wet-mount myxospores. D: drawing of myxospore. Scale bars: 5 μm (A-D).

A whitish, spherical plasmodia, diameter < 1.0 mm and containing immature and mature myxospores was found in the liver of *S. franciscanus*. The myxospores were oval with the anterior region aculiform in frontal view, comprising two symmetrical, equal, and biconvex shell valves adhering together along the sutural line. Myxospore measurements were as follows: 11.4–14.2 (12.8 ± 0.8 [*n* = 36]) μm long, 7.7–9.9 (8.7 ± 0.6 [*n* = 37]) μm wide, and 6.5–7.5 (6.9 ± 0.4 [*n* = 4]) μm thick. The two polar capsules had equal size with a rounded posterior extremity that converged anteriorly into tapered ends and occupied slightly more than half of the myxospore, which measured 4.9–7.4 (5.9 ± 0.5 [n = 37]) μm long, 2.3–3.5 (3.0 ± 0.2 [n = 37]) μm wide (Table 2) (Fig. 1A–D). Ultrastructure analysis showed the absence of connective tissue capsule of host origin surrounding the plasmodia. Pinocytotic canals connected the outside of the plasmodia to the ectoplasm zone and numerous mitochondria were observed in the ectoplasm zone (Fig. 3A and B). Below the ectoplasm were generative cells in early stages of sporogenesis, and advanced spore developmental stages were observed (Fig. 3A–C). Binucleated sporoplasm contained numerous sporoplasmosomes were observed in the young myxospores (Fig. 3A). Mature myxospores were observed in a deeper region of the plasmodia. Inside the polar capsules, polar tubules with 8–9 turns (seldom 7) arranged perpendicularly to the longitudinal axis (Figs. 1A–D and 3D).

Remarks

When compared morphologically to the *Myxobolus* spp. found infecting bryconids (Table 2), the *M. aureus* myxospores presented the greatest similarity to *M. iecoris* n. sp., and the morphometric data of the myxospores also overlapped in several parameters in these two species.

Table 2

Comparison of *Myxobolus* species parasites of South America bryconid fish. Dimensions are given in micrometers by the range and followed by the mean ± standard deviation. PCL: polar capsules length, PCW: polar capsules width, NCT: number of polar tubule coils, dashes: no data.

Species	Spore length	Spore width	Thickness	PCL	PCW	NCT	Site of infection	Host	Locality
Myxobolus iecoris n. sp. (this study)	11.4–14.2 12.8 ± 0.8	7.7–9.9 8.7 ± 0.6	6.5–7.5 6.9 ± 0.4	4.9–7.4 5.9 ± 0.5	2.3–3.5 3.0 ± 0.2	8–9	Liver	*Salminus franciscanus*	São Francisco River, Brazil
Myxobolus lienis n. sp. (this study)	10.3–13.8 12.0 ± 0.6	6.8–9.3 8.3 ± 0.5	6.9–7.0 7.0 ± 0.6	3.9–5.8 4.6 ± 0.5	2.0–3.5 2.8 ± 0.3	5–6	Spleen	*Brycon orthotaenia*	São Francisco River, Brazil
Myxobolus batalhensis [11]	14.0–15.4 15.2 ± 0.8	8.0–8.7 8.5 ± 0.5	5.0–5.2 5.2 ± 0.3	5.0–5.5 5.2 ± 0.3	2.6–3.0 2.8 ± 0.2	6–9	Ovary/Liver	*Salminus hilarii*	Batalha River, Brazil
Myxobolus hilarii [10]	9.8–13.4 11.5 ± 0.8	9.7–12.4 11.0 ± 0.7	6.7–9.0 7.6 ± 1.0	6.0–7.2 6.5 ± 0.4	3.6–5.3 4.0 ± 0.2	5–7	Kidney	*Brycon hilarii*	Fish farm, São Paulo, Brazil
Myxobolus filamentum [9]	7.4–9.7 9.0 ± 0.3	5.1–7.3 6.2 ± 0.4	4.8–5.7 5.3 ± 0.3	3.7–5.4 4.7 ± 0.3	1.2–2.2 1.7 ± 0.1	10	Gill filaments	*Brycon orthotaenia*	São Francisco River, Brazil
Myxobolus aureus [31]	12.6 ± 0.5	8.3 ± 0.3	5.5 ± 0.3	5.7 ± 0.3	2.9 ± 0.2	7–8	Liver	*Salminus brasiliensis*	Pantanal wetland, Brazil
Myxobolus pantanalis [31]	9.3 ± 0.4	6.5 ± 0.4	–	4.2 ± 0.5	2.0 ± 0.1	4–5	Gill filaments	*Salminus brasiliensis*	Pantanal wetland, Brazil
Myxobolus umidus [31]	13.5 ± 0.7	7.8 ± 0.4	7.7 ± 0.1	5.1 ± 0.4	2.7 ± 0.3	4–5	Spleen	*Brycon hilarii*	Pantanal wetland, Brazil
Myxobolus piraputangae [31]	10.1 ± 0.5	8.7 ± 0.5	6.7 ± 0.3	5.2 ± 0.4	3.0 ± 0.3	4–5	Kidney	*Brycon hilarii*	Pantanal wetland, Brazil
Myxobolus brycon [51]	6.5–7.2 6.9	3.9–4.8 4.2	1.9–2.8 2.5	3.8–4.7 4.2	1.7–2.5 1.9	8–9	Gill filaments	*Brycon hilarii*	Pantanal wetland, Brazil
Myxobolus oliveirai [8]	11.2 ± 0.4	7.4 ± 0.5	4.6 ± 0.6	5.6 ± 0.2	2.3 ± 0.2	6–8	Gill filaments	*Brycon hilarii*	Pantanal wetland, Brazil
Myxobolus salminus [36]	10.1 ± 0.4	6.1 ± 0.4	5.0 ± 0.6	4.6 ± 0.2	1.7 ± 0.1	7–8	Gill filaments	*Salminus brasiliensis*	Pantanal wetland, Brazil
Myxobolus macroplasmodialis [52]	10.5–12 11	8–9 8.5	5–5.5 5.2	4–5 4.5	2–3 2.8	6	Abdominal cavity	*Salminus maxillosus* = *S. brasiliensis*	Mogi Guaçu River, Brazil
Myxobolus paranensis [53]	12–15	7–8	–	6–7	2.5	8–11	Gonads	*Salminus maxillosus* = *S. brasiliensis*	Mogi Guaçu River, Brazil

