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Morphological, ultrastructural and phylogenetic analyses of *Myxobolus hilarii* n. sp. (Myxozoa, Myxosporea), a renal parasite of farmed *Brycon hilarii* in Brazil

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ABSTRACT

Myxobolus hilarii n. sp. was described, based on morphology, histology, ultrastructure and 18S rDNA sequencing, infecting the kidney of *Brycon hilarii* (Valenciennes 1850) (Characiformes: Bryconidae) taken from fish farms in the state of São Paulo, Brazil. Thirteen specimens of *B. hilarii* were examined and 100% had round, white plasmodia in the kidney. The mature myxospores were rounded, measuring 11.5 ± 0.8 (9.8–13.4) μm in length, 11.0 ± 0.7 (9.7–12.4) μm in width and 7.6 ± 1.0 (6.7–9.0) μm in thickness. Polar capsules were elongated and of equal size, with 6.5 ± 0.4 (6.0–7.2) μm in length and 4.0 ± 0.2 (3.6–5.3) μm in width and their polar filaments had 5 to 7 coils. Histological analysis revealed plasmodial development in the renal tubules, causing compression and deformation of adjacent tissues and destruction of renal tubule cells. Ultrastructural analysis showed direct contact between the plasmodial wall and the host tissue and asynchronous plasmodial development. The phylogenetic analysis of South American myxobolids, based on 18S rDNA sequencing, showed the myxosporeans grouping into two main clades. *M. hilarii* n. sp. appears as sister species of *Myxobolus piraputangae*.
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1. Introduction

Myxozoans are among the most abundant parasites in nature [1]). Their main vertebrate hosts are fish, from both natural and farmed environments ([2,3,1]). The genus *Myxobolus* Bütschli, 1882, is the most specious of myxozoans, with over 800 known species [4,5,6], of which 38 have been found in South American freshwater fish [6]. Of the South American *Myxobolus* species, four *Myxobolus oliveirai* Milanin, Eiras, Arana, Maia, Alves, Silva, Carriero, Ceccarelli and Adriano, 2010; *Myxobolus brycon* Azevedo, Casal, Marques, Silva and Matos, 2011; *Myxobolus piraputangae* Carriero, Adriano, Silva, Ceccarelli and Maia, 2013; and *Myxobolus umidus* Carriero, Adriano, Silva, Ceccarelli and Maia, 2013 and *Myxobolus filamentum* Naldoni, Zatti, Capodifoglio, Milanin, Maia, Silva and Adriano, 2015, have been found infecting species of the genus *Brycon* Müller and Troschel, 1844, in natural environments [7,8,9,10].

Brycon hilarii (Valenciennes, 1850) (Syn.: *Brycon microlepis* Perugia, 1897) is a characiform from the Bryconidae family and is endemic in Paraguay Basin [11]. It is popularly known in Brazil as "piraputanga" and may reach up to 50 cm in length and 3.4 kg in weight [12]. Due to its commercial popularity and rapid growth rate, *B. hilarii* is valuable to Brazilian fish farmers [13], with 265,000 kg of the fish being produced in 2011 [14].

The present study, which is a part of an ongoing investigation into the myxosporean parasites of Brazilian freshwater fish, used morphological, histological, ultrastructural and molecular data to describe a new myxosporean species found infecting the kidneys of farmed *B. hilarii* in São Paulo State, Brazil.

2. Materials and methods

Thirteen specimens of *B. hilarii* were captured using nets from a fish farm in São Paulo State, Brazil, in 2012. After being captured, the fish were immediately transported alive to the laboratory. The fish were euthanized with an overdose of benzocaine, in accordance with Brazilian law (Federal Law No. 11.794 dated October 8, 2008, and Federal Decree No. 6899 dated July 15, 2009), measured and examined, searching for plasmodia. For morphological analysis, the plasmodia was separated from the host tissue, placed on a slide and ruptured. Thirty-four mature myxospores were photographed using a computer equipped with Axivision 4.1 image-capture software coupled to an Axioplan 2 Zeiss Microscope. The spore dimensions were obtained in accordance with the procedures described by Lom and Arthur [15] and recorded in micrometers as mean ± standard deviation (SD).

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2.1. Histology and ultrastructural analysis

For histological analysis, fragments of the infected tissue were fixed in 10% buffered formalin and embedded in paraffin. Serial 4-μm-thick sections were stained with hematoxylin–eosin. For transmission electron microscopy, the plasmodia were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 12 h, washed in glucose–saline solution for 2 h and post-fixed in OsO_4. All of these procedures were performed at 4 °C. After dehydration using an acetone series, the sample was embedded in EMbed 812 resin (Electron Microscopy Sciences, Hatfield, PA, USA). Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined using a LEO 906 electron microscope at 60 kV.

