Taxonomy and 18S rDNA-based phylogeny of *Henneguya multiradiatus* n. sp. (Cnidaria: Myxobolidae) a parasite of *Brochis multiradiatus* from Peruvian Amazon

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ABSTRACT

A new myxozoan species belonging to the genus *Henneguya* was isolated from the serous membrane of the visceral cavity of the hognosed catfish *Brochis multiradiatus* from Peruvian Amazon. Whitish plasmodia, macroscopically visible, were found in four of the thirty examined fishes. Mature myxospores were ellipsoidal in shape in frontal view and had a total length of 44.5 ± 0.6 μm (43.9–45.1), spore body measured 18.7 ± 0.9 μm (16.8–19.6) in length, 7.1 ± 0.2 μm (6.6–7.4) in width and 5.5 ± 0.3 μm (4.9–5.6) in thickness. The two polar capsules were elongated and equal in size, measuring 9.1 ± 0.1 μm (8.8–9.4) in length and 1.7 ± 0.1 μm (1.6–1.8) in width, occupying half of the myxospore body. Polar tubules coiled in 10 to 11 turns perpendicular to the long axis of the polar capsule. The caudal appendage was not bifurcated and measured 25.8 ± 0.6 μm (24.7–26.5) in length. The sequencing of the 18S rDNA gene resulted in 1400 bp and this sequence did not match any of the myxozoans available in GenBank. Phylogenetic analysis placed the new species in a well-supported subclade of *Henneguya* spp. infecting callichthyid fishes, with *Henneguya loretoensis* being the closest species. This study is the first description of a myxozoan species, *Henneguya multiradiatus* n. sp. from a fish of the genus *Brochis*.

Keywords: Cnidaria, Endocnidozoa, Myxosporean, Hognosed catfish, 18S rDNA sequencing, Peru

1. Introduction

Myxozoans are microscopic endoparasites with complex life cycles involving invertebrate and vertebrate hosts [1]. Recently, due to synapomorphies and cnidarian-specific genes, myxozoans were placed within the phylum Cnidaria and currently represent about 20% of the known cnidarian species diversity around the world [1]. While

predominantly known to infect freshwater and marine fishes, myxozoans can also infect other groups of vertebrates such as amphibians, reptiles, birds, and terrestrial small mammals [2]. Within fish-infecting myxozoans, *Henneguya* Thélohan, 1892, is one of the largest genera, with more than 195 described species within a wide geographical range [3]. *Henneguya* spp. are histozoic parasites and they show high host-specificity and organ- or tissue tropism, with some species inducing severe henneguyosis in wild and cultured fish [4–7].

It is well known that the growing global pet trade, including aquarium market, plays a role in the spread of pathogens [8]. According to Hallett et al. [9], the aquatic pet trade would very likely stand as a primary mode for international transport of myxozoan parasites. In fact, there are some reports about the introduction of myxosporeans through commercial transfers of pets with some cases of ecological and economic impacts [10–12]. As such, there is a growing need for myxozoans screening during parasitological surveys in these aquatic pet animals.

The armored catfish corydoradines are members of the family Callichthyidae. With over 190 described species, it is one of the most diverse siluriform assemblages in the Neotropics [13]. These neotropical freshwater catfishes are endemic to South America [13]. Among these corydoradines, *Brochis multiradiatus* Orcés V., 1960 is found in the Napo River in Ecuador and Peru [14]. It can reach up to 6.7 cm in length and is largely marketed in the aquarium trade, being one of the most popular in the Chinese ornamental fish market [15]. Although corydoradines are highly appreciated in the international aquarium trade, there are few parasitological surveys about this fish, especially those concerning myxozoan parasites.

To our knowledge, nothing is known about myxozoan parasites infecting any member of the genus *Brochis*. Herein, based on morphological, biological/ecological traits and

molecular data, we described a new *Henneguya* species infecting the ornamental fish *B. multiradiatus* from the Amazon region of Peru, a key supply region of wild ornamental fishes for the international aquarium trade.

2. Material and methods

In September 2019, thirty wild specimens of *B. multiradiatus* were collected by a local fisherman. The fishes were caught in the Napo River, in an adjacent area of the village Cabo Pantoja (0°57′25″S, 75°27′11″W), Department of Loreto, Peru. The fish were transported alive to the field laboratory where they were euthanized by neural pithing and examined. The procedures were approved by the Federal University of São Paulo-UNIFESP Ethics Committee (CEUA No. 9209080214), in accordance with Brazilian law for scientific use of animals (Federal Law No. 11794, dated 8 October 2008). The family and current status of the host fish (valid species name or synonym) were reviewed using FishBase [14].