However, differences were observed regarding the myxospores thickness (6.5–7.5 μm for *M. iecoris* n. sp. and 5.5 μm for *M. aureus*), myxospores width (7.7–9.9 μm for *M. iecoris* n. sp. and 8.3 μm for *M. aureus*), and number of polar tubule turns (8–9 for *M. iecoris* n. sp. and 7–8 for *M. aureus*). In addition to these morphometric differences, it was also observed that the polar capsules of *M. iecoris* n. sp. occupy proportional smaller space in relation to the length of the myxospores than those of *M. aureus*. Both species were found infecting the liver. *M. iecoris* n. sp. infected *S. franciscanus*, an endemic fish from the São Francisco River basin, while *M. aureus* was described parasitizing the congeneric species *Salminus brasiliensis* Cuvier, 1816, that is endemic to the La Plata River Basin, where it was caught in the Pantanal Brazilian Wetland, Mato Grosso do Sul State. Associated with these morphologic/morphometric differences, *M. iecoris* n. sp. and *M. aureus* also showed 2.0% of genetic divergence based on ssrDNA sequences. The myxospores of *M. iecoris* n. sp. also morphologically resembled to those of *Myxobolus filamentum* Naldoni, Zatti, Capodifoglio, Milanin, Maia, Silva, and Adriano, 2015, both showing oval shaped myxospores. However, *M. iecoris* n. sp. was larger than *M. filamentum* in all myxospores dimensions, displayed less number of turns of polar tubules (8–9 versus 10), and occurred in distinct infection sites and host species (liver of *S. franciscanus* for *M. iecoris* n. sp. versus gill filaments of *B. orthotaenia* for *M. filamentum*). Addionally, these two species showed genetic divergence of 19.6%. The *Myxobolus lienis* n. sp., which is also described in this study infecting the spleen of *B. orthotaenia,* showed morphometric similarity in the length, width, and thickness myxospores. However, these species may be morphologically distinguished by the characteristic anterior aculiform region of the myxospores and the presence of 8–9 polar tubules turns for *M. iecoris* n. sp., while only 5–6 turns for *M. lienis* n. sp. (Table 2, Figs. 1A–D and 2A–E). These two species also showed genetic divergence of 12.4% in their ssrDNA.

Myxobolus spp. also parasitizes other South American fish, such as the *M. iecoris* n. sp., in which myxospores showed resemblances to those of *Myxobolus metynnis* Casal, Graça and Azevedo, 2006, parasite of an Amazonian characiform fish. However, the new species had thicker myxospores (6.5–7.5 μm while 3.4–4.5 μm for *M. metynnis*), and parasitizes the liver of *S. franciscanus* from the São Francisco River basin, whereas *M. metynnis* infects the connective subcutaneous tissues of the orbicular region of *Metynnis argenteus* from the Amazon River. Unfortunately, there is no DNA sequence of *M. metynnis* with which to perform a molecular comparison. Notably, considering the morphometric, morphologic, and molecular data, and biological traits, *M.*

