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Henneguya correai n. sp. (Cnidaria, Myxozoa) Parasitizing the Fins of the Amazonian Fish *Semaprochilodus insignis* †

Maria I. Müller [1,*], Rayline T. A. Figueredo [2], Stephen D. Atkinson [3], Jerri L. Bartholomew [3] and Edson A. Adriano [1,2]

[1] Department of Ecology and Evolution, University of São Paulo (UNIFESP), Diadema 09972-270, Brazil
[2] Institute of Biology, State University of Campinas (UNICAMP), Campinas 13083-862, Brazil
[3] Department of Microbiology, Oregon State University (OSU), Corvallis, OR 97331, USA
* Correspondence: mariaisabel.muller@gmail.com
† urn:lsid:zoobank.org:pub:77381F9A-D67D-4137-AB4F-04763FA5E9CD.

Abstract: We used a combination of morphological, molecular and biological data to characterize a novel *Henneguya* (Myxozoa) species infecting the Amazonian prochilodontid *Semaprochilodus insignis* or "kissing prochilodus", a popular food fish and aquarium species in the Brazilian Amazon. Twenty-one *S. insignis* were caught live from the Tapajós river, Pará State, Brazil, then examined for myxozoan infections. Cysts of a novel *Henneguya* species were observed in the connective tissue of the fins. Myxospores measured 48 ± 4.9 (39.5–60.8) µm total length, of which caudal appendages were 33 ± 4.5 (26.4–45.2) µm and spore body was 15 ± 1.6 (12.4–20.5) µm. The spore body was 4.0 ± 0.6 (2.7–5.3) µm wide × 3.2 ± 0.4 (2.7–3.6) µm thick, with two unequal polar capsules (nematocysts) 7.2 ±0.8 (5.2–8.3) × 1.5 ± 0.3 (1.0–2.2) µm for the larger capsule and 5 ± 0.7 (4.0–6.3) × 1.4 ± 0.2 (1.0–1.8) µm for the smaller capsule. Polar tubules had 8–13 turns. Generative cells, immature and mature myxospores were observed within plasmodia. Ultrastructure showed plasmodia surrounded by collagen fibers, with the plasmodial membrane having pinocytotic channels. Phylogenetic analysis of small subunit ribosomal DNA sequences showed that the new *Henneguya* species clustered as a sister taxon to *Henneguya tietensis*, a parasite of the gills of the prochilodontid fish *Prochilodus lineatus*, from the geographically distant Paraná–Paraguai River basin.

Keywords: Prochilodontidae; cnidarian parasites; Myxozoa; freshwater fish; morphology; ultrastructure; phylogeny

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1. Introduction

The Tapajós River is one of the largest tributaries in the Brazilian Amazon, and its drainage has an enormous fish diversity encompassing more than 490 species, of which about 17% are endemic [1]. The Tapajós River drainage is ~489,000 km^2, being the fifth largest tributary of the Amazon basin [1]. Among the fish diversity in the Tapajós basin, Prochilodontidae are detritivorous freshwater fish popularly known as *curimbatá* and *jaraqui* in Portuguese, *bocachicos* in Spanish and flannel-mouth characiforms in English [2]. This fish family harbors three genera: *Ichthyoelephas* Posada, 1909, distributed from the rivers of Andes in Colombia and Ecuador; *Prochilodus* Agassiz in Spix and Agassiz, 1829, distributed along all major South American rivers on both sides of the Andes; and the *Semaprochilodus* Fowler, 1941, broadly distributed east of the Andes through the Amazon, Tocantins and Orinoco basins and some coastal rivers draining the Guiana shield [3]. *Semaprochilodus insignis* (Jardine, 1841) along *Semaprochilodus taeniurus* (Valenciennes, 1821) are important protein sources for people in the Central Amazon [4].

