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Novos dados morfológicos e diagnóstico molecular de *Henneguya friderici* (Myxozoa: Myxobolidae), parasito de *Leporinus friderici* (Osteichthyes: Anostomidae) do sudeste do Brasil

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Abstract

The myxozoan *Henneguya friderici* is a parasite of the gills, intestine, kidney and liver of *Leporinus friderici*, a characiform fish belonging to the family Anostomidae. Forty-two specimens of *L. friderici* that had been caught in the Mogi Guaçu River, state of São Paulo, were studied. Elongated white plasmodia were found in the gill filaments of 10 host specimens (24%). The mature spores had an ellipsoidal body with polar capsules of equal size and caudal length greater than body length. This study also described 18S rDNA sequencing of *H. friderici* infecting the gill filaments. This produced a sequence of 1050 bp that demonstrated significant genetic differences with previously described species of *Henneguya*. Similarity analysis using sequences from species that clustered closest to those produced by this study showed that the species with greatest genetic similarity to *H. friderici* was *H. leporinicola*, with 94% similarity.

Keywords: Myxosporea, Characiformes, 18S rDNA, phylogeny.

Resumo

O myxozoa *Henneguya friderici* é um parasito encontrado nas brânquias, fígado, intestino e rins de *Leporinus friderici*, (Characiformes: Anostomidae). Foram capturados e examinados quarenta e dois espécimes de *L. friderici* oriundos do Rio Mogi Guaçu, estado de São Paulo. Cistos alongados e brancos foram encontrados nos filamentos branquiais de 10 (24%) hospedeiros. Os esporos maduros apresentaram o corpo alongado com as cápsulas polares em tamanhos iguais e o comprimento caudal maior do que o comprimento corporal. Com isso, o presente trabalho, descreve o sequenciamento de 1050 pb do gene 18S rDNA de *H. friderici* infectando os filamentos branquiais, o que demonstrou diferenças genéticas significativas em comparação com espécies previamente descritas de *Henneguya*. A análise de similaridade utilizando as sequências de espécies que se agruparam mais próximas às produzidas por este estudo mostrou que a espécie com maior semelhança genética com *H. friderici* foi *H. leporinicola*, com 94% de similaridade.

Palavras-chave: Myxosporea, Characiformes, 18S rDNA, filogenia.

Introduction

The diversity of known myxozoans has grown greatly since the early work of Kudo (1919). Around 2.200 species have now been described (LOM & DYKOVÁ, 2006) and these represent around 18% of cnidarian species diversity, as far as is currently known (OKAMURA et al., 2015). *Henneguya* Thélohan, 1892,

is one of the most diverse genera of Myxosporea and currently includes more than 200 known and described species (LOM & DYKOVÁ, 2006). This widespread genus includes typical coelozoic and histozoic species and predominantly infects marine and freshwater fish (EIRAS & ADRIANO, 2012).

Currently, more than 44 species of *Henneguya* are known to infect South America fish (EIRAS, 2002; EIRAS & ADRIANO, 2012; CARRIERO et al., 2013; NALDONI et al., 2014).

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Of these, around 28 have been found to infect fish species of the order Characiformes.

Identification of the species in this genus, like those in other genera of myxozoans, is based almost exclusively on spore morphology. In the class Myxosporidia, morphology has been the main criterion for classification of species (KUDO, 1933; MOLNÁR, 1994). In fact, this method has always failed to identify highly similar species that are found in the same infection site and host and which only have subtle differences in spore structures (YE et al., 2012). Fortunately, this problem has been solved through molecular approaches (SMOTHERS et al., 1994; ANDREE et al., 1999; HOLZER et al., 2004). 18S rDNA is the molecular marker that has most commonly been used for detection, identification and phylogenetic analysis on myxozoans (HOLZER et al., 2006). The difficulties of relying on spore morphology for species identification have led authors to recommend that SSU rDNA sequencing should be included when new species are described (ANDREE et al., 1999; KENT et al., 2001; LOM & DYKOVÁ, 2006).