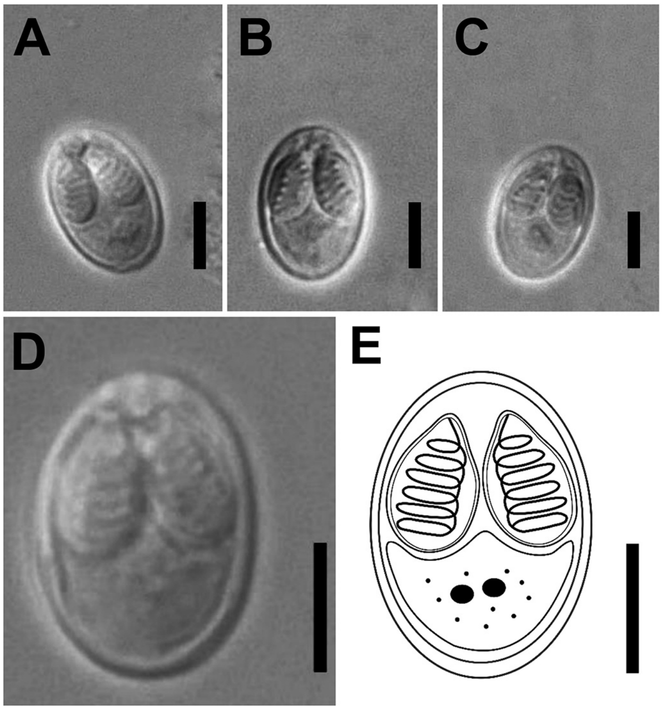

Fig. 2. Myxospores of *Myxobolus lienis* n. sp., parasite of spleen of *Brycon orhtotaenia.* A–D: photomicrographs using DIC of wet-mount myxospores. E: drawing of myxospore. Scale bars: 5 μm (A-E).

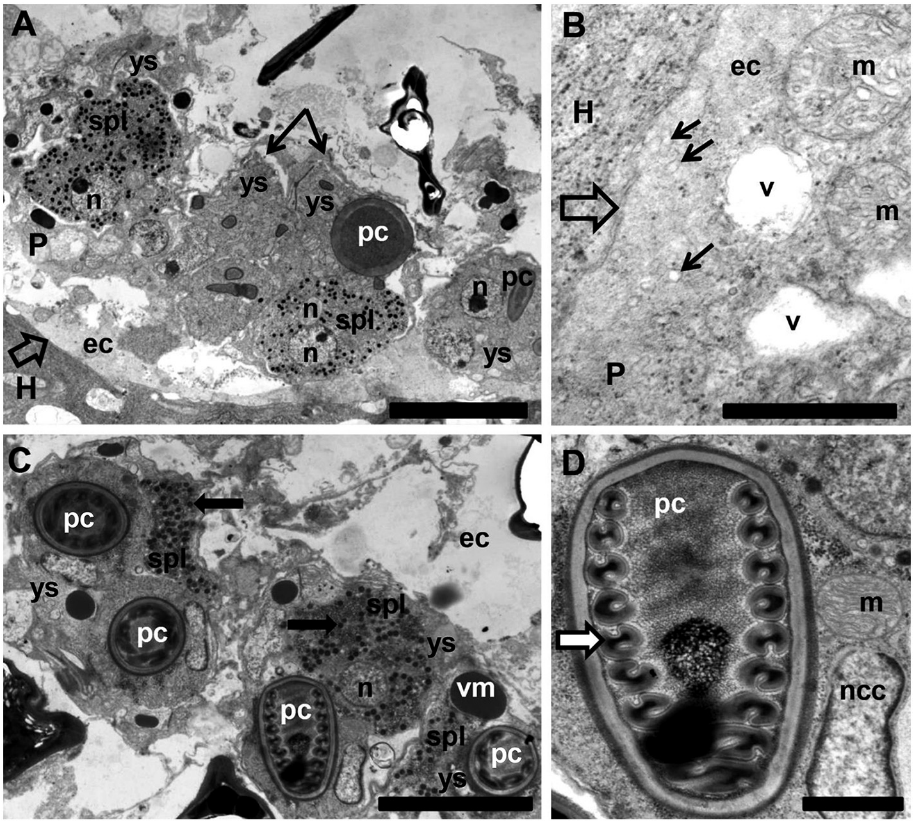

Fig. 3. Transmission electron micrographs of liver of *Salminus franciscanus* infected by *M. iecoris* n. sp. A: host–parasite interface showing the host tissue (H) and the plasmodial wall (empty arrow). Note, inside the plasmodium (P), a thin ectoplasm layer (ec), disporic pansporoblast (thin black arrows) containing young myxospores (ys) showing polar capsule (pc) with polar tubules not yet internalized, sporoplasms (spl) and their nuclei (n). Scale bar = 5 μm. B: Amplified portion of the host-parasite interface showing the host tissue (H), the plasmodial wall (empty arrow), ectoplasm (ec) with pinocytotic canals (thin black arrows), mitochondria (m) and several vacuoles (v). Scale bar: 1 μm. C: Young myxospores (ys) showing polar capsules (pc) with internalized polar tubules and sporoplasm (spl) with numerous small sporoplasmosomes (large black arrow). Scale bar: 5 μm. D: Details of a polar capsule (pc) with its polar tubule (arrow). Note nucleus of capsulogenic cell (ncc) and a mitochondria (m). Scale bar: 1 μm.

iecoris n. sp. differs from all other *Myxobolus* spp. of freshwater fish from other continents in at least one of these characters [6,7].

