

Myxobolus freitasi n. sp. (Myxozoa: Bivalvulida), a parasite of the brain of the electric knifefish in the Brazilian Amazon region

Myxobolus freitasi n. sp. (Myxozoa: Bivalvulida), parasita do cérebro do peixe-faca elétrico na Amazônia brasileira

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Abstract

A total of 30 specimens of the Amazonian electric knifefish, *Brachyhyppopomus beebei* Schultz, 1944 (Gymnotiformes: Hypopomidae), were collected from the Peixe-Boi River in the state of Pará, Brazil (1°06'59" S; 47°18'26" W). Fragments of the brain tissue were extracted for analysis via optical microscopy, and 18 specimens (60%) presented microparasites of the genus *Myxobolus*, with unequal capsules. The spores were 18.6 µm (17.7-19.8 µm) long and 8.6 µm (8.4-9.0 µm) wide; the largest polar capsule was 13.0 µm (12.4-13.4 µm) long and 5.6 µm (5.3-6.0 µm) wide, and the smallest capsule was 5.0 µm (4.5-5.3 µm) long and 2.5 µm (2.3-2.6 µm) wide. Infected brain fragments were extracted for histological processing and staining with hematoxylin-eosin and Ziehl-Neelsen. Some fragments were conserved in ethanol for molecular genetics analysis. A partial sequence of the 18S DNA gene was obtained from the spores, which did not correspond to any other sequences deposited in GenBank, although it did form a clade with other *Myxobolus* parasites of the nervous system. The morphological data, together with molecular phylogeny, supported the designation of a new species *Myxobolus freitasi* n. sp.

Keywords: Cnidaria, Myxozoa, molecular biology, histology.

Resumo

Um total de 30 espécimes do peixe-faca elétrico da Amazônia, *Brachyhyppopomus beebei* Schultz, 1944 (Gymnotiformes: Hypopomidae), foram coletados no rio Peixe-Mani, no estado do Pará, Brasil (1 ° 06'59 "S; 47 ° 18 ' 26 "W). Fragmentos de tecido cerebral foram extraídos para análise em microscopia óptica, sendo que 18 espécimes (60%) apresentavam microparasitos do gênero *Myxobolus*, com cápsulas desiguais. Os esporos apresentavam 18,6 µm (17,7-19,8 µm) de comprimento e 8,6 µm (8,4-9,0 µm) de largura; a maior cápsula polar tinha 13,0 µm (12,4-13,4 µm) de comprimento e 5,6 µm (5,3-6,0 µm) de largura, e a menor cápsula tinha 5,0 µm (4,5-5,3 µm) de comprimento e 2,5 µm (2,3-2,6 µm) de largura. Fragmentos cerebrais infectados foram extraídos para processamento histológico e coloração com hematoxilina-eosina e Ziehl-Neelsen. Alguns fragmentos foram conservados em etanol para análise genética molecular. Dos esporos, foi obtida uma sequência parcial do gene 18S do DNA, que não correspondeu a nenhuma outra sequência depositada no GenBank, embora tenha formado um clado com outros parasitas do gênero *Myxobolus* do sistema nervoso. Os dados morfológicos, juntamente com a filogenia molecular, apoiaram a designação de uma nova espécie *Myxobolus freitasi* n. sp.

Palavras-chave: Cnidaria, Myxozoa, biologia molecular, histologia.

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Introduction

The hydrographic basin of the Peixe-Boi River is located in the northeastern part of the state of Pará, Brazil, and has a total area of 1,044.32 km² (Silva & Lima, 2000). It is located primarily within the municipality of Peixe-Boi, an area characterized by unregulated settlement, which has caused extensive environmental degradation and modification of the local ecosystems. This has interfered with the biota of the municipality's rivers. These pressures are a stress factor for the local fish and may, in particular, reduce their immunological response (Dean et al., 2001), thus leading to increased incidence of infectious diseases (Pavanelli et al., 2002).

