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Myxobolus marajoensis sp. n. (Myxosporea: Myxobolidae), parasite of the freshwater catfish *Rhamdia quelen* from the Brazilian Amazon region

Myxobolus marajoensis sp. n. (Myxosporea: Myxobolidae), parasita do bagre de água doce *Rhamdia quelen* da região da Amazônia brasileira

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Abstract

This study provides morphological and molecular data of a new parasite species found in the muscle layer of the intestinal tract of the South American silver catfish, *Rhamdia quelen* from Marajó Island region (Pará State, Brazil), an important fishery resource with recognized potential for fish farming. The morphology of these parasites was reanalyzed and phylogenetic analyses were run on their 18S rDNA gene sequences. The spores were morphologically distinct from those of other *Myxobolus* species described previously. The obtained partial sequence of the 18S rDNA gene sequences of the new species were compared to those of 24 other *Myxobolus* and *Henneguya* species available in GenBank. The results of morphological and molecular analyses indicated clearly the existence of a new species, *Myxobolus marajoensis* sp. n.

Keywords: Amazonia, fish, Siluriform, Myxozoa, intestine, Marajó Island.

Resumo

Este estudo fornece dados morfológicos e moleculares de um novo parasita encontrado na parede intestinal do jandiá, *Rhamdia quelen* coletado na região da ilha do Marajó (Estado do Pará, Brasil), um importante recurso pesqueiro com potencial para aquicultura. Foram realizadas comparações morfológicas deste parasita e análises filogenéticas da região do gene 18S rDNA sequenciada. Os esporos foram morfologicamente distintos das espécies de outros *Myxobolus* descritos anteriormente. A sequência parcial obtida do gene 18S rDNA da nova espécie foi comparada com outras 24 espécies de *Myxobolus* e *Henneguya* retiradas do GenBank. Os resultados de análises morfológica e molecular indicaram claramente a existência de uma nova espécie, *Myxobolus marajoensis* sp. n.

Palavras-chave: Amazônia, peixe, Siluriforme, Myxozoa, intestino, Ilha do Marajó.

Introduction

Myxobolus Bütschli, 1882 is an important microparasite genus, with approximately 856 species. Some of these species have a considerable impact on fishery productivity by provoking diseases in both the natural environment and the farmed fish (GILBERT & GRANATH, 2001; EIRAS et al., 2005, 2014; LOM & DIKOVÁ, 2006).

In the catfish (Siluriformes), these parasites have been found infecting a variety of organs, such as the gills of the bandit corydoras, *Corydoras melini* Lönnberg and Rendahl, 1930 (MATHEWS et al., 2016) and the jau, *Zungaro jahu* Ihering, 1898 (ADRIANO et al., 2009), the intestine of the Amur catfish *Silurus asotus* Linnaeus, 1758 (LIU et al., 2016) and the Philippine catfish *Clarias batrachus* Linnaeus, 1758 (NARASIMHAMURTI & KALAVATI, 1986), the intestine and gills of the European chub, *Leuciscus cephalus* Linnaeus, 1758 (MOLNÁR et al., 2007) and the spleen of the striped catfish, *Pangasianodon hypophthalmus* Sauvage, 1878 (BASKA et al., 2009).

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In Brazil, the South American silver catfish *Rhamdia quelen* Quoy and Gaimard, 1824 is an important fishery resource, as well as being a potentially valuable species for fish farming in southern Brazil (BALDISSEROTO & RADUNZ, 2004; BRASIL, 2011). Despite being exploited widely as a source of human food, few studies are available on the parasitism of this species, except for Matos et al. (2005), who described the myxosporean *Henneguya rhamdia*, found in the gills of this catfish. More recently, Abrunhosa et al. (2016) reported on the occurrence of cysts caused by *Myxobolus*, which caused inflammatory processes in the intestine of the *R. quelen*.