Myxobolus lienis n. sp. (Fig. 2A–E).

Type host: *Brycon orthotaenia* Günther, 1864, Characiformes, Bryconidae.

Prevalence: 65.8% (27/41).

Site of infection: Spleen.

Intensity: up to three plasmodia per host.

Type-locality: São Francisco River, municipality of Pirapora, Minas Gerais State, Brazil (coordinates: 17°12′ 06.05”S, 44°50′16.35” W).

Etymology: The specific name refers to the organ where the parasite develops (spleen). Latin, “*lienis*” = from spleen.

Type material

A glass slide with stained spores (syntype) was deposited in the collection of the Museum of Zoology “Adão José Cardoso” of University of Campinas (UNICAMP), São Paulo, Brazil (accession number ZUEC MYX 81). The ssrDNA sequence was deposited in GenBank, with accession number MH500003.

Description

Whitish and spherical plasmodia, measuring up to 1.0 mm and containing immature and mature myxospores were found infecting the spleen of *B. orthotaenia*. Myxospores were round to oval in frontal view, comprising two symmetrical, equal and biconvex in lateral view shell valves that adhered together along the sutural line. Myxospore measurements were as follows: 10.3–13.8 (12.0 ± 0.6 [*n* = 41]) μm long, 6.8–9.3 (8.3 ± 0.5 [n = 41]) μm wide, 6.9–7.0 (7.0 ± 0.6 [*n* = 5]) μm thick. Two oval polar capsules of equal size measured 3.9–5.8 (4.6 ± 0.5 [n = 41]) μm long, 2.0–3.5 (2.8 ± 0.3 [n = 41]) μm wide and occupied half of the myxospore (Table 2) (Fig. 2A–E). Ultrastructure analysis demonstrated that a connective tissue layer composed of fibroblasts surrounded the plasmodium (Fig. 4A–B). The plasmodial membrane had a few calibrous pinocytotic canals connecting the outside of the plasmodia to the ectoplasm zone (Fig. 4B). Generative cells occurred below the ectoplasm. Early sporogonic stages were observed in the subsequent layer and mature myxospores were located in the central zone of the plasmodia (Fig. 4A and C). Sporoplasm with its nucleus and numerous sporoplasmosomes were observed in the young myxospores (Figs. 4A and 4C). Inside the polar capsules, polar tubules with 5–6 turns were perpendicularly arranged to the longitudinal axis of the polar capsule (Figs. 2A-E and 4C).

Remarks

Compared to other *Myxobolus* spp. found infecting hosts belonging to Bryconidae, *M. lienis* n. sp. myxospores morphologically resemble those of *M. umidus*. However, in *M. lienis* n. sp., the myxospores were round to oval-shaped, while in *M. umidus* the myxospores were ellipsoidal shaped. The morphometric data overlapped among almost all the structures of these two myxospore species, although differences were observed in the myxospore thickness and the number of polar tubule turns. Typically, *M. lienis* n. sp. myxospores were less thick (6.9–7.0 versus 7.7), and the polar capsules showed a greater number of polar tubule turns (5–6 versus 4–5). Another subtle, but important, difference in the morphometry of the myxospores of these two species is that in *M. lienis* n. sp. the range length was of 10.3 up to 13.8 μm, while the mean length of 13.5 μm provided to *M. umidus* is in the limit of the larger dimension displayed to this new species. This can be easily observed in the morphologic comparison, where it is possible to see the myxospores of *M. umidus* more elongated. Further, the polar capsules of these two species, despite having the same mean length, occupy half of the extension of the myxospores in *M. lienis* n. sp., while in *M. umidus*, they clearly occupy less than half of the myxospores length (for comparison