2.2. DNA extraction, amplification, sequencing and phylogeny

For molecular analysis, two plasmodia were isolated from the tissue of specimens of *B. hilarii*, placed in separate tubes and fixed in absolute ethanol. DNA was extracted using the DNeasy® Blood & Tissue Kit (QIAGEN, USA), according to the manufacturer's instructions and quantified using a spectrophotometer at 260 nm. Amplification of the 18S rDNA was performed in a final volume of 25 μl, which contained 10–40 ng of extracted DNA, 1× PCR Buffer (Sigma-Aldrich), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U of Taq DNA Polymerase (Sigma-Aldrich), 0.2 pmol of each primer and ultrapure water (Barnstead/Thermolyne, Dubuque, IA, USA). For amplification of the 18S rDNA of each plasmodium the primer pairs Erib1 (forward): 5′-ACCTGGTTGATCCTGCCAG-3′ [16], Act1r (reverse): 5′-ATTTCACCTCTCGCTGCCA-3′ [17] and Tedf (forward): 5′-AATTACCCAATCCAGACAAT-3′ [18], Erib10 (reverse): 5′-CTTC CGCAGGTTCACCTACGG-3′ [16] were used for PCR performed in a Thermocycler (Eppendorf, Hamburg, Germany) with an initial 5 min denaturation step at 95 °C. This was followed by 35 cycles of denaturation at 95 °C for 60 s, annealing at 58–64 °C for 60 s, extension at 72 °C for 90–120 s and a final extended elongation step at 72 °C for 5 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis in a TAE buffer (Tris–Acetate EDTA, Tris 40 mM, Acetic Acid 20 mM, EDTA 1 mM), stained with Sybr Safe DNA gel stain (Invitrogen by Life Technologies, CA, USA) and analyzed with a Stratagene 2020E transilluminator. The sizes of the amplified fragments were estimated by comparison with the 1 Kb Plus DNA Ladder (Invitrogen by Life Technologies, CA, USA). The PCR products of the two amplified samples were

Fig. 1. Photomicrograph of fresh mature myxospores of *Myxobolus hilarii* n. sp. from the kidney of *Brycon hilarii*. Bar: 10 μm.

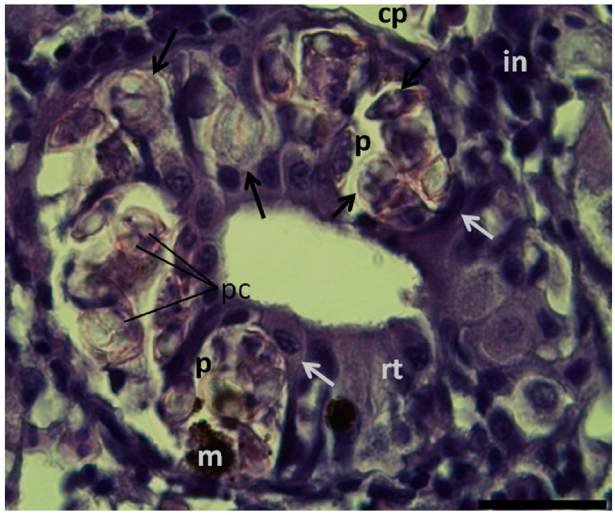

Fig. 2. Photomicrographs of histological sections from renal tubule (rt) of *Brycon hilarii* infected with *Myxobolus hilarii* n. sp. Note small plasmodia (p) with myxospores (black arrows) and they polar capsules (pc) and deformation of epithelial cells of the renal tubule (white arrows). Interstitium (in), melanomacrophage (m). Bar: 20 μm.

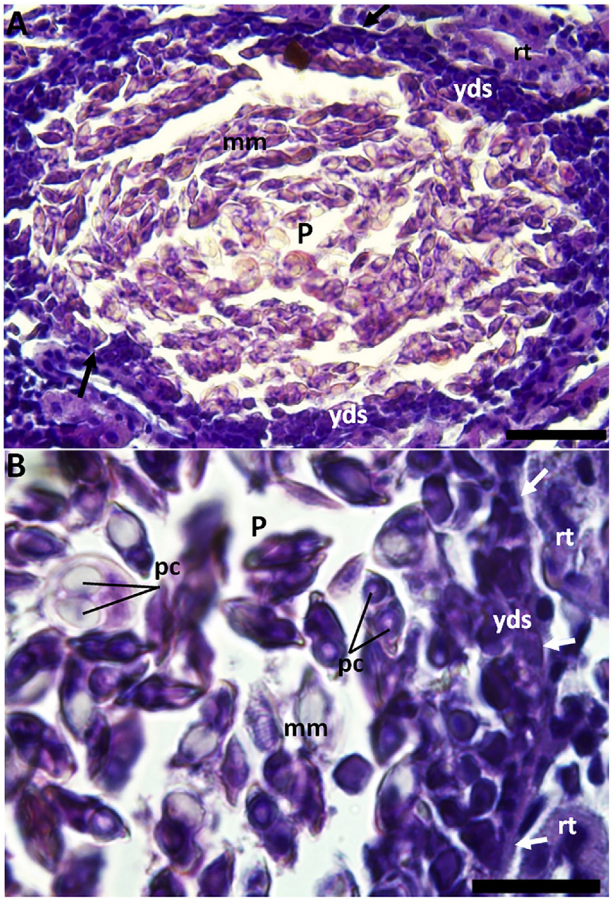

Fig. 3. Photomicrographs of histological sections from kidney of *Brycon hilarii* infected with *Myxobolus hilarii* n. sp. (A) Large plasmodium (P) causing compression and deformation of the renal tubule (rt). Note the presence of young developmental stages (yds) near to the border of the plasmodium (black arrows) and mature myxospores (mm) in the central area. Bar: 40 μm. (B) Amplified area of A showing the thin wall of the plasmodium (white arrows), young developmental stages (yds) in the periphery and mature myxospores (mm) with their polar capsules in the deeper area of the plasmodium (P). Bar: 20 μm.

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