Myxosporeans are a diverse group of cnidarian endoparasites of aquatic animals (mostly) with more than 2600 described species [5]. The genus *Henneguya* Thélohan, 1892 harbors 254 species with worldwide distribution. They infect freshwater and marine

fishes, and some species can cause important diseases in their host fish [6–9]. In neotropical, freshwater habitats of South America, species of Myxobolidae are the most commonly encountered and described myxozoans [8–12].

Three myxozoans are known to infect *S. insignis*: *Myxobolus insignis* Eiras, Malta, Varella and Pavanelli, 2005; *Myxobolus maiai* Müller, Naldoni, Corrêa and Adriano, 2022; and *Myxobolus iarakiensis* Müller, Naldoni, Corrêa and Adriano, 2022, all of which sporulate in gills [13,14]. Herein, we characterize a novel *Henneguya* species, which sporulates in plasmodia in the fins of *S. insignis*. We describe the parasite using morphological and ultrastructural characters, and small subunit ribosomal DNA (ssrDNA) sequence and phylogenetic analysis.

2. Material and Methods

2.1. Sampling

Twenty-one *S. insignis* were sampled from the Tapajós River, in the municipality of Santarém, in the state of Pará, Brazil, in October 2021. The fish were caught using seine nets, and sampling was authorized by the Brazilian Ministry of the Environment (SISBIO # 66053-1 and SisGen (Como, Italy) # A656D8E). Fish were transported alive to a field laboratory on the shore of the river. The methodology was approved by the Ethics Research Committee of the Federal University of São Paulo (CEUA # 6549290920), in accordance with Brazilian law (Federal Law No. 11794, 8 October 2008). Fish were killed, measured and necropsied, and the organs were examined in fresh mounts under stereo- and compound microscopes. Tissue fragments up to 10 mm containing plasmodia were fixed in 10% neutral-buffered formalin for morphological and histological analysis [15], buffered 2.5% glutaraldehyde for ultrastructural analysis and in 100% ethanol for DNA analysis.

2.2. Morphological and Ultrastructural Analysis

For morphological analysis, 30 fixed mature myxospores were measured using a Carl Zeiss Axio Imager A2 light microscope equipped with an Axio Cam, and AxioVision AxioVs 40V4.8.2 software, following the guidelines of Lom and Arthur [15] and Sellyei et al. [16]. All measurements are expressed in µm as mean ± standard deviation (SD) and range in parentheses. For ultrastructural analysis, tissue fragments containing plasmodia were fixed in 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer (pH 7.4) for at least 12 h, then washed in the same buffer, post-fixed in OsO_4 for 1 h, and dehydrated in an acetone series. All of these processes were performed at 4 °C. The material was then embedded in EMbed 812 resin (Sigma-Aldrich, St. Louis, MO, USA). Semi-thin sections were stained with toluidine blue solution and examined using light microscopy. Ultrathin sections were double-stained with uranyl acetate and lead citrate, and examined in a LEO 906 electron microscope at 60 kV in the Electron Microscopy Laboratory of the University of Campinas (UNICAMP), São Paulo, Brazil.

2.3. DNA Extraction, PCR and Sequencing

Total genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, and adjusted to a final volume of 50 µL. For PCR, 10–50 ng of genomic DNA, 12.5 µL of 2× Dream Taq Green PCR Master Mix (Thermo Scientific—Carlsbad, CA, USA), 0.2 µM of each specific primer, and nuclease-free water (Thermo Scientific—Carlsbad, CA, USA) were used in a final reaction volume of 25 µL. ssrDNA was amplified, using the primer pairs and conditions described by Capodifoglio et al. [17], in which ssrDNA was amplified in two parts: ~1000 bp using ERIB1 [18] and ACT1r [19]; and ~1200 bp using primers TEDf [17] and ERIB10 [18]. Identity of the fish host was confirmed by PCR and sequencing of the mitochondrial COI gene using published methods [20].