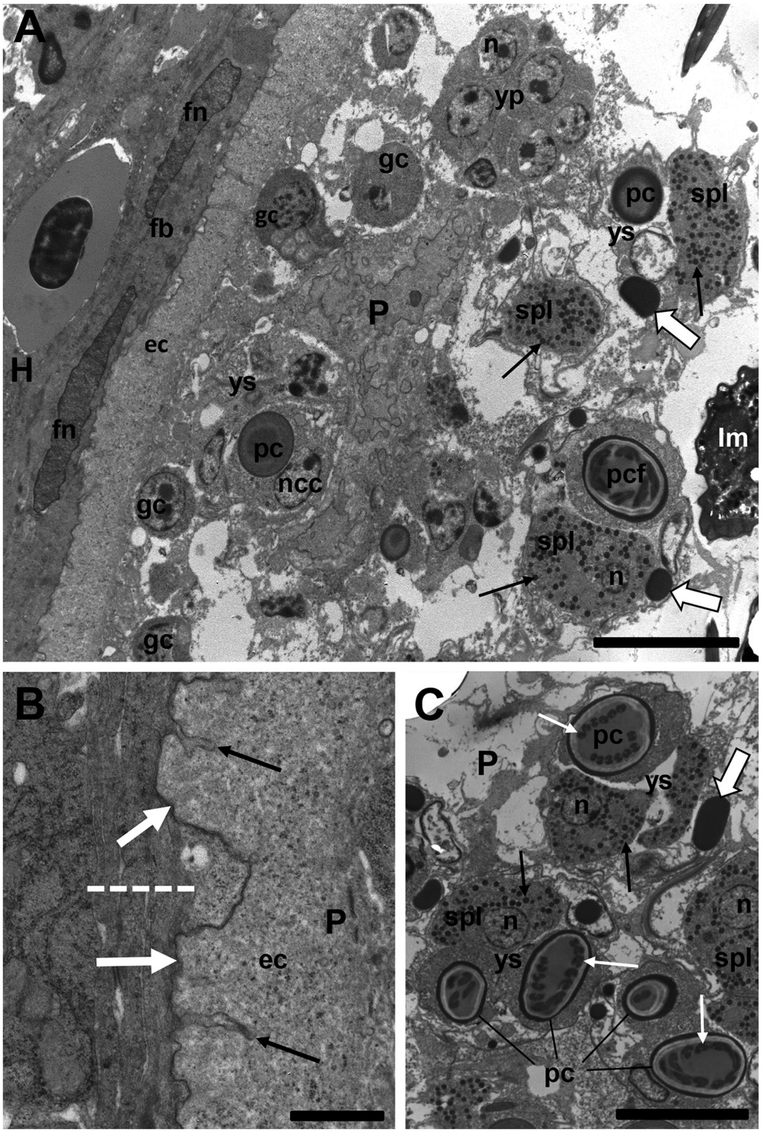

Fig. 4. Transmission electron micrographs of spleen of *Brycon orhtotaenia* infected by *M. lienis* n. sp. A: host–parasite interface showing a connective tissue layer composed by fibroblast (fb) surrounding the plasmodium (P). Inside the plasmodium can be seen the ectoplasm layer (ec), generative cells (gc), pansporoblast in early developmental stage (yp) with several nuclei (n), young myxospores (ys) with their polar capsules (pc) containing polar tubule not yet internalized and nucleus of capsulogenic cells (ncc), young myxospores with polar capsule and its internalized polar tubule (pcf), sporoplasm (spl) with nucleus (n) and numerous sporoplasmosomes (thin black arrows), valve-forming material (large white arrows: fn = fibroblasts nuclei, Im = immature myxospores. Scale bar: 5 μm. B: Amplified portion of host-parasite interface showing the connective tissue layer (white dashed line) surrounding the plasmodium (P), plasmodial wall (white arrows) and with few pinocytotic canals (black arrows) in the ectoplasm (ec). Scale bar: 1 μm. C: Deep region of the plasmodium (P) showing young myxospores (ys) with their polar capsules (pc) with internalized polar tubules (white arrows), spororoplasm (spl) with nuclei (n) and numerous sporoplasmosomes (thin black arrow). Scale bar: 2.5 μm.

see Fig. 2 and reference [31]). Regarding the biological traits, *M. lienis* n. sp. and *M. umidus* were both found in the spleen. The former parasitizes *B. orthotaenia,* which is an endemic fish from the São Francisco River basin. The latter parasitizes *Brycon hilarii* Valenciennes, 1850*,* which is an endemic fish from the Brazilian Pantanal Wetland. Notably, these species presented genetic divergence of 1.5% in their ssrDNA sequences.

Considering *M. filamentum*, which infects the same host species, *M. lienis* n. sp. myxospores showed larger morphometrics dimensions among all myxospore structures. They also had a fewer number of polar tubule turns (5–6 for the new species while 10 for *M. filamentum*), differerent sites of infection (gill filaments and spleen respectively) and genetic divergence of 19.8% in the ssrDNA sequences. The comparison of the new species with all the nominal *Myxobolus* spp. parasitizing fish from different geographic localization, according to the checklists of Eiras et al. [6,7], showed that despite any similarities to some species, *M. lienis* n. sp. differed at least in one of the morphometric/morphological, biological traits or molecular characters.