Fish are the most ancient and diverse group of vertebrates, and they include more than half of the vertebrate species described to date, i.e. approximately 28,000 out of an estimated total of 54,711 vertebrate species (Nelson, 2006). The aquatic lifestyle of these animals facilitates dispersal and transmission of parasites (Ahid et al., 2009).

In the Amazon region, Gymnotiformes fish are small organisms, with commercial importance in aquarium, the species of the freshwater benthopelagic genus *Brachyhypopomus* Schultz, 1944 (Hypopomidae) present a variety of pathogens, which exercise different levels of pathogenicity in relation to their natural habitat. It is due to homeostasis between the nutritional and physiological status of the fish being in balance with the environment (Mendoza-Franco & Reina, 2008; Azevedo et al., 2011; Casal et al., 2016).

One important group of fish parasites are those of the phylum Cnidaria Hatschek, 1888, which belong to the unclassified subphylum Myxozoa (Cnidaria), a group that includes approximately 2402 species, in 64 genera (Fiala et al., 2015; Zhang, 2011). Fish are the intermediate vertebrate hosts of most myxozoans. These parasites are responsible for myxosporidiosis, a disease that causes high mortality rates and which is found worldwide (Lom & Dyková, 2006). *Myxobolus* Bütschli, 1882, is the most diverse group of this phylum, with approximately 850 species described to date (Abdel-Ghaffar et al., 2017). This genus is a member of the order Bivalvulida Shulman, 1959, and is characterized by an ellipsoidal, ovoid or rounded body in the valvular view, which is biconvex in the sutural view. It comprises two valves surrounding two pyriform polar capsules that are sometimes unequal in size, and it has binucleated sporoplasm (Matos et al., 2001; Kent et al., 2001; Lom & Dyková, 2002; Lom & Dyková, 2006).

Parasites of the genus *Myxobolus* infect a wide range of organs, such as the gills, liver, musculature, skin and nervous system. They are of considerable clinical interest, given their potential as a source of disease, as in the case of *Myxobolus cerebralis* (Hoffer, 1903), which causes whirling disease (Gilbert & Granath, 2001) and may have been responsible for the decline in the population of *Oncorhynchus mykiss* Walbaum, 1792, in the western United States (Nehring et al., 2003).

Given the importance of *Myxobolus*, parasites of this genus that were found in specimens of the electric knife fish, *Brachyhypopomus beebei* Schultz, 1944, were analyzed in the present study. This species has considerable potential as an ornamental fish, due to its behavior, body size and attractiveness.

Material and Methods

A total of 30 specimens of *Brachyhypopomus beebei* were collected from the Peixe-Boi River in the municipality of Peixe-Boi, located in the northeastern extremity of the state of Pará, in northern Brazil (1°06'59" S; 47°18'26" W), in 2015 and 2016. The specimens were collected using fishing nets, and were transported live in aerated plastic bags containing river water to the Carlos Azevedo Research Laboratory (LPCA-UFRA) and the Edilson Matos Research Laboratory (LPEM-UFPA), both in Belém, Pará, Brazil. In these laboratories, the specimens were transferred to aquaria containing water from the species' natural habitat at temperatures of 28-30°C, as observed in the field, for two days (Ethic Committee Approval number 013/2014 CEUA/UFRA). The fish were analyzed, anesthetized using tricainemethanesulfonate (MS222 Sigma) at a concentration of 50 mgL⁻¹ and then euthanized by means of myelotomy and necropsied to detect the presence of parasites using a stereomicroscope.

During this analysis, the braincase was opened and small fragments of the cerebral tissue were extracted for observation via optical microscopy. After confirmation of the presence of *Myxobolus* spores, images were obtained using a Zeiss AxioCam ICc 5 camera attached to a Zeiss Primo Star microscope. A total of 30 spores were measured using the AxioVision LE software.

For the histological analyses, small fragments were extracted from infected organ and fixed in Davidson solution for 24 h. The fragments were then embedded in paraffin in order to cut sections of thickness 5 µm. These were then stained using standard (hematoxylin-eosin, HE) and special (Ziehl-Neelsen) histological techniques (Luna, 1968).