This study aims to describe a new myxosporean species found in the intestinal tissue samples of the *R. quelen* based on the morphological features and in the data of the partial sequence of the DNA of the 18S small ribosomal subunit in order, to verify the phylogenetic position.

Materials and Methods

Fish sampling

Samples were obtained from 20 adult specimens (body length 15-23 cm) of *R. quelen* captured using nylon gill nets on the Paracauari River, in the municipality of Salvaterra (00°45' S; 48°31' W) on Marajó Island (Pará State), northern Brazil, in the Amazon region. The specimens were anesthetized with 50 mg/L of tricaine methanesulfonate (MS222 SIGMA) for transportation to the laboratory, where they were maintained in aquaria until necropsy. Before dissection, the fish were anesthetized until death, according to the rules of the UFRA ethics committee for animal experimentation (CEUA: 013/2014).

Sample preparation and histological analysis

The organs were examined under a ZEISS stereomicroscope. Once detected in the intestinal tract of the hosts, the cysts were extracted carefully for the preparation of slides and analyzed by light microscopy (Olympus CX41).

From fresh examination, the cysts were removed from the muscular layer of the intestine using tweezers and placed on glass slides covered with cover slips. The spores were released from the cysts smeared on the slides with a few drops of distilled water. Fresh plasmodia with mature spores were examined morphologically and morphometrically by light microscopy and the photomicrographs were obtained with DFC310 FX camera (Leica) under light microscopy conjugated with DIC (DM2500, 40 objective, Leica). The cysts were photographed under a Zeiss Primo Star stereomicroscope equipped with a Zeiss AxioCam ERc 5s camera and the AxioVision 5.1 software. For the histological procedure, small fragments of the parasitized tissue extracted from the intestinal were then fixed in Davidson's solution for 24 h before being processed and stained using the Hematoxylin and Eosin technique (LUNA, 1968).

Molecular characterization and phylogenetic analysis

For the molecular and phylogenetic analyses, the cysts were removed from the intestine and preserved in 80% alcohol. The analyses included samples of the intestines of 10 *R. quelen* specimens infected by *Myxobolus* held by the Carlos Azevedo Research Laboratory at UFRA. These specimens were collected from the Paracauari River in the municipality of Salvaterra (00°45'S; 48°31'W), Marajó Island, in the Brazilian Amazon region, between March and September 2016.

The DNA was extracted using a PureLink® Genomic DNA Mini kit (Invitrogen), following the maker's protocol. The DNA content was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific) at 260 nm and then diluted to 5ng/µL. The MC5 (forward) 5'-CCTGAGAAACGGCTACCACATCCA-3' and MC3 (reverse) 5'-GATTAGCCTGACAGATCACTCCACGA-3 primers (MOLNÁR, 2002) were used to amplify the 18S (SSU rDNA) gene by Polymerase Chain Reaction (PCR) in the SimpliAmpli Thermal Cycler (Applied Biosystems).

The final volume of the PCR was 25 µL, which contained approximately 5-10 ng of the DNA template, 2 mM $MgCl_2$, 4 mM of dNTP mix (Invitrogen), 5 pmol of each primer and 1.2 units of Taq DNA polymerase (Invitrogen®). The amplification protocol consisted of denaturation at 95 °C for 1 min, followed by for 35 cycles 66 °C for 1 min followed by 72 °C for 2 min and then by 5 min at 95 °C, followed by a final extension at 72 °C for 30 min.

Aliquots (3 µL) of the PCR products were visualized with Sybr® safe DNA gel stain (Invitrogen) after electrophoresis on 1% agarose gel, and purified using GFX PCR DNA and a Gel Purification kit (GE Healthcare), according to the manufacturer's instructions. The samples were sequenced using an ABI 3130 automatic DNA analyzer (Applied Biosystems) with BigDye® (Applied Biosystems) Terminator v3.1, following the manufacturer's specifications. The MC5 and MC3 primers used to obtain the amplicons were also used in the sequencing process. The nucleotide sequences obtained here were edited and aligned using the BioEdit software (HALL, 2007).