3.2. Molecular analysis

We generated a partial ssrDNA sequence of 1580 bp for *M. iecoris* n. sp. (GenBank accession number MH500002) and of 1886 bp for *M. lienis* n. sp. (GenBank accession number MH500003). According to a BLAST search, these sequences showed 98% and 98.5% of affinity to *Myxobolus aureus* Carriero, Adriano, Silva, Ceccarelli, and Maia, 2013, and *Myxobolus umidus* Carriero, Adriano, Silva, Ceccarelli, and Maia, 2013, respectively. The similarity matrix of the ssrDNA sequences, taking into account only *Myxobolus* ssp. of Bryconidae, showed that the smallest genetic distance 1.5% was observed between *M. lienis* n. sp. and *M. umidus* and the largest, 23.7% was between *Myxobolus batalhensis* Vieira, Alama-Bermejo, Bartholomew, Abdallah, and Azevedo, 2017, and *Myxobolus pantanalis* Carriero, Adriano, Silva, Ceccarelli and Maia, 2013 (Table 3).

3.3. Phylogenetic analysis

ML and BI phylogenetic analyses were performed on an alignment of 31 ssrDNA sequences from the most closely related myxozoans, as determined by the BLAST search, and included all *Myxobolus/Henneguya* sequences from South America available from the NCBI database (accession numbers are indicated in the phylogenetic tree). Once the trees had nearly identical topology, the ML with both support values for nodes was revealed. The phylogenetic analyses showed grouping into two main clades, which were named as A and B, both containing myxobolid of several host families. Clade A was largely