For the molecular analyses, fragments were removed from the brain and fixed in 80% alcohol. The total DNA was extracted using PureLink® Genomic DNA mini-kit (Invitrogen, Carlsbad, California, USA), following the manufacturer's instructions. The small subunit ribosomal DNA (18s DNA) was amplified using the MX5/MX3 and MC5/MC3 primers (Andree et al., 1999; Molnár, 2002). The final volume of the polymerase chain reaction (PCR) was 25 µL, containing 5-10 ng of the template DNA, 20 mM of Tris (pH 8.4), 50 mM of KCl, 4 mM of dNTPs (Invitrogen®), 2 mM of MgCl₂, 5 pmol of each primer and 1.2 units of Taq DNA polymerase (Invitrogen®). The amplification protocol consisted of 35 cycles of 1 min at 95°C, 1 min at 66°C and 2 min at 72°C, preceded by 5 min at 95°C, and followed by 5 min at 72°C.

The amplicons were electrophoresed on 1.5% agarose gel, purified using GFX PCR DNA and the gel purification kit (GE Healthcare, Chicago, Illinois, USA), following the manufacturer's instructions. The samples were then sequenced in an ABI 3130 automatic DNA analyzer (Applied Biosystems™, Foster City, California, USA) with BigDye® Terminator v3.1, following the manufacturer's specifications. The MC5 and MC3 primers, which were used to obtain the amplicons, were also used for sequencing. The nucleotide sequences were edited and aligned using the BioEdit software (Hall, 2007).

Following a BLAST search of GenBank sequences, the partial SSU rDNA sequence obtained from the samples was aligned with 42 *Myxobolus*, *Thelohanellus* and *Henneguya* sequences with similarity greater than 85%, which are available in GenBank. *Sphaeromyxa kenti* (JX443489) and *Sphaeromyxa zaharoni* (AY538662) sequences were also included as outgroups of the database for the phylogenetic analysis, just as they were used by Zatti et al. (2018).

Bayesian inference (BI) analysis was used based on Markov Chain Monte Carlo (MCMC) tree searches, which were run in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Two parallel runs of four simultaneous MCMC searches, each with five million generations, were conducted, with one tree being sampled every 500 generations. The results from the first 1000 trees were discarded as burn-in. The remaining trees were analyzed in MrBayes to estimate the posterior probability of each node in the phylogenetic reconstruction. Tracer v1.4.1 (Rambaut et al., 2018) was used to check the stationarity of all the parameters sampled in the chains. The BI analysis was based on the GTR + G model of nucleotide substitution, as indicated by jModelTest 2.0.2 (Posada, 2008). This model has base frequencies of A = 0.2614, C = 0.2007, G = 0.2869 and T = 0.251; a substitution model of AC = 1.4849, AG = 3.7832, AT = 3.0822, CG = 0.627, CT = 6.5267 and GT = 1; and variable site rates (G = 0.2840) that follow the gamma distribution. Genetic distances were determined using PAUP 4.0b (Swofford, 1998) with SSU rDNA gene sequences from only one fish parasite, *Myxobolus* sp., which was grouped in the same clade as *Myxobolus freitasi* n. sp.

Results

In the present study, a 60% prevalence of infection was recorded for myxozoa in the central nervous system (CNS) of the host, *Brachyhypopomus beebei*. The spore morphology was suggestive of myxosporids of the genus *Myxobolus* (Figure 1A-B). Hematoxylin-eosin staining (Figure 1C) revealed the cysts and their proximity to the pyramidal neuron layer, while Ziehl-Neelsen staining (Figure 1D) provided a more detailed view of the arrangement of the spores and, in particular, the polar capsules.

Description of *Myxobolus freitasi* n. sp.

Vegetative stages - The histological analyses revealed the presence of irregular cysts in the cerebral parenchyma, with fine fibrous capsules containing spores. The cysts were 130-770 µm (N = 8) in length. The cerebral tissue was replaced by a mass of *Myxobolus* spores, with multifocal necrosis in the areas adjacent to the cyst.