PCR products were run on a 1.5% agarose gel with TBE buffer (0.045 M Tris-borate, 0.001 M EDTA pH 8.0), stained with Sybr Safe DNA gel stain (Thermo Scientific—Carlsbad, CA, USA) and analyzed in a MiniBis Pro transilluminator. The sizes of the amplified

fragments were estimated by comparison with 1 kb Plus DNA Ladder (Thermo Scientific—Carlsbad, CA, USA). Amplicons were purified using the QIAquick PCR Purification kit (Qiagen) and sequenced in both directions with PCR primers using the BigDye v.3.1 Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA). Sequences were read with a Life Technologies—Applied Biosystems ABI 3730 DNA genetic analyzer, and were assembled and edited to obtain a single consensus sequence for each specimen using Sequencer v.5.2.4 (Gene Codes, Ann Arbor, MI, USA), then submitted to GenBank.

2.4. Sequence Alignment and Phylogenetic Analyses

Alignment was carried out using ssrDNA sequences of myxozoans (*Myxobolus/Henneguya/Thelohanellus* sequences) that were at least 80% similar to the novel *Henneguya* sequence, plus myxobolid parasites of South American Prochilodontidae. The outgroups chosen were *Myxidium peruviensis* Espinoza, Mertins, Gama, Pata and Mathews, 2014 (KY996746) and *Myxidium turturibus* Aguiar, Adriano and Mathews, 2017 (KX611144). Sequences were aligned using the default parameters of the Muscle algorithm (Edgar, 2004) performed in Geneious 7.1.3 [21]. To evaluate substitution saturation, the aligned matrix was tested and the Iss index estimated using DAMBE 5 [22].

The best-fit model of nucleotide evolution in the resulting matrixes was determined to be GTR + G + I using the Akaike information criterion in jModelTest [23]. Phylogenetic analyses were performed using Bayesian Inference (BI) and Maximum Likelihood inference (ML) using resources available in the CIPRES Science Gateway [24]. Bayesian analysis employed the following nucleotide substitution model settings for both datasets: lset nst = 6, rates = invariable, ncat = 4, shape = estimate, inferrates = yes and basefreq = empirical. For the Markov chain Monte Carlo (MCMC) search, chains were run with 10,000,000 generations, saving one tree every 1500 generations. The first 25% of generations were discarded as burn-in, and the consensus tree (majority rule) was estimated using the remaining topologies; only nodes with posterior probabilities >90% were considered well supported. Maximum Likelihood inference (ML) was implemented using RAxML [25], with bootstrap support values of 1000 repetitions, and only nodes with bootstrap values > 70% were considered well supported. The trees were visualized using FigTree v.1.3.1 [26].

3. Results

Of the 21 *S. insignis* adult specimens examined (average length 20.4 ± 1.8 (16–23) cm), 9 (43%) had plasmodia of a *Henneguya* species in the fins; this parasite is described herein. We also observed plasmodia and myxospores of a *Myxobolus* species (six fish were infected; 28%) in the fins; however, insufficient material was available to make a formal description at this time.

Description: *Henneguya correai* n. sp. (Figures 1A,B and 2A,B).

Formalin-fixed, mature myxospores (n = 30) in frontal view measured 47.9 ± 4.9 (39.5–60.8) µm total length, 14.4 ± 1.6 (12.4–20.5) µm spore body length, 4.0 ± 0.6 (2.7–5.3) µm wide and 33.7 ± 4.5 (26.4–45.2) caudal appendages length. In lateral view, the valves were asymmetrical and myxospores were 3.1 ± 0.4 (2.7–3.6) (n = 4) µm thick. Polar capsules (nematocysts) were unequal: the larger was 7.2 ± 0.8 (5.2–8.3) µm long and 1.5 ± 0.3 (1.0–2.2) µm wide and contained a polar tubule with 11–13 turns; the smaller capsule was 5.6 ± 0.7 (4.0–6.3) µm long, 1.4 ± 0.2 (1.0–1.8) µm wide and contained a tubule with 8–9 turns. (Table 1, Figure 1A,B).