The partial sequence of the 18S rDNA gene of the *Myxobolus* specimens obtained from the intestinal tissue of *R. quelen* was aligned using the BioEdit software (HALL, 2007) for comparisons with the 24 sequences of closely related species of *Myxobolus* and *Henneguya* (obtained from both freshwater and marine fish) available in blast search and GenBank sequences. The outgroup was *Zschokkella nova*, GenBank sequences DQ377688.

Phylogenetic relationships were determined through Bayesian Inference (BI), using Markov Chain Monte Carlo (MCMC) tree searches in MrBayes 3.1.2 (RONQUIST & HUELSENBECK, 2003). The most appropriate evolutionary model was determined using jModelTest 2.0.2 (DARRIBA et al., 2012), based on the Akaike Information Criterion (AIC). We performed two parallel runs of four simultaneous MCMC searches of 5 million generations each, sampling one tree every 500 generations, and discarding the results of the first 1000 trees as burn-in. The remaining trees were used by MrBayes to estimate the posterior probability of each node in the phylogenetic reconstruction. Tracer v1.4.1 (RAMBAUT et al., 2008) was used to check the stationarity of all

the parameters sampled by the chains, as indicated by jModelTest 2.0.2 (DARRIBA et al., 2012). Genetic distances (*p*) in relation to other *Myxobolus* species were determined using PAUP 4.0b (SWOFFORD, 1998).

Results

Description of Myxobolus marajoensis sp. n.

Type host: Rhamdia quelen Quoy and Gaimard, 1824

Type locality: Paracauri River, municipality of Salvaterra (00°45'S, 48°31'W) on Marajó Island, Pará State, Brazil.

Site of tissue development: muscular layer of the intestine.

Prevalence: 20% (4/20).

Representative sequence: The 18S rDNA sequence of *M. marajoensis* sp. n. (Figures 1c and d) is deposited in GenBank under accession number KX857727.

Etymology: The specific epithet *marajoensis* refers to the collecting locality, Marajó Island, in Brazil.

Description

Cyst: The cysts were found ellipsoidal to oval-shaped showing a whitish colour and averaging 345 µm (213-408) in length and 195 µm (122-245) in width (Figure 1a).

Histology: In histological sections, cysts were found in the intestinal layer between luminar mucosa and the serosal layer (Figure 1b).