Mature spores - The spores had a piriform body and two polar capsules of unequal size, (Figure 2), which were 18.6 µm (17.7-19.8 µm) long and 8.6 µm (8.4-9.0 µm) wide. The larger polar capsule was 13.0 µm (12.4-13.4 µm) long and 5.6 µm (5.3-6.0 µm) wide, while the smaller polar capsule was 5.0 µm (4.5-5.3 µm) long and 2.5 µm (2.3-2.6 µm) wide. A helicoidal filament was observed within each capsule, with 14-15 coils in the larger capsule and 4-5 coils in the smaller capsule. Comparisons with the measurements of other *Myxobolus* species with unequal capsules are shown in Table 1.

Type locality - Peixe-Boi River (01°7' S; 47°18' W) in the municipality of Peixe-Boi, in northeastern Pará, Brazil.

Voucher specimens - Histological sections, on glass slides with coverslips, of the brain of *B. beebei*, containing spores of *Myxobolus freitasi* n. sp., are deposited in the International Biological Collection of Protozoan Type Slides at the Brazilian National Institute of Amazon Research (INPA), Manaus, state of Amazonas, Brazil (INPA 28).

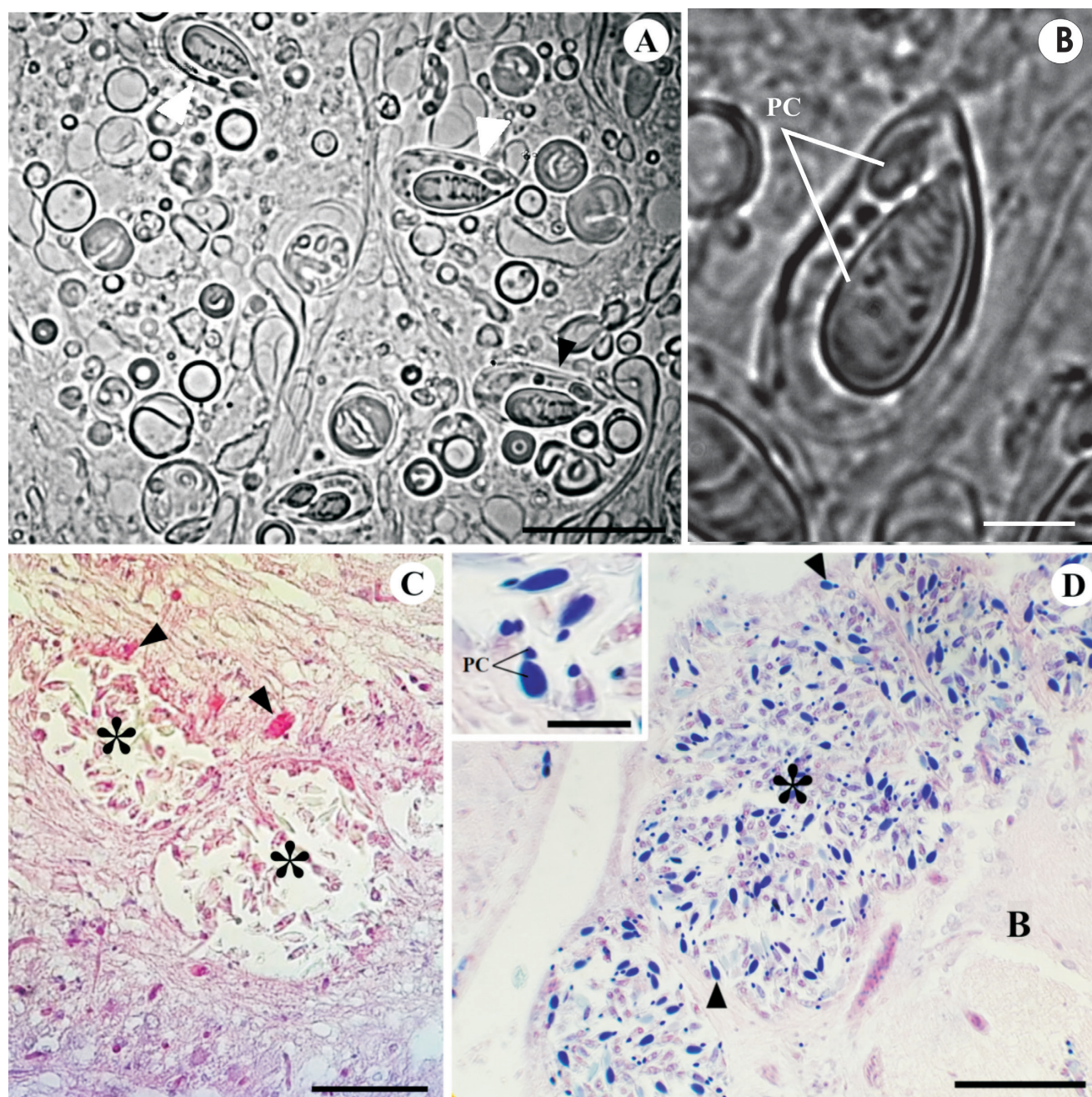


Figure 1. A and B - Fresh *Myxobolus freitasi* n. sp. spores (arrowhead), showing the larger and smaller polar capsules (PC). Scale bars: 20 μ m (A) and 5 μ m (B); C - Photomicrograph of histological sections stained with Haematoxylin and Eosin of cysts with spores