Leporinus friderici (Bloch, 1794) is a characiform fish belonging to the family Anostomidae that is, popularly known in Brazil as “piau”. It is widely distributed in the Amazon and Paraguay river basins (FROESE & PAULY, 2016). Among the species of *Henneguya*, only *Henneguya friderici* (CASAL et al., 2003) has been reported from *L. friderici*.

Henneguya friderici was found infecting the gills, intestine, kidney and liver of “piau” from an estuarine region of the Amazon River, in the state of Pará, Brazil. Relative organelle preservation occurred in the liver tissue and, in some cases, development of the parasite caused gradual and generalized degeneration in the intestine, gills and kidney (CASAL et al., 2003).

The present paper supplements the original description of *H. friderici* with new data on morphology and 18S rDNA sequencing on samples from gill filaments of *L. friderici* from the Mogi Guaçu River, state of São Paulo, Brazil. The new data support the original diagnosis by Casal et al. (2003).

Materials and Methods

Forty-two specimens of *L. friderici* were caught by local fishermen with nets and hooks in the Mogi Guaçu River near Pirassununga, state of São Paulo, Brazil (21°55'36" S; 47°22'6" W), between January 2014 and January 2016. Gills extracted from the fish were placed in Petri dishes with tap water and were examined for myxozoans using a dissecting microscope. Infected gill filaments were preserved using two different methods: frozen (for spore measurements) and in 95% ethanol (for DNA analysis).

Parasitological examinations were conducted using standard methods with the aid of an optical microscope (Olympus BX51) with differential interference contrast (DIC). Images were captured using a 3.2 mp UC30 digital camera and were analyzed by means of photomicrography software (Cell^D 3.4, Olympus Soft Imaging Solutions GmbH, Germany). At least 30 measurements were made for each relevant spore dimension, following the guidelines of Lom & Arthur (1989).

Gill filaments from three hosts were used for DNA extraction by means of the DNeasy Blood & Tissue Kit, following the manufacturer's instructions (QIAGEN Inc., California, USA). The polymerase chain reaction (PCR) was performed as described by Whipps et al. (2015) in 50 µl reaction volumes of the Quick-Load Taq 23 Master Mix (New England Biolabs, Ipswich, Massachusetts, USA), with 0.5 µM of each primer and 3 µl of template DNA. A first round of amplification targeting the small subunit (SSU) rDNA was performed using the primers 18E and 18R (WHIPPS et al., 2003), followed by a second round of PCR with 18E and Myxgen2R (KENT et al., 2000) or with 18R and Myxgen3F (KENT et al., 2000). The amplifications were performed in a C1000™ thermal cycler (BioRad Laboratories, Hercules, California, USA) with initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 45 s and 68 °C for 90 s, and a final extension at 72 °C for 7 min. Product amplification was evaluated by observation on 1% agarose gel, and the remainder of the sample was purified using the E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek, Norcross, Georgia, USA). DNA was quantified using a DNA spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). Sequencing reactions were carried out by means of the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit version 3.1, using the ABI3730xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Contiguous sequences were assembled in Geneious (Geneious version 9, created by Biomatters, available from (<http://www.geneious.com/>) and were deposited in GenBank (Table 1).

Alignments were subjected to maximum likelihood (ML) and Bayesian inference (BI) (rates = invgamma) analyses; additionally, Tamura & Nei (TRN) distance values were performed using Geneious. ML and BI trees were calculated under the TRN + I + G model for the sequences of the rDNA 18S, using PHYML (GUINDON & GASCUEL, 2003) and MrBayes (HUELSENBECK & RONQUIST, 2001) Geneious plug-ins for ML and BI, respectively. These models were selected using jModelTest2 (DARRIBA et al., 2012). Nucleotide frequencies were estimated from the data (A = 0.2824, C = 0.1634, G = 0.2826, T = 0.2715). Six rates of nucleotide substitution were (AC) = 1.0000, (AG) = 3.2212, (AT) = 1.0000, (CG) = 1.0000, (CT) = 6.0419, (GT) = 1.0000; proportion of invariable sites = 0.1460; gamma distribution = 0.3960 estimated with 4 rate categories. ML nodal support was estimated by 1000 nonparametric bootstrap replications. Bayesian posterior probability were determined running the Markov chains (two runs and four chains) for 4×10^6 generations, discarding the initial 1/4 of sampled trees (trees sample every 4×10^3 generations) as burn in fraction. Phylogenetic trees were rooted using *Ceratonova shasta* (Noble, 1950) as outgroup based upon previous Myxobolidae phylogenies (ADRIANO et al., 2009; CAPODIFOGGIO et al., 2015; NALDONI et al., 2015).