Host type: *Semaprochilodus insignis* (Jardine, 1841), Characiformes: Prochilodontidae (GenBank accession number OK413187, Müller et al. [14]).

Locality: Tapajós River, municipality of Santarém, Pará state, Brazil (2°26′22.53″ S; 54°53′34.72″ W).

Type material: Syntypes-air-dried, stained with Giemsa solution and mounted in medium on permanent slides (accession number ZUEC MYX 116).

Prevalence: 9/21 (43%).

Site of Infection: Connective tissue in fins.

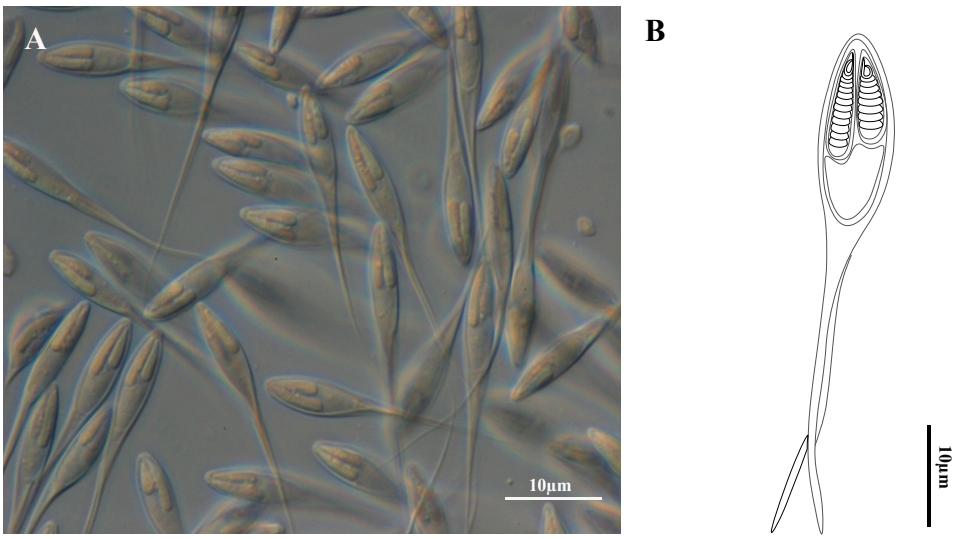

Figure 1. *Henneguya correai* n. sp. parasite of the fins of *Semaprochilodus insignis*. (**A**) Photomicrograph of mature myxospores (frontal view) under Differential Interference Contrast. (**B**) Schematic drawing of a myxospore in frontal view.

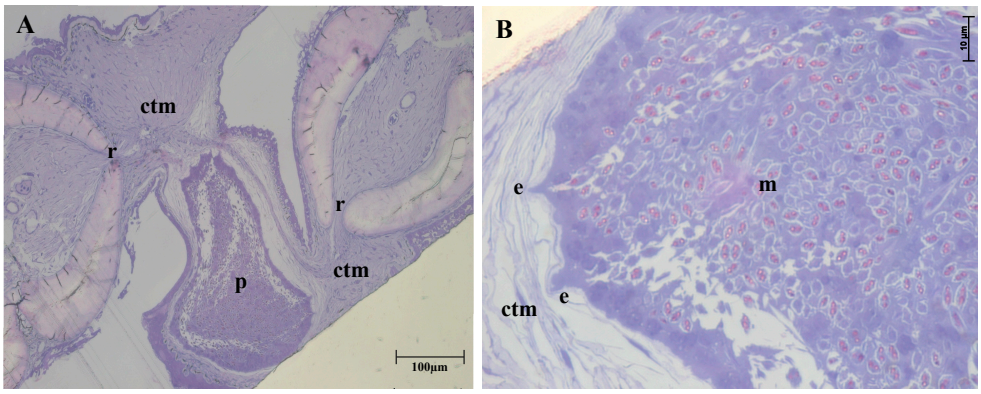

Figure 2. Histological thin section of fin of *Semaprochilodus insignis* with plasmodium of *Henneguya correai* n. sp. (**A**): Parasite plasmodium (p) in host connective tissue (ctm) between the rays (r). (**B**): Enlarged portion of A showing host–parasite boundary plasmodial expansions (e) toward the layered host connective tissue (ctm) and maturing myxospores within (m). Toluidine blue stain.