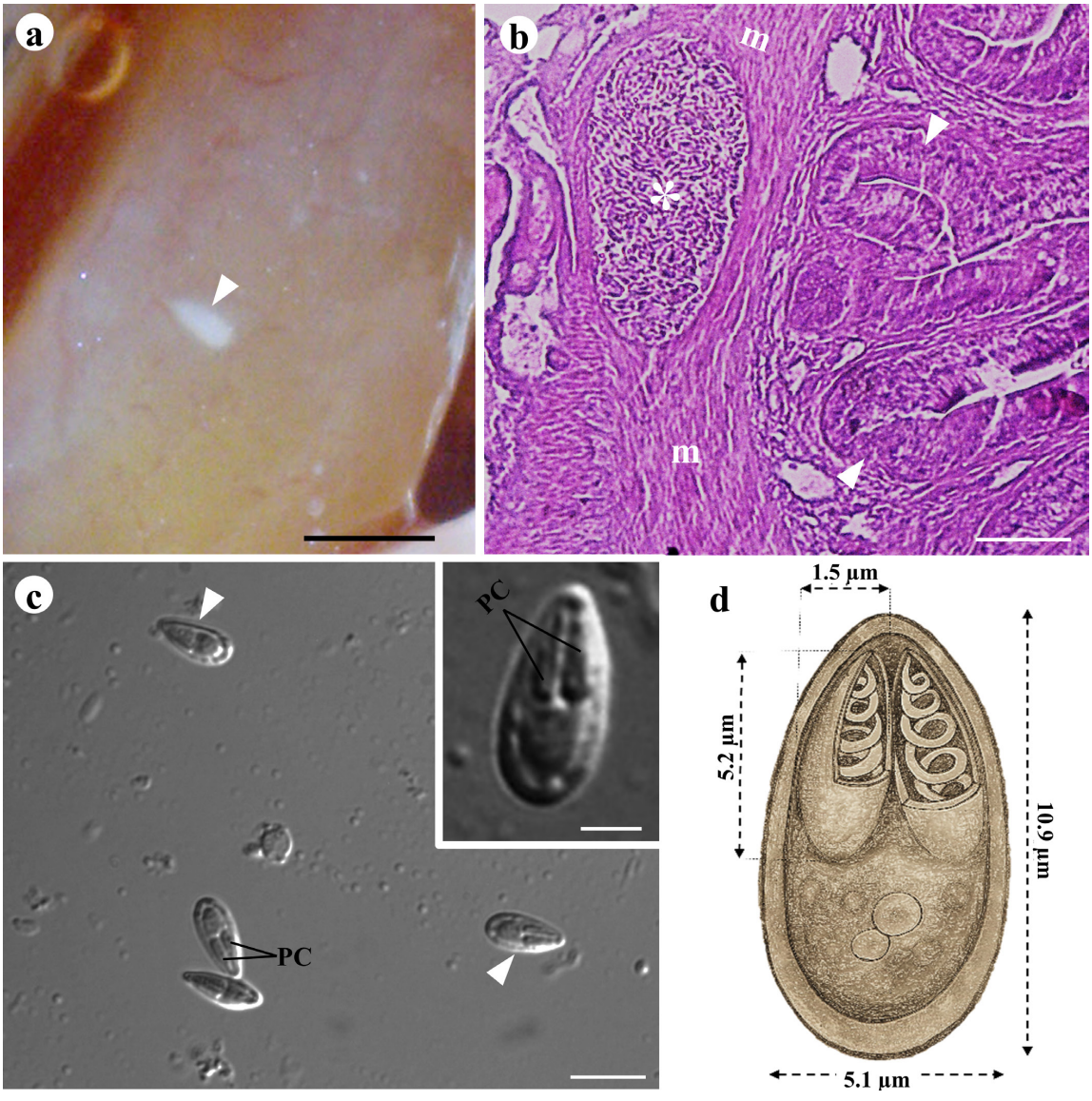

Figure 1. *Myxobolus marajoensis* sp. n. of *Rhamdia quelen*. (a) Intestine with a cyst (arrow head); Scale bar = 2000 µm; (b) Histological section of the intestine stained with Hematoxylin and Eosin, showing the cyst (*) in the muscle layer (m), detail of the intestinal mucosa (arrow head); Scale Bar = 75 µm; (c) Spores (arrow head) with polar capsules (PC); Scale bar = 15 µm; Inset; Detail of fresh spores, with polar capsules (PC) under Differential Interference Contrast (DIC); Scale bar = 3 µm; (d) Schematic drawing of a spore, found in the intestine; Spore in valvular view, showing its internal organization.

Mature spores: Mature pyriform spores (Figure 1c) were observed with a mean length of 10.9 µm (10.0-11.6) and mean width of 5.1 µm (4.2-5.4). Each spore bears two polar capsules of equal size, 5.3 ± 0.6 µm long and 1.6 ± 0.36 m wide (Table 1).

Type material: Slides containing spores were obtained from the muscle layer of the intestine, processed using the paraffin technique, stained with Gutierrez (Figure 1b), and mounted by the low viscosity method. These specimens were deposited in the International Protozoan Type Specimen Collection at the Brazilian National Institute of Amazonian Research (INPA) in Manaus, Amazonas, Brazil (catalog number: INPA 026).