Results

Henneguya friderici cysts were found in gill filaments from ten piau specimens, i.e. 24% of the total examined. The elongated white plasmodia measured approximately 2 mm in length. The mature spores were ellipsoid in frontal view and the valves were symmetrical

Table 1. List of myxozoan whose sequences were used for analyses and the obtained in the present study.

Parasite	GenBank accession No.	Host	Country	Reference
<i>Ceratomyxa shasta</i>	AF001579	<i>Oncorhynchus mykiss</i>	USA	Bartholomew et al. (1997)
<i>Henneguya adiposa</i>	EU492929	<i>Ictalurus punctatus</i>	USA	Griffin et al. (2009)
<i>Henneguya bulbosus</i>	KM000055	<i>Ictalurus punctatus</i>	USA	Rosser et al. (2014)
<i>Henneguya. cerebrealis</i>	JX131380	<i>Thymallus nigrescens</i>	Mongolia	Batueva et al. (2013)
<i>Henneguya. corruscans</i>	JQ654971	<i>Pseudoplatystoma corruscans</i>	Brazil	Adriano et al. (2012)
<i>Henneguya creplini</i>	EU732597	<i>Zingel zingel</i>	Hungary	Eszterbauer et al. (2006)
<i>Henneguya cuniculator</i>	KF732840	<i>Pseudoplatystoma corruscans</i>	Brazil	Naldoni et al. (2014)
<i>Henneguya cutanea</i>	AY676460	<i>Abramis brama</i>	Hungary	Kallert et al. (2005)
<i>Henneguya dogieli</i>	KJ725078	<i>Siniperca chuatsi</i>	China	Unpublished
<i>Henneguya doneci</i>	LC011456	<i>Carassius gibelio</i>	China	Li et al. (2015)
<i>Henneguya doneci</i>	EU344898	<i>Carassius auratus</i>	China	Unpublished
<i>Henneguya doneci</i>	HM146129	<i>Carassius gibelio</i>	China	Ye et al. (2012)
<i>Henneguya doori</i>	H DU37549	<i>Perca fluviatilis</i>	Canada	Siddall et al. (1995)
<i>Henneguya exilis</i>	AF021881	<i>Ictalurus punctatus</i>	USA	Lin et al. (1999)
<i>Henneguya friderici</i>	KY315824	<i>Leporinus friderici</i>	Brazil	Present study
<i>Henneguya gurlei</i>	DQ673465	<i>Ameiurus nebulosus</i>	USA	Iwanowicz et al. (2008)
<i>Henneguya ictaluri</i>	AF195510	<i>Ictalurus punctatus</i>	USA	Pote et al. (2000)
<i>Henneguya jocu</i>	KF264964	<i>Lutjanus jocu</i>	Portugal	Azevedo et al. (2014)
<i>Henneguya leporinicola</i>	KP980550	<i>Leporinus macrocephalus</i>	Brazil	Capodifoglio et al. (2015)