Representative ssrDNA sequence: GenBank accession number OR036989.

Etymology: The specific epithet is in honor of Prof. Dr. Lincoln Lima Corrêa for his important support and contribution to myxozoan parasite research in the Amazon.

Histology of semi-thin sections (Figure 2) showed parasite plasmodia developing in the connective tissue of fins, between host fin rays. The plasmodia had pseudopodia extending into layered host tissue (Figure 2B) without compromising the adjacent epithelial layer. Ultrastructural analysis revealed that plasmodia of *H. correai* n. sp. had numerous pinocytotic channels in the plasmodial membrane linking the outside to the ectoplasm zone of the plasmodium, while generative cells, immature and mature myxospores were observed deeper in the plasmodium (Figure 3A–C).

Sequencing of *H. correai* n. sp. ssrDNA yielded 1763 bp, and the BLASTn search of the NCBI database showed the closest sequence was *Henneguya tietensis* Vieira, Rangel, Tagliavini, Abdallah, Santos and Azevedo, 2020 (MN653131) with 88.2% similarity [12]. ML and BI phylogenetic analysis based on 46 taxa over 950 bp revealed similar topologies of South American myxobolids, recovering two primary clades well supported by posterior probability (PP) and bootstrap (B) (Figure 4). In the clade A, which comprised *Myxobolus*

and *Henneguya* species from several fish families, the new species appears as a sister species of *H. tietensis*. Clade B comprises *Myxobolus* species parasites from different fish families and *Thelohanellus marginatus* Rocha, Casal, Velasco, Alves, Matos, Al-Quraishy and Azevedo, 2014, a parasite of a pimelodid (Figure 4) [27].

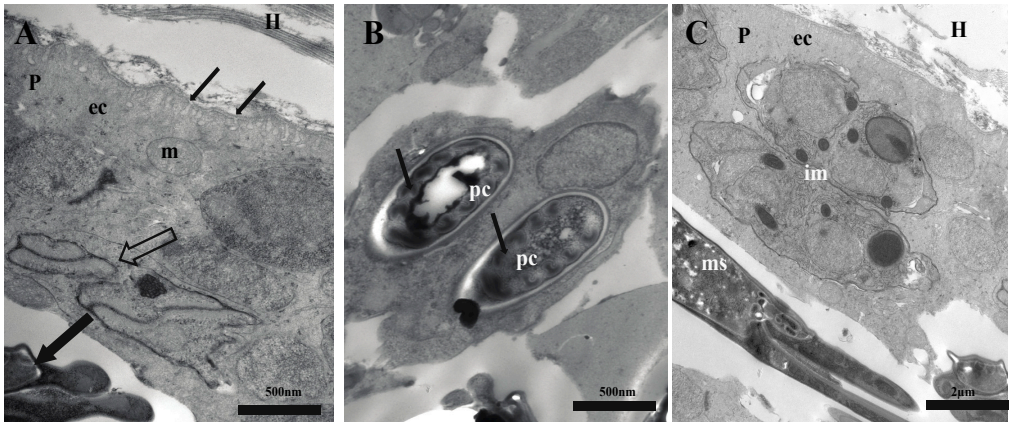

Figure 3. Electron micrographs of sections of *Henneguya correai* n. sp. in fins of *Semaprochilodus insignis*. (**A**) Host–parasite interface showing the plasmodium (p) developing in host (H) connective tissue; large empty arrow indicates immature myxospores, and large black arrow shows mature myxospore. The parasite ectoplasm (ec) has pinocytic channels, thin black arrows, with a mitochondrion (m); (**B**) mature myxospore with polar capsules (pc) and its coiled polar tubule (thin black arrows); (**C**) plasmodium showing longitudinal sections of immature myxospores (im) in the periphery and mature myxospores (ms) within.