of *Myxobolus freitasi* n. sp. (*) and pyramidal neurones (arrowhead) in the cerebral parenchyma of the *B. beebei*. Scale bar: 80 μ m; D - Histological sections stained with Ziehl-Neelsen of cyst (*) with spores of *Myxobolus freitasi* n. sp. (arrowhead) infecting the cerebral parenchyma (B), highlighting spores (inset) with large and small polar capsules (PC). Scale bar: 150 μ m (D) and 80 μ m (inset).

Prevalence - A total of 18 (60%) of the 30 specimens analyzed were infected.

Etymology - The species epithet honors Pedro Gonçalves de Freitas, an employee of the Federal Rural University of Amazonia, in Belém, for his invaluable contribution, through 30 years of specimen collection, to the work of the two parasitological research laboratories (LPEM-UFPA and LPCA-UFRA) in which the present study was developed.

Phylogeny - The partial 18S DNA gene sequence obtained here contains approximately 1,400 bp (GenBank accession number MG250286). The Bayesian analysis indicated that the species of the genera *Myxobolus*,

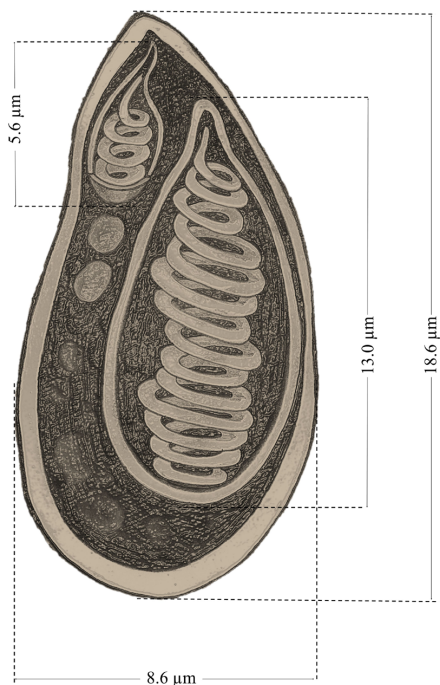


Figure 2. Diagram of *Myxobolus freitasi* n. sp. spore, in the valvular view.

Table 1. Comparison of the measurements of the spore of *Myxobolus freitasi* n. sp. with those of the other *Myxobolus* species that have unequal capsules. All measurements are given in micrometers.