Molecular data

Based on the alignment of 929 bps, the 18S rDNA sequence of *Myxobolus marajoensis* sp. n. was distinct from all the other *Myxobolus* sequences, obtained from other siluriform catfish, and from all other myxozoan species. The pairwise *p* distances recorded in relation to the other *Myxobolus* species that parasitize siluriforms were all relatively high, with the lowest value (13.0%) being recorded for *M. flavus*, reinforcing the existence of a new species (Table 2).

The phylogenetic tree is formed by two clades, A and B, with both clades including two subclades (A1 and A2, and B1 and B2). Subclade A1 encompasses the *Myxobolus* and *Henneguya* species found in freshwater siluriforms and characiforms, while subclade A2 includes the *Myxobolus* species that infect marine mugiliforms. Subclade B1 included species that parasitize siluriform and cypriniform hosts, while subclade B2 contained parasites of siluriform and characiform hosts (*M. plasmodialis* and *M. aureus*), which were allocated to different branches (Figure 2).

The phylogenetic analyses indicated that the *Myxobolus* species of clade A are paraphyletic, grouping with four *Henneguya* species, *H. eirasi*, *H. maculosos*, *H. visibilis* and *H. pellucida*. *Myxobolus marajoensis* sp. n. forms a subclade isolated from the other taxa of clade A. Phylogenetically, *Myxobolus marajoensis* sp. n. is associated with subclade A1, and was placed closest to *M. flavus* in the Bayesian inference (Figure 2).

Discussion

In the present study, the parasites were investigated in more detail, based on the analysis of their structural and morphometric characteristics (Figures 1c and d), as well as phylogenetic criteria (Figure 2), and the sum of the evidence indicated conclusively the existence of a new species, denominated *M. marajoensis* sp. n.

The morphology of this species is similar to that of *M. miyarii* (LIU et al., 2016) and *M. cunhai* (PENIDO, 1927), given the pyriform shape of the spores, which are distinct from the ovoid spores of *M. pangasii* (MOLNÁR et al., 2006), the elliptical spores of *M. hakyi* Baska et al. (2009) and *M. gayerae* Molnár et al. (2007), and the spherical spores of *M. bivacuolatus* (NARASIMHAMURTI & KALAVATI, 1986) (Table 1).

The spores of *Myxobolus marajoensis* sp. n. (Figures 1c and d) are smaller in size than those of most other *Myxobolus* species found in catfish, except for *M. cunhai*, which infects the catfish *Pimelodus clarias maculatus* Lacepède, 1803 (PENIDO, 1927), and *M. bivacuolatus*, which have spores similar in size to those of *Myxobolus marajoensis* sp. n. (Table 1). Even so, these two species can be differentiated on the basis of the relative size of the polar capsules, with two polar capsules of equal size in *Myxobolus marajoensis* sp. n., whereas in *M. cunhai*, these structures are extremely elongated and unequal in size.

Table 1. Comparison of the parameters (mean measurements in µm) of the spores of *Myxobolus* spp. described for different freshwater and marine catfish species (Siluriformes).

***Myxobolus* species**	**Hosts**	**Infection site**	**Spore shape**	**SL**	**SW**	**PCL**	**PCW**	**Size of the polar capsules**	**Country**
M. hakyi (BASKA et al., 2009)	*Pangasius hypohthalmus*	Epiderm	Elipsoidal	15.9	6.6	6.3	2.3	Equal	Thailand
M. pangasii (MOLNÁR et al., 2006)	*Pangasius hypophthalmus*	Spleen	Ovoid	14.3	7.03	6.4	1.7	Equal	Malaysia
M. miyarii (LIU et al., 2016)	*Silurus asotus*	Intestine	Pyriform	13.3	6.6	6.5	1.9	Equal	Japan
M. cunhai (PENIDO, 1927)	*Pimelodus clarias*	Intestine	Pyriform	10.0	5.0	-	-	Unequal	Brazil
M. bivacuolatus (NARASIMHAMURTI & KALAVATI, 1986)	*Clarias batrachus*	Intestine	Spherical	9.0	-	4.2	3.0	Equal	India
M. marajoensis sp. n. (Present study)	*Rhamdia quelen*	Intestine	Pyriform	10.9	5.1	5.2	1.5	Equal	Brazil

SL: spore length, SW: spore width, PCL: polar capsule length, PCW: polar capsule width.