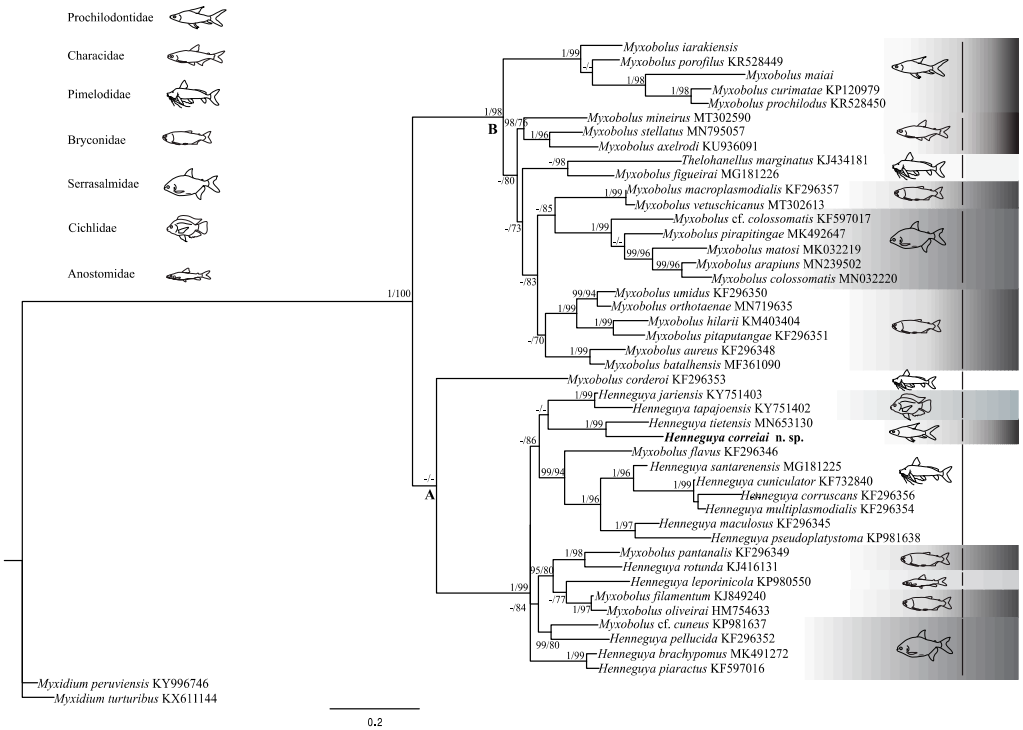

Figure 4. Maximum Likelihood tree based on ssrDNA sequences of myxobolids most closely related to *Henneguya correai* n. sp. and selected myxobolids from South America. GenBank accession numbers are given after species names. Nodal support values are shown for posterior probability (PP) and bootstrap (B). Values for weakly supported nodes (<0.9 PP and <70 B) are not shown. Gray scale rectangles differentiate fish families and their schematic fish figures.

Table 1. Morphometric comparison of *Henneguya correai n.* sp. with other species of *Henneguya* infecting Prochilodontidae.