<i>Henneguya lobosa</i>	EU732600	<i>Esox lucius</i>	Germany	Eszterbauer et al. (2006)
<i>Henneguya maculosus</i>	KF296344	<i>Pseudoplatystoma corruscans</i>	Brazil	Carriero et al. (2013)
<i>Henneguya mississippiensis</i>	KP404438	<i>Ictalurus punctatus</i>	USA	Rosser et al. (2015)
<i>Henneguya multiplasmodialis</i>	JQ654969	<i>Pseudoplatystoma corruscans</i>	Brazil	Adriano et al. (2012)
<i>Henneguya pellis</i>	FJ468488	<i>Ictalurus punctatus</i>	USA	Griffin et al. (2009)
<i>Henneguya pellucida</i>	KF296352	<i>Piaractus mesopotamicus</i>	Brazil	Carriero et al. (2013)
<i>Henneguya piaractus</i>	KF597016	<i>Piaractus mesopotamicus</i>	Brazil	Müller et al. (2013)
<i>Henneguya pseudoplatystoma</i>	KP981638	<i>Pseudoplatystoma corruscans</i>	Brazil	Milanin et al. (2015)
<i>Henneguya pseudorhinogobii</i>	AB447994	<i>Rhinogobius</i> sp.	Japan	Kageyama et al. (2009)
<i>Henneguya psorospermica</i>	EU732602	<i>Esox lucius</i>	Germany	Eszterbauer et al. (2006)
<i>Henneguya rhinogobii</i>	AB447992	<i>Rhinogobius</i> sp.	Japan	Kageyama et al. (2009)
<i>Henneguya rotunda</i>	KJ416130	<i>Salminus brasiliensis</i>	Brazil	Moreira et al. (2014a)
<i>Henneguya salminicola</i>	AF031411	<i>Oncorhynchus kisutch</i>	Canada	Hervio et al. (1997)
<i>Henneguya</i> sp.	JQ411297	<i>Oncorhynchus masou masou</i>	Japan	Yokoyama et al. (2012)
<i>Henneguya</i> sp.	KR704889	<i>Cirrhinus mrigala</i>	India	Unpublished
<i>Henneguya</i> sp.	EU732601	<i>Esox lucius</i>	Hungary	Eszterbauer et al. (2006)
<i>Henneguya</i> sp.	EU732599	<i>Perca fluviatilis</i>	Hungary	Eszterbauer et al. (2006)
<i>Henneguya</i> sp.	JQ690355	<i>Carassius auratus</i>	China	Unpublished
<i>Henneguya sutherlandi</i>	EF191200	<i>Ictalurus punctatus</i>	USA	Griffin et al. (2008)
<i>Henneguya visibilis</i>	KC771143	<i>Leporinus obtusidens</i>	Brazil	Moreira et al. (2014b)
<i>Henneguya zikaweiensis</i>	KR020026	<i>Carassius auratus</i>	China	Zhang et al. (2015)
<i>Henneguya zschokkei</i>	HZU13827	<i>Prosopium williamsonii</i>	USA	Smothers et al. (1994)
<i>Henneguya zschokkei</i>	AF378344	<i>Prosopium williamsonii</i>	Canada	Kent et al. (2001)