Species	Spore		Larger Polar Capsule		Smaller Polar Capsule		Long polar filament coils	Short polar filament coils	References
	length	width	length	width	length	width			
<i>Myxobolus freitasi</i> n. sp*	18.6 (17.7-19.8)	8.6 (8.4-9.0)	13.0 (12.5-13.4)	5.6 (5.3-6.0)	5.0 (4.5-5.3)	2.5 (2.3-6)	14-15	04-05	Present study
<i>M. desaequalis</i> *	18.3 (17.6-19.1)	11.2 (10.6-11.9)	11.2 (10.7-11.9)	4.9 (4.5-5.2)	4.6 (4.1-4.8)	2.8 (2.5-3.1)	11-12	04-05	Azevedo et al. (2002)
<i>M. axelrodi</i> *	20.5 (19.0-21.8)	6.6 (5.7-7.9)	9.9 (8.0-11.2)	3.8 (3.2-4.8)	4.1 (3.5-4.5)	2.0 (1.8-2.3)	15-17	05-06	Camus et al. (2017)
<i>M. stellatus</i> *	18.4 (17.0-19.4)	8.8 (8.2-9.3)	9.9 (9.1 - 10.7)	5.4 (4.9-6.3)	5.0 (4.3 - 5.9)	2.6 (2.2 - 3.1)	8-10	04-06	Stilwell et al. (2020)
<i>M. inaequus</i> *	19.8 (15.6-22.0)	8.6 (7.8-9.3)	11.8 (9.4-13.0)	3.6 (3.1-3.9)	4.8 (3.9-5.5)	-	14	06	Kent & Hoffman (1984)
<i>M. absonus</i> *	15.7	10.2	6.4	3.6	4.2	2.5	05	03	Cellere et al. (2002)
<i>M. toyamai</i> *	14.3 (13.5-15.8)	5.5 (4.5-6.3)	5.8 (5.0-6.8)	3.5 (2.3-4.5)	3.4 (2.7-4.5)	0.8 (0.5-1.4)	07	-	Yokoyama & Ogawa (2015)
<i>M. brycon</i> **	6.9 (6.5-7.2)	4.2 (3.9-4.8)	4.2 (3.8-4.7)	1.9 (1.7 - 2.5)	-	-	08-09	-	Azevedo et al. (2011)
<i>M. franciscoi</i> **	6.4 (6.0-6.9)	6.0 (5.8-6.4)	2.0	1.5	-	-	03	-	Eiras et al. (2010)
<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)	-	-	04-05	-	Azevedo et al. (2009)
<i>M. oliveirai</i> **	11.2	7.4	5.6	2.3	-	-	06-08	-	Milanin et al. (2010)
<i>M. sciades</i> **	9.2	4.3	4.4	1.4	-	-	04-05	-	Azevedo et al. (2010)
<i>M. testicularis</i> **	8.6 (8.2-9.1)	7.2 (6.7-7.5)	3.5 (3.3-3.8)	1.7 (1.3-2.0)	-	-	04-05	-	Tajdari et al. (2005)

Myxobolus* with unequal polar capsules; *Myxobolus* with equal polar capsules.

Table 2. Genetic distances (p-distances) between all *Myxobolus* species of Clade A (Figure 3).