Species	TL	BL	CA	BW	PCL	PCW	T	PT	Host	Site of Infection	Locality	Reference
Henneguya correiai sp. *nov.*	47.9 ± 4.9 (39.5–60.8)	14.4 ± 1.6 (12.4–20.5)	33.7 ± 4.5 (26.4–45.2)	4.0 ± 0.59 (2.71–5.3)	7.2 ± 0.8 (5.2–8.3)	1.5 ± 0.3 (1.0–2.2)	3.15 ± 0.4 (2.67–3.55)	8–13	*Semaprochilodus insignis*	Fins	Tapajós river, Amazon basin, PA, Brazil	This study
Henneguya tietensis	55.5 ± 2.1 (53.5–57.0)	16.2 ± 1.1 (15.0–16.9)	39.0 ± 2.0 (35.2–39.7)	5.5 ± 0.1 (5.4–5.6)	7.3 ± 0.2 (7.1–7.5)	1.7 ± 0.2 (1.3–1.7)	3.8 ± 0.3 (3.7–4.2)	11–13	*Prochilodus lineatus*	Gills	Paraná river basin, Brazil	[12]
Henneguya caudalongula	71.0 ± 1.4	16.6 ± 0.5	52.6 ± 1.5	4.6 ± 0.2	6.1 ± 0.2	1.6 ± 0.2	_	10–11	*Prochilodus lineatus*	Gills	Pirassununga, SP, Brazil	[28]
Henneguya paranaensis	33.3 ± 1.5	11.4 ± 0.3	24.1 ± 1.5	4.1 ± 0.4	8.4	2.0	_	10–12	*Prochilodus lineatus*	Gills	Paraná river basin, Brazil	[29]

TL—total length, BL—body length, CA—caudal appendages length, BW—body width, PCL—polar capsule length, PCW—polar capsule width, T—thickness, PT—polar tubule turns. All measurements are given as mean ± standard deviation in μm.

4. Discussions

Semaprochilodus insignis is an ecologically and economically important fish in the Amazon region, being harvested as a food source and traded as a tropical aquarium fish [4,30]. Juveniles provide around 45% of biomass consumed by piscivores cichlids [31]. Thus, the value of this species has stimulated research into its parasite fauna, and their potential disease impacts, with recent work identifying three novel myxosporean species: *M. insignis*; *M. maiai*; and *M. iarakiensis* [1,14].

Herein, we describe another novel myxozoan parasite of *S. insignis*, *Henneguya correai* n. sp. This taxon could be distinguished from all other described species on the basis of morphological and morphometric characters and ssrDNA sequence. Ultrastructural analysis revealed pinocytic channels at the host–parasite interface (Figure 3), which are common among Myxobolidae and considered to be related to nutrition and sporogonic development [10,11,19,28,32–35].

Comparison of parasite myxospores with other freshwater myxobolids [6,13,36,37], showed that *H. correai* n. sp. was morphologically and genetically most closely related to *H. tietensis*, a parasite of *Prochilodus lineatus* (Valenciennes, 1837), another South American prochilondontid. Target tissue differs between these species, with *H. correai* n. sp. sporulating in the fins, while *H. tietensis* matures in gills. Morphometric differences between the taxa include: different size polar capsules (nematocysts)—*H. correai* n. sp. has unequal capsules versus equal capsules in *H. tietensis*; smaller total length (47.9 µm for *H. correai* n. sp. versus 55.5 µm for *H. tietensis*), caudal appendages length (33.7 vs. 39.0 µm), spore body width (4.0 vs. 5.5 µm) and occasionally fewer polar tubule coils (8–13 vs. 11–13) (Table 1).

In comparison with other *Henneguya* species that parasitize prochilodontids, the novel species shares some morphological features with *H. caudalonga* Adriano, Arana and Cordeiro, 2005, and *H. paranaensis* Eiras, Pavanelli and Takemoto, 2004, such as body width and polar capsule width [28,29]. However, the new species differed morphometrically from *H. caudalonga* in multiple features, such as total length (47.9 vs. 71.0 µm), polar capsule length (7.2 vs. 6.1 µm) and polar tubule turns (8–13 vs. 10–11) (Table 1). Considering *H. paranaensis*, morphometric differences included total length (47.9 µm for *H. correai* n. sp. versus 33.3 µm for *H. paranaensis*), spore body length (14.4 vs. 11.4 µm), capsule length (7.2 vs. 8.4 µm) and number of polar tubule turns (8–13 vs. 10–12) (Table 1). Comparisons of *H. correai* n. sp. with species parasitizing non-prochilodontid hosts showed differences in at least one of the following characters: shape and/or size of the plasmodia and myxospores, number of polar tubule turns, host, site of infection or genetic differences [6,38].