and convex in lateral view. The polar capsules were elongated and equal in size and occupied a little less than half of the spore body (Figure 1). Spores ($N = 30$) were 12.8 ± 2.1 (7.4-14.8) μm in length, 4.4 ± 0.4 (3.4-5.2) μm in width and 32.8 ± 2.6 (2.49-40) μm in total length. The bifurcated caudal processes were cylindrical, equal in size, 19.6 ± 2.2 (16.1-24.4) μm in length, and extended behind the spore. Two equal capsules were pyriform, tapering toward their anterior end and occupying nearly half of the spore, and they measured 5.1 ± 0.5 (3.7-5.9) μm in length and 1.5 ± 0.1 (1.2-1.8) μm in width (Figure 1). Table 2 provides a comparison

between the data on the spore dimensions, infection sites and host of *H. friderici* obtained in this study and the data from the original descriptions.

The 18S rDNA sequencing on *H. friderici* spores resulted in a sequence containing 1050 bp, which was deposited in the GenBank database under accession number KY315824. This sequence was used for phylogenetic analysis. A BLAST comparison between the sequence obtained and other myxosporean sequences available in GenBank revealed that the 18S rDNA sequence of *H. friderici* had 92% similarity to that of *Henneguya leporinicola* Martins,

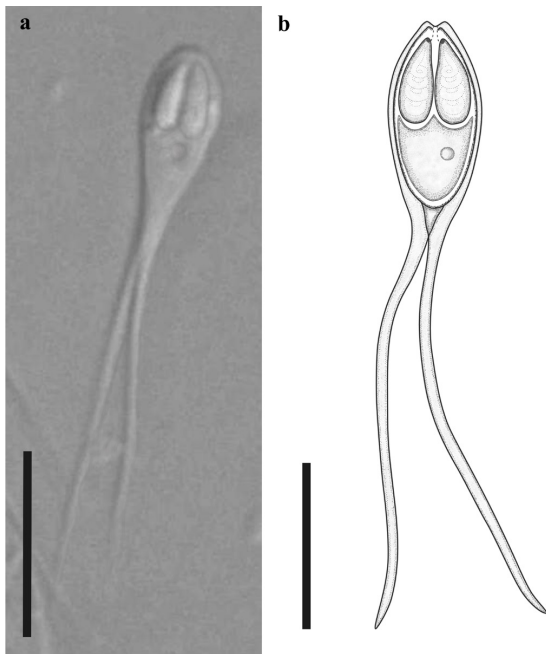


Figure 1. (a) Mature spore of *Henneguya friderici* parasite of gill filaments of *Leporinus friderici* in frontal view with Nomarski interference contrast; (b) schematic of *Henneguya friderici* myxospore demonstrating the polar capsule, spore capsule, and caudal processes. Scale bar 10 µm.

Souza, Moraes & Moraes, 1999 (KP980550) and 89% similarity to that of *H. bulbosus* Rosser, Griffin, Quiniu, Khoo & Pote, 2014 (KM000055).

Similarity analysis using sequences from species that clustered closest to those produced by the present study showed that the species with greatest genetic similarity to *H. friderici* was *H. leporinicola*, with 94% similarity. The ML and BI phylogenetic tree (Figure 2) showed that *H. friderici* appears as a sister species of *H. leporinicola* in a subclade composed mainly of myxosporean parasites of Characiformes and Esociformes.

Discussion

Henneguya friderici was described by Casal et al. (2003) infecting the gills, intestine, kidney and liver of *L. friderici* in the Amazon River, near Belém, state of Pará, Brazil. Its description was based on morphological and ultrastructural data. This was, in the past, the main method for characterization and identification of myxosporeans (MOLNÁR, 2002). However, Kent et al. (2001) and Lom & Dyková (2006) suggested that amplification of 18S rDNA is fundamental for describing new species of myxosporeans, because of the difficulties of characterizing the spores morphologically.

The present study provided 18S rDNA sequencing on *H. friderici* that was found infecting the gill filaments of host caught in the Mogi Guaçu River in the state of São Paulo. This enabled phylogenetic