Table 2. P distance of some freshwater species of *Myxobolus* described in Siluriformes showing Genbank accessions after species name.

		1	**2**	**3**	**4**	**5**	**6**
1	*Myxobolus marajoensis* (KX857727)						
2	*Myxobolus flavus* (KF296346)	0.130					
3	*Myxobolus hakyi* (FJ816269)	0.135	0.115				
4	*Myxobolus miyarii* (KT001495)	0.142	0.130	0.135			
5	*Myxobolus* sp.1 (KP990667)	0.233	0.216	0.221	0.233		
6	*Myxobolus gayerae* (DQ439809)	0.241	0.243	0.255	0.248	0.241	
7	*Myxobolus muelleri* (DQ439806)	0.243	0.115	0.245	0.235	0.250	0.075

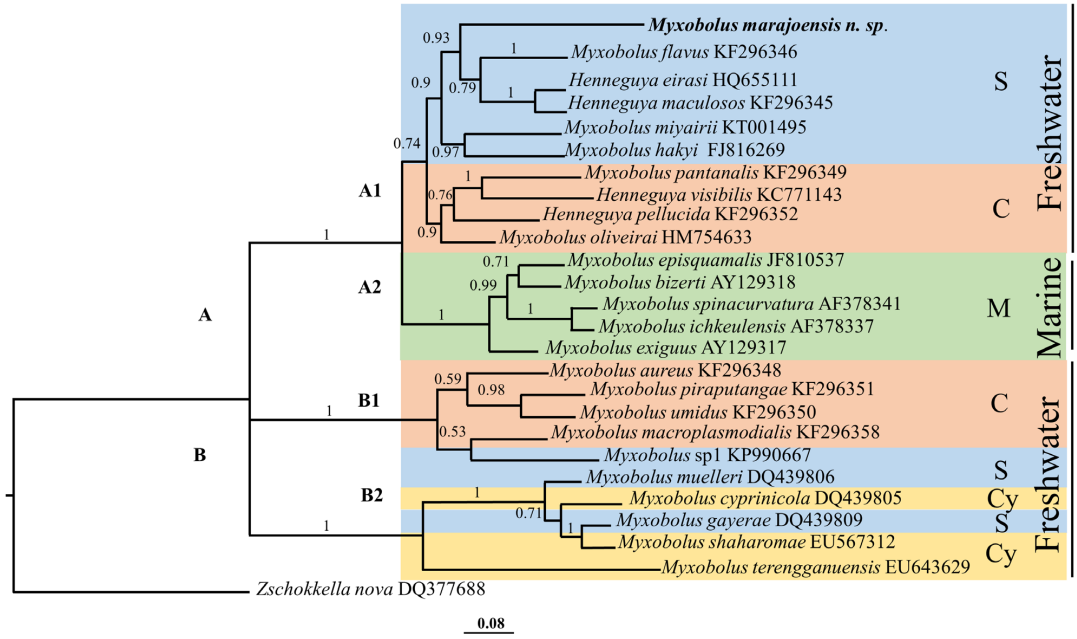

Figure 2. Phylogenetic tree of *Myxobolus marajoensis* sp. n. inferred by Bayesian analysis using a SSU rDNA data set. The values associated with each branch are their posterior probabilities. The species names are followed by their GenBank accession numbers. S = Siluriformes; C = Characiformes; M = Mugiliformes; Cy = Cypriniformes.