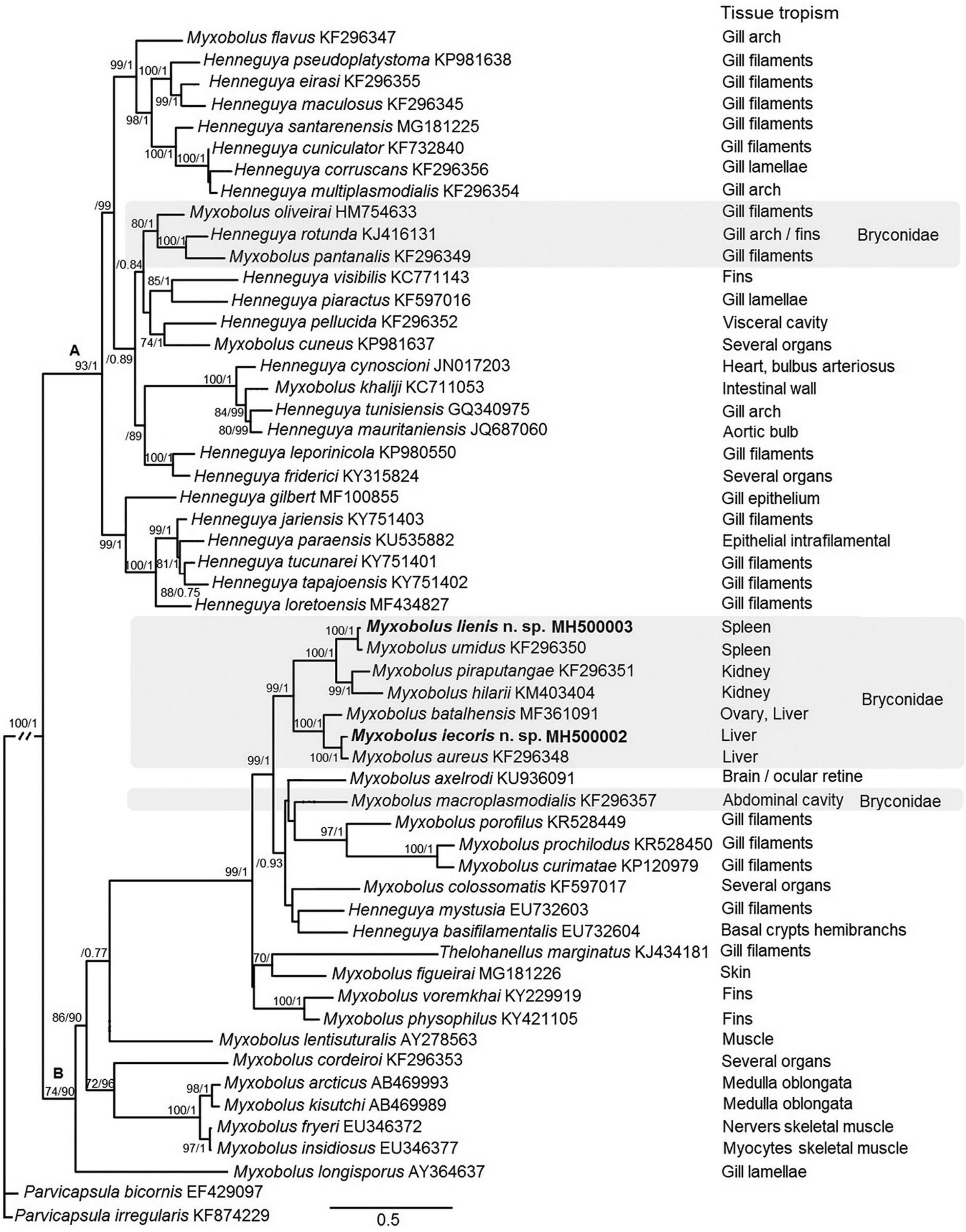

Fig. 5. Consensus ML phylogenetic tree based on ssrDNA sequences of selected myxobolids species. GenBank accession numbers are given in after species' name. The tissue tropism is shown for each species. Nodal supports are indicated for ML with a bootstrap of 1000 replicates, and BI with posterior probabilities, respectively. Values for weakly supported nodes (< 50) are not shown.

composed of *Henneguya* species, while the majority of clade B was formed of *Myxobolus* spp. In clade B, *M. lienis* n. sp. and *M. iecoris* n. sp. clustered in a well supported subclade formed by bryconid parasites, where *M. iecoris* n. sp. appears as a sister species of *M. aureus* and *M. lienis* n. sp. as a sister of *M. umidus* (Fig. 5).

4. Discussion

To date, of the twelve myxosporeans species reported parasitizing bryconids, only *M. filamentum* had been found in the São Francisco River basin [9]. In this study, we describe two novel *Myxobolus* species,

Table 3
Pairwise genetic identities of ssrDNA sequences from selected *Myxobolus* species adjusted for missing data. The upper triangular matrix shows the number of differences of nucleotides and the lower triangular matrix shows the differences in terms of percentage of nucleotides.

Species	1	2	3	4	5	6	7	8	9	10
1. *Myxobolus iecoris* n. sp.	–	195	100	206	307	31	333	191	188	280
2. *Myxobolus lienis* n. sp.	12.4	–	236	168	354	239	395	19	126	328
3. *Myxobolus batalhensis*	6.30	13.5	–	236	371	127	415	175	169	325
4. *Myxobolus hilarii*	13.1	9.2	13.8	–	341	243	377	139	107	329
5. *Myxobolus filamentum*	19.6	19.8	21.5	19.4	–	359	229	255	259	68
6. *Myxobolus aureus*	2.00	13.7	7.50	14.0	20.7	–	364	193	196	310
7. *Myxobolus pantanalis*	21.3	21.7	23.7	21.3	12.4	21.1	–	281	285	215
8. *Myxobolus umidus*	14.7	1.5	13.5	10.7	19.7	14.8	21.7	–	126	232
9. *Myxobolus piraputangae*	14.5	9.8	13.1	8.3	20.1	15.2	22.3	9.8	–	253
10. *Myxobolus oliveirai*	20.9	22.1	22.1	22.1	4.5	20.0	14.2	21.2	22.9	–

significantly increasing the myxosporeans diversity for infecting bryconids in the São Francisco River basin.

In the ultrastructural analysis, the absence of a connective capsule involving the plasmodium, as observed for *Myxobolus iecoris* n. sp., was reported for some myxosporean species [32–40], and according to Current et al. [32], it may have some importance in the parasite pathogenicity. On the other hand, the presence of a connective capsule surrounding the plasmodium, as observed for *M. lienis* n. sp., is commonly observed in myxosporean infections, which could be a host reaction to isolate the plasmodium and prevent its dispersal to adjacent tissues. In addition, the plasmodium encapsulation process observed for *M. lienis* n. sp. may also be related to a chronic response, while the absence of an encapsulation process observed for *M. iecoris* n. sp. may be related to earlier infection. The presence of conspicuous pinocytotic canals connecting the outside to the ectoplasm zone, observed in both species described in this study, are features also observed in many other myxosporean studies [32,33,36,39,40].