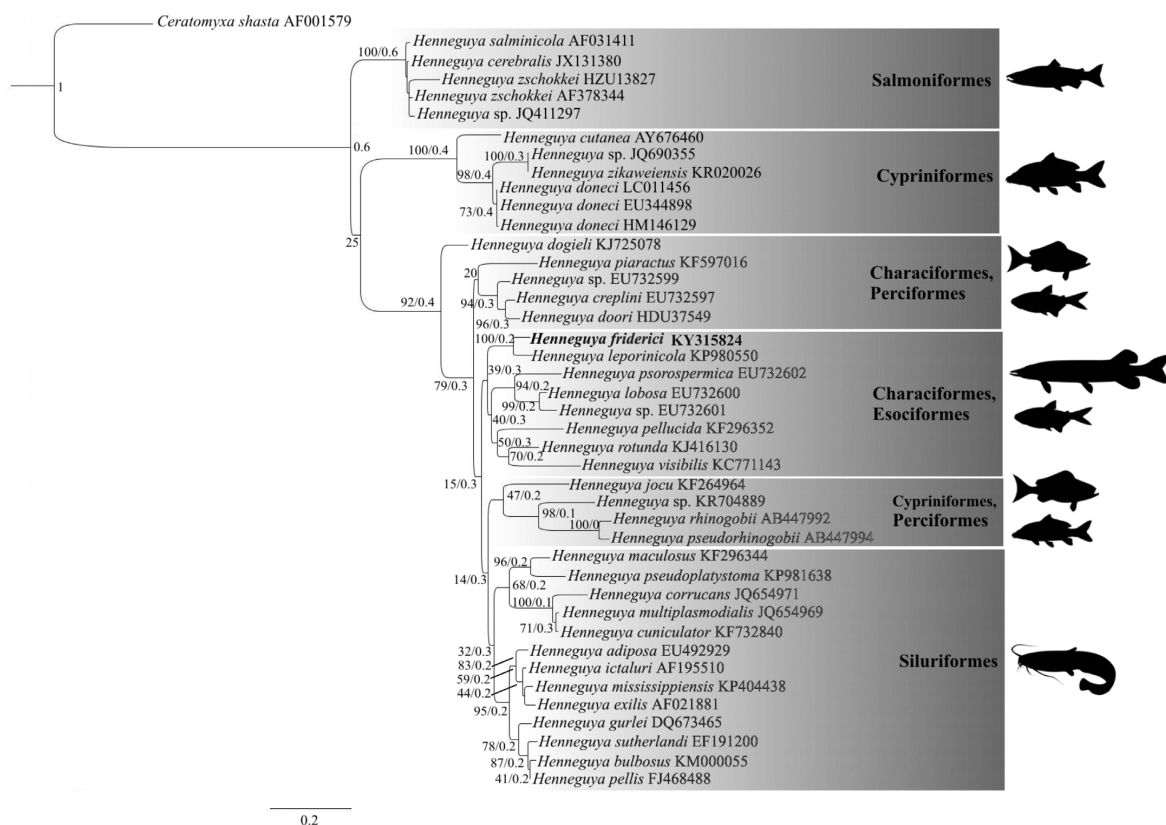


Figure 2. Maximum Likelihood from phylogenetic analysis of the sequences of 18S rDNA gene of *Henneguya friderici* associated with the closest species indicated by the analysis of Max Score by BLAST of the NCBI platform. First number of nodal support is from maximum likelihood bootstrap (1000 replications), the second number shows Bayesian posterior probability (for 4×10^6 generations; burn-in = 4×10^3). Sample from the present study is in **bold**.

Table 2. Comparison of the characteristics of *Henneguya friderici* with similar species.

Species	LS	WS	AL	TL	PCL	PCW	Host	Site	Reference
<i>Henneguya leporinicola</i>	7.6(5.5-8.7)	4.2 (3.6-4.9)	21.8 (12.9-32.2)	-	3.0 (2.0-3.6)	1.6 (1.2-2.0)	<i>Leporinus macrocephalus</i>	Gills	Martins et al. (1999)
<i>Henneguya azevedoi</i>	12.0(11-13)	3.2 (3-4)	39.4 (37-40)	56.4 (52-58)	6.3 (6-7)	2.1 (2-3)	<i>Leporinus obusidens</i>	Gill lamellae	Barassa et al. (2012)
<i>Henneguya caudicula</i>	11.3 (11-12)	5.4 (5-6)	3.4 (3-4)	14.7 (14-16)	3.7 (3-4)	1.5	<i>Leporinus lacustris</i>	Gill filament	Eiras et al. (2008)
<i>Henneguya friderici</i>	10.4 (9.6-11.8)	5.7 (4.8-6.6)	23.3 (19.1-28.7)	33.8 (28.7-39.3)	4.9 (4.2-5.9)	2.1 (1.5-2.6)	<i>Leporinus friderici</i>	Gills	Casal et al. (2003)
<i>Henneguya schizodon</i>	13.1 (12-14)	3.3 (3-4)	16.3 (15-17)	28.9 (27-30)	5.4 (5-6)	1.3 (1-1.5)	<i>Schizodon fasciatus</i>	Kidney	Eiras et al. (2004)
<i>Henneguya visibilis</i>	10.8 ± 0.6	3.9 ± 0.2	18 ± 1.2	26.8 ± 1.1	4.9 ± 0.3	1.4 ± 0.1	<i>Leporinus obusidens</i>	Connective tissue	Moreira et al. (2014b)
<i>Henneguya friderici</i>	12.8 ± 2.1 (7.4-14.8)	4.4 ± 0.4 (3.4-5.2)	19.6 ± 2.2 (16.1-24.4)	32.8 ± 2.6 (2.49-40)	5.1 ± 0.5 (3.7-5.9)	1.5 ± 0.1 (1.2-1.8)	<i>Leporinus friderici</i>	Gill filament	Present study