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)
(01) <i>Myxobolus freitasi</i> n. sp. (MG250286)	-																
(02) <i>M. neurotropus</i> (DQ846661)	0.098	-															
(03) <i>Myxobolus</i> sp. (AF378342)	0.100	0.000	-														
(04) <i>M. arcticus</i> (AB469993)	0.100	0.004	0.004	-													
(05) <i>M. kisutchi</i> (AB469988)	0.102	0.006	0.006	0.003	-												
(06) <i>M. neurobius</i> (AB469986)	0.101	0.004	0.004	0.006	0.008	-											
(07) <i>M. insidiosus</i> (EU346377)	0.102	0.010	0.010	0.013	0.012	0.011	-										
(08) <i>M. fryeri</i> (EU346372)	0.102	0.010	0.010	0.013	0.013	0.011	0.000	-									
(09) <i>M. squamalis</i> (MSU96495)	0.117	0.110	0.110	0.112	0.114	0.112	0.108	0.105	-								
(10) <i>Myxobolus</i> sp. (AB469984)	0.103	0.011	0.011	0.014	0.014	0.013	0.003	0.002	0.106	-							
(11) <i>M. silentisuturalis</i> (AY278563)	0.141	0.126	0.125	0.125	0.129	0.127	0.125	0.125	0.118	0.125	-						
(12) <i>M. cordeiroi</i> (KF296353)	0.120	0.129	0.129	0.132	0.134	0.130	0.130	0.131	0.137	0.131	0.117	-					
(13) <i>M. ellipsoides</i> (AF085178)	0.119	0.109	0.109	0.114	0.114	0.112	0.108	0.106	0.007	0.108	0.119	0.134	-				
(14) <i>M. djragini</i> (AF085179)	0.122	0.112	0.112	0.116	0.116	0.114	0.110	0.108	0.010	0.111	0.129	0.137	0.004	-			
(15) <i>M. squamalis</i> (U96495)	0.112	0.030	0.030	0.033	0.033	0.031	0.022	0.021	0.118	0.023	0.134	0.140	0.118	0.122	-		
(16) <i>M. cerebralis</i> (EF370479)	0.114	0.109	0.109	0.111	0.112	0.110	0.108	0.105	0.010	0.106	0.117	0.135	0.013	0.016	0.117	-	
(17) <i>M. toyamai</i> (LC010115)	0.131	0.130	0.129	0.131	0.131	0.133	0.126	0.127	0.110	0.127	0.123	0.137	0.112	0.115	0.141	0.111	-
(18) <i>M. minutus</i> (KU232372)	0.130	0.135	0.135	0.136	0.135	0.138	0.134	0.130	0.110	0.130	0.142	0.146	0.112	0.117	0.142	0.114	0.068

Discussion

Myxobolus freitasi n. sp. has a shape similar to that described for other myxozoans of this genus, such as *M. axelrodi*, described by Camus et al. (2017) infecting *Paracheirodon axelrodi*, *M. toyamai*, found in *Cyprinus carpio* in Japan (Yokoyama & Ogawa, 2015) and *M. stellatus* infecting *Thoracocharax stellatus* from Colombia (Stilwell et al., 2020).

The presence of unequal polar capsules in *M. freitasi* n. sp. is a characteristic that had already been observed in other parasites of the genus *Myxobolus* described in previous studies: *M. inaequus* in *Eigenmannia virescens* (Kent&Hoffman, 1984), *M. absonus* in *Pimelodus maculatus* (Cellere et al., 2002) and *M. desaequalis* in *Apteronotus albifrons* (Azevedo et al., 2002). These three *Myxobolus* species with unequal polar capsules were most similar to *M. freitasi* n. sp. in terms of the width and length of the spore, although they were considerably different in terms of the dimensions of the polar capsules, with *M. freitasi* n. sp. having by far the largest capsules. The number of coils in the polar filament of *M. axelrodi* was within the range of values recorded in *M. freitasi* n. sp., in both capsules. In this context, it may be important to note that both of these species are parasites of the CNS.

Azevedo et al. (2011) also described a myxosporean, *Henneguya torpedo*, in the CNS of *Brachyhyppopomus*, with a prevalence of 33.3%. Azevedo et al. (2018) subsequently described the species *H. lepturus* and *Thelohanellus lepturus*, which are both myxosporean parasites of the CNS of gymnotiform fishes from the Brazilian Amazon region, with a prevalence of 29.6%. In all cases, the prevalence recorded in the previous studies was lower than that found in the present study on *B. beebei* (60%). Despite the prevalence of *M. freitasi* n. sp. infection and the extent of the lesions in the cerebral tissue, the host did not present clinical symptoms. Infections of the CNS by myxosporeans have been reported from many regions of the world, and typically result in direct or indirect damage to the health of the host (Lom et al., 1987; Meng et al., 2011; Sindeaux et al., 2016). This highlights the importance of the present study for understanding myxosporidiosis in the neural tissue of Amazonian fish.

Taken together, the morphological, morphometric and molecular data obtained in the present study support the description of the new species, denominated *Myxobolus freitasi* n. sp. This taxon is clearly distinct from all other forms described previously in the genus *Myxobolus*.

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