Myxospore morphology, fish host family and tissue tropism often correlate strongly with ssrDNA phylogenetic patterns in Myxozoa [12,39–42]. The Myxobolidae, however, have many exceptions to these correlations, particularly in the case of myxospore morphology, where molecular data generally do not support the morphological distinctions of the genera *Myxobolus* and *Henneguya* based on the presence of caudal processes [40–44], or distinction of *Myxobolus* and *Thelohanellus* on the basis of polar capsule number [45]. Our phylogenetic analysis of South American Myxobolidae revealed clades with differing morphological heterogeneity: while clade A is a mixture of *Henneguya* and *Myxobolus* species, therein illustrating the polyphyletic nature of the group, Clade B consists of almost exclusively *Myxobolus* species, with one *Thelohanellus* species (Figure 4). This pattern of myxobolid sub-clades and lineages being morphologically similar has been observed before [41,45], and shows that some lineages can be exclusively one morphotype, while others have morphological plasticity (e.g., both with and without caudal projections—a mix of *Myxobolus* and *Henneguya* types). A deeper understanding of correlations between myxospore morphotypes and phylogeny might only be possible when the target alternate host annelids are known, to better characterize the evolutionary pressures driving myxospore morphology.

Regarding those myxobolid species that parasitize prochilodontid fish, our analysis showed clustering based on myxospore morphology. The *Myxobolus* species *M. iarakiensis*, *M. porofilus* Adriano, Arana, Ceccarelli and Cordeiro, 2002, *M. maiai*, *M. curimatae* Zatti,

Naldoni, Silva, Maia and Adriano, 2015 and *M. prochilodus* Azevedo, Vieira, Vieira, Silva, Matos and Abdallah, 2014 comprise a lineage in clade B, while the two *Henneguya* parasites *Henneguya correai* n. sp. and *H. tietensis* cluster together in clade A [14,34,45,46]. These data reveal that despite overall polyphyly of myxobolid parasites of prochilodontids, there exist monophyletic sub-groups of the *Henneguya* and *Myxobolus* species. Furthermore, our analysis showed *H. correai* n. sp. clustered as a sister taxon to *H. tietensis* from *Prochilodus lineatus*, another prochilodontid fish from a sister genus to *Semaprochilodus* [3]. Despite being sister taxa that share a host family, these parasites have different tissue tropism: respectively, fins and gills. Thus, these two parasites lie within a broader clade of *Henneguya* species from gills and fins, but from cichlids and pimelodids. This illustrates that different host/parasite characters correlate with tree topology at different levels.

The Prochilodontidae are a characiform family comprising detritivorous migratory freshwater fishes that occur throughout South America [3]. *Prochilodus lineatus* occurs in the second largest South American watershed, the La Plata basin. It is hypothesized that in the geologic past there was connection between the Amazon and La Plata basins, either during marine incursions in the Miocene period, or as a result of episodic headwater connections between their upper tributaries during the Miocene and Pliocene [47–50]. We therefore postulate that the common ancestor of *H. correai* n. sp. and *H. tietensis* infected an ancestral prochilodontid in this connected system, with subsequent geographic separation driving speciation of host and parasite. Thus, it would be interesting in the future to collect more samples of *Henneguya* spp. in other rivers/basins and from other prochilondontids.

In summary, here we provide a taxonomic description of a novel *Henneguya* species from an economically and ecologically important prochilodontid fish from the Brazilian Amazon. Although no apparent disease was caused by *Henneguya correiai* n. sp., given that *S. insignis* is important in the ornamental fish trade, there is potential that if the parasite were to become established under aquarium conditions that this could impact fish health; however, knowledge of the parasite's life cycle is needed to better assess these risk factors.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional of Ethics Research Committee of the Federal University of São Paulo (CEUA # 6549290920—17/12/2020).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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