LS: length of the spore; WS: width of the tail; AL: length of the tail; TL: total length of the spore; PCL: polar capsules length; PCW: polar capsules width.

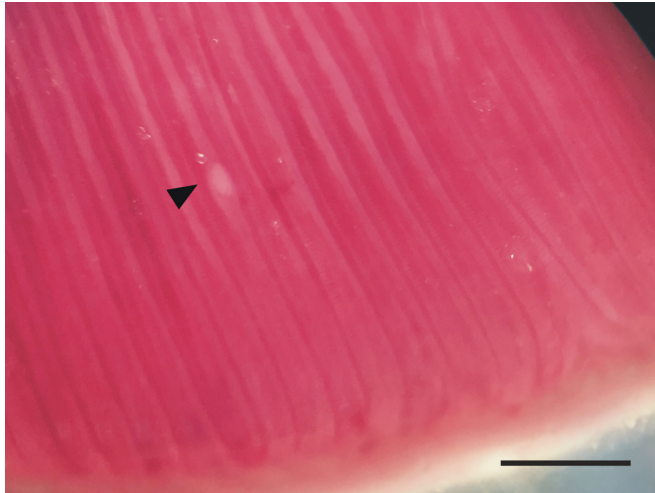


Figure 3. Plasmodia of *Henneguya friderici* infecting the gill filaments of *Leporinus friderici*. Scale bar = 10 mm.

analysis on this parasite. The 18S rDNA gene is used in molecular systematics for determining relationships among myxozoans because it is highly variable between very closely related species (KENT et al., 2001). The morphometric and morphological data obtained in the present study clearly confirmed the identification of the species as *H. friderici*, which was originally described by Casal et al. (2003) (Table 2).

Molnár (2002) divided the formation of gill-located myxosporean plasmodia into three types: (1) lamellar; (2) filamental; and (3) gill arch. Among these, the filamental type is subdivided into four types: (1) vascular; (2) epithelial; (3) intrachondral; and (4) basifilamental. In the present study, the *H. friderici* plasmodia developed on the filamental epithelium of the gills and deformed the gill filaments (Figure 3).

The prevalence of *H. friderici* in piau was 24%. This was close to the 30% reported by Casal et al. (2003), considering all the infected organs of *L. friderici*. However, in fish from the Mogi Guaçu River, infection was only observed in the gill filaments. Furthermore, these results corroborated data from other studies conducted in South America in which species of *Henneguya* were found at the same infection site (NALDONI et al., 2009, 2014).

These supplementary data on the morphology, 18S rDNA sequencing and phylogeny of *H. friderici* may facilitate accurate diagnoses and better understanding of the phylogenetic relationships of this parasite. Fiala (2006) indicated that host preference is very important and that myxosporean species could group together according to fish host species. Although host geographical origin is particularly important, tissue tropism in myxosporean evolution has also been revealed in numerous phylogenetic studies (ANDREE et al., 1999; KENT et al., 2001; ESZTERBAUER, 2004; FIALA, 2006).

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