In this study, we combined all relevant data to study two *Myxobolus* spp. parasitizing bryconids from the São Francisco River basin. As pointed out in the remarks section, *M. iecoris* n. sp. and the previously described species *M. aureus* have marginally distinct myxospores in morphology (see Table 2). However, these species were found in distinct host species and watersheds: *M. iecoris* n. sp. parasitizing *S. franciscanus* in the São Francisco River basin and *M. aureus* parasitizing *S. brasiliensis* in the Brazilian Pantanal Wetland. They showed a slight genetic divergence of 2% between their ssrDNA sequences. Similarly, *M. lienis* n. sp. and *M. umidus* showed marginally different morphology between them (see Table 2). However, these species were found in distinct host species and watersheds: the former parasitizes *B. orthotaenia* from the São Francisco River basin, and the latter parasitizes *B. hilarii* from the Brazilian Pantanal Wetland. They also showed a genetic divergence of 1.5% between their ssrDNA sequences. The comparisons herein of the morphologic/morphometrics and ssrDNA data of the *Myxobolus* spp. with their closest species, in conjunction with their biological traits (such as host species and distinct geographic watersheds), suggest that *M. iecoris* n. sp. and *M. lienis* n. sp. are evolving in separate lineages, evidencing that they are a different species [41], giving support to the establishment of the new taxa. The accurate identification of species is crucial to research in all areas of biology, but the statement of a species is not always a simple task [42,43]. Lom and Arthur [16] delineated the criteria necessary to accurately describe myxosporeans with respect to the data of the hosts, the vegetative stages, and the spores. Given the development of molecular biology tools in last decades and the limitations of using morphologic/biologic traits alone, researchers have increasingly incorporated molecular biology DNA sequences as additional data for reliable identification of myxosporeans species [44,45]. Nevertheless, molecular data alone cannot always clearly identify species boundaries. According to Nolan and Cribb [46] the level of genetic variation that equates to species differentiation should be assessed on a case by case basis employing a range of additional evidence to define species boundaries. In this context, ssrDNA data are the most commonly used genetic information in myxozoans taxonomy [45]. There is however no universal criterion regarding what level of genetic variation in the ssrDNA sequence is sufficient to distinguish species in this parasite group. According to Gunter and Adlard [47], the genetic variations, when combined with the morphological and/or ecological data as delineated by Lom and Arthur [16], are compelling evidence for the characterization of new species.

Lom and Arthur [16] in their guidelines established that host species and locality of collection are indispensable characteristics in describing new myxosporean species, since specific host and geographic locality are traits that may play an important role in speciation. The distance between the two points of sampling of *M aureus* and *M. umidus* from the Pantanal (Carriero et al. [31]) and *M. iecoris* n. sp. and *M. lienis* n. sp. from the São Francisco River (this study) is around a 1300 km straight line. These two biomes inhabit distinct watersheds, one being the La Plata River and other the São Francisco River basins. Studies have suggested that a connection between these two watersheds ceased around 1.8 Ma ago [48], and the split between lineages of *S. brasiliensis* in the La Plata River basin and *S. franciscanus* in the São Francisco River basin may have occurred at 5.9 ± 1.8 Ma [49]. Thus, the geological events leading to the split of these large-scale Brazilian watersheds, besides having influenced the radiation of the fish, may also have been crucial in the inter-related genetic and evolutionary patterns observed in their parasites. The slight morphological/morphometric and genetic divergences of the ssrDNA sequences between the *M. iecoris* n. sp. and *M. aureus* (2.0%) and between the *M. lienis* n. sp. and *M. umidus* (1.5%) may reflect the relatively recent geological separation of these host-parasite systems when their watersheds split, as has been noted previously in other myxosporeans of these biomes [50].

Phylogenetic analyses using partial sequences of the ssrDNA of *M. iecoris* n. sp. and *M. lienis* n. sp. with their most closely related species, showed *Henneguya/Myxobolus* clustering in two main lineages: A, which was composed mainly of *Henneguya* species and B, which clustered mainly *Myxobolus* species. In general, the clusters are in concordance with previous studies showing that host affinity is an important phylogenetic signal within the Myxobolidae (e.g. Carriero et al. [31]). Our tree also demonstrates that tissue tropism has phylogenetic influence in some places; especially when considering species parasitizing hosts phylogenetically close (see Fig. 5). The *Myxobolus iecoris* n. sp. and *M. lienis* n. sp. clustered in a well-supported subclade composed exclusively of parasites in bryconid hosts. The *M. iecoris* n. sp. clustered as sister to *M. aureus* and *M. lienis* n. sp. as sister to *M. umidus.* These phylogenetic proximities are in conformity with the genetic distances observed between their respective species (see Table 3), although our analysis showed that myxobolid parasites of bryconid are polyphyletic. In addition to the subclade formed by the seven *Myxobolus* spp., parasites of the bryconid in clade B, the *M. oliveirai*, *Henneguya rotunda* Moreira, Adriano, Silva, Ceccarelli, and Maia, 2014, and *Myxobolus pantanalis* Carriero Adriano, Silva, Ceccarelli, and Maia, 2013, formed a small lineage of bryconid parasites within clade A. This is similar to the *Myxobolus macroplasmodialis* Molnár, Ranzani-Paiva, Eiras, and Rodrigues, 1998, which is a parasite of the bryconid *S. brasiliensis* and appears as a sister species of a lineage of parasites of prochilodontid hosts in clade B. This result shows that splits observed in the myxobolid parasites of bryconids, as well as those in other families, seem to reflect evidence of switching between hosts throughout evolutionary history. Alternatively, this result could reflect limitations of current sampling, since many taxa are still unsampled, especially in South America.

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