In comparison with the *Myxobolus* species found in the intestinal tracts of other catfish species, *Myxobolus marajoensis* sp. n. is closest in size to *M. cunhai*, which infects the catfish *P. clarias maculatus* (PENIDO, 1927), *M. bivacuolatus* and *M. pangasii*, found in *Pangasius hypophthalmus* (MOLNÁR et al., 2006; BASKA et al., 2009), and *M. miyarii*, a parasite of *S. asotus* (LIU et al., 2016) (Table 1). *Myxobolus marajoensis* sp. n. is smaller than the *Myxobolus* species found infecting cyprinid hosts, such as *M. gayerae* in *L. cephalus* (MOLNÁR et al., 2007), *M. nodulointestinalis* in *Barbus sharpeyi* Gunther, 1874 (MASOUMIAN et al., 1996), and *M. cyprinicola* in *Cyprinus carpio* Linnaeus, 1758 (MOLNÁR, 2002).

The phylogenetic analysis, based on comparisons with the 18S rDNA gene of 24 *Myxobolus* and *Henneguya* species obtained from GenBank, provided clear evidence of the existence of a distinct new species. A number of other *Myxobolus* species found in siluriforms have been described based on the analysis of the 18S rDNA gene, including *M. myarii*, found in the *S. asotus* by Liu et al. (2016). Using this gene, Baska et al. (2009) confirmed the presence of *M. hakyi* in the epidermis of the *P. hypophthalmus*, while Molnár et al. (2006) found *M. pangasii* infecting its spleen.

The phylogenetic relatedness of the *Myxobolus* and *Henneguya* species reflects a tendency for the formation of parasite clades related closely to their host species (CARRIERO et al., 2013), indicating coevolutionary relationships between the ancestral parasites and their hosts (BROUGHTON et al., 2013). In fact, the cladogram (Figure 2) reflects a grouping associated more closely with the hosts than the morphological or biogeographic characteristics of the parasites themselves.

Fish parasitized by the myxozoan species tend to form groups based on the type of environment (freshwater or marine) and taxon (FIALA, 2006; FERGUSON et al., 2008; ADRIANO et al., 2012; CARRIERO et al., 2013; MOREIRA et al., 2014). The lowest *p* distance recorded between *M. marajoensis* sp. n. and a congener was 13.0%, in the case of *M. flavus* (Table 2); increasing progressively in relation to the other *Myxobolus* clades that together with the morphological data presented here, support the classification of the new myxosporidean species. New microparasite species are described regularly in the Amazon region, based on histological and molecular evidence, such as the description of the morphological features of *M. niger*, found in the gill rake of the bandit corydoras (*C. melini*) by Mathews et al. (2016). Rocha et al. (2016) provide molecular data on *Kudoa* Meglitsch, 1947 found infecting the blue discus, *Symphysodon aequifasciatus* Pellegrin, 1904. Adriano et al. (2009) described *M. cordeiroi* from the gills of the siluriform *Z. jahu*, based on a phylogenetic topology similar to that described in the present study, with a fundamental division between the freshwater and marine species of *Myxobolus*, indicating a systematic relationship with the type of habitat occupied by the host.

The results of the present study provide important insights into the myxosporidean infections found in *R. quelen*, which may cause significant tissue damage (MARTINS et al., 1999; ADRIANO et al., 2005a, b, 2006; FEIST & LONGSHAW, 2006) or even death (MARTINS et al., 1999; FEIST & LONGSHAW, 2006). A better understanding of this freshwater microparasite fauna will be essential for the prevention and control of diseases in both wild and farmed stocks. This will be important not only to guarantee the health of stocks, but also the quality of the end product for human consumption.

Based on the morphological description and the molecular and phylogenetic analyses (18S rDNA sequences) comparing 24 sequences of *Myxobolus* and *Henneguya* species that parasitize freshwater and marine siluriforms, cypriniforms, mugiliforms, and characiforms,

the results of the present study provide conclusive evidence of the existence of a new species, *Myxobolus marajoensis* sp. n., from the Brazilian Amazon region.

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