Morphometric analyses were performed on mature myxospores following the criteria outlined by Lom and Arthur [16]. Measurements and photographs were taken from 30 myxospores using a computer equipped with Axiovision 4.1 image capture software coupled to an Axioplan 2 Zeiss microscope (Carl Zeiss AG, Oberkochen, Germany). The dimensions of the myxospores were given in micrometers (μm) and expressed as the mean ± standard deviation, followed by the range in parentheses. Prevalence of infection [(number of infected fish/total number of fishes examined) ×100] was calculated according to Bush et al. [17]. Smears containing free myxospores were air-dried, fixed with methanol and stained with Ziehl-Neelsen to mount on permanent slides that were deposited in the cnidarian collection of the zoology Museum of the University of São Paulo - USP, São Paulo, Brazil (MZUSP).

For molecular studies, a plasmodium was dissected from the host tissue and fixed in absolute ethanol. The genomic DNA was extracted from a single plasmodium using DNeasy® Blood & Tissue Kit (Qiagen, Valencia, USA), in accordance with the manufacturer's instructions. The DNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA). Polymerase chain reactions (PCRs) were performed in accordance with Milanin et al. [18] and conducted in a final volume reaction of 25 μL, which comprised 10–50 ng of extracted DNA, 0.2 pmol for each primer, 12.5 μL of Dream Taq Green PCR Master Mix (Thermo Scientific) and nuclease-free water. Partial 18S rDNA sequence was amplified using the primer pairs ERIB1 [19] - ACT1r [20] and Myxgen4F [21] - ERIB10 [19] (Table 1), which amplified two fragments containing approximately 1.000 and 1.200 bp respectively. PCR amplification was done by initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, 64 °C (ERIB1-ACT1r) or 58 °C (Myxgen4F-ERIB10) for 1 min, 72 °C for 2 min, and then final elongation at 72 °C for 5 min. PCRs were performed in an AG22331 Hamburg Thermocycler (Eppendorf, Hamburg, Germany). PCR products were subjected to electrophoresis in 2.0% agarose gel (BioAmerica, Irvine, USA) 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, Carlsbad, USA), and then analyzed with a Stratagene 2020E trans illuminator (Stratagene California, San Diego, USA). The size of the amplicons was estimated by comparison with the 1 Kb Plus DNA Ladder (Invitrogen by Life Technologies). PCR products were purified using USB® ExoSap-IT® (Thermo Fisher Scientific) in accordance to the manufacturer's instructions and sequenced using the same PCR primers plus two additionally MC5 and MC3 primers used to overlap the obtained amplification fragments [22]. Sequencing was performed at Paulista Medical School, Federal University of São Paulo, using a BigDye® Terminator

v3.1 Cycle Sequencing kit (Applied Biosystems Inc., California, USA) in an ABI 3130 automatic DNA analyzer (Applied Biosystems Inc.™). The obtained sequence was visualized, assembled and edited using BioEdit 7.1.3.0 software [23]. A standard nucleotide BLAST (blastn) search was conducted to verify the similarity of the sequence obtained in this study with other sequences available in GenBank [24]. Phylogenetic analysis was conducted using the most closely related myxozoans sequences with similarity > 80%. The sequences were aligned with ClustalW within BioEdit version 7.1.3.0 [23]. Phylogenetic analysis was performed using Maximum Likelihood (ML) method with a Kimura 2-parameter (K2P) evolution sequence model in MEGA 6.0 [25]. Bootstrap analysis (1000 replicates) was employed to assess the relative robustness of the branches in ML tree. *Ceratonova shasta* sequence was used as outgroup. The pairwise method with the p-distance model in MEGA 6.0 [25] was performed to evaluate the genetic distance between the myxozoan species clustering together with the new sequence obtained.

3. Results

Whitish plasmodia macroscopically visible measuring 2.1 to 3 mm in diameter and containing large quantities of myxospores belonging to the genus *Henneguya* were found in the serous membrane of the visceral cavity of wild specimens of *B. multiradiatus* (Fig. 1A). The prevalence of the myxozoan parasites in these fish hosts was 13.3% (4/30) and no signs of diseases were observed in the infected individuals.

Henneguya multiradiatus n. sp.

Taxonomic summary

Phylum: Cnidaria Verrill, 1865

Class: Myxosporea Bütschli, 1881

Order: Bivalvulida Shulman, 1959

Family: Myxobolidae Thélohan, 1892

Genus: *Henneguya* Thélohan, 1892

Type host: *Brochis multiradiatus* (Orcés V., 1960) (Siluriformes: Callichthyidae)

Site of infection: Serous membrane of the visceral cavity

Type locality: Napo River, adjacent area of the village Cabo Pantoja (0°57′25″S, 75°27′11″W), Department of Loreto, Peru.

Prevalence: 13.3 % (4/30).

Type of material: Syntypes-air-dried slide stained with Ziehl-Neelsen deposited in the cnidarian collection of Zoology Museum of the University of São Paulo - USP, São Paulo, Brazil (MZUSP 8508). Partial 18S rDNA sequence (1400bp) was deposited in GenBank under accession number MT415832.

Etymology: The specific name (*H. multiradiatus*) is based on host species name.

Morphological characterization

Mature myxospores were ellipsoidal in shape from the frontal view, measuring 44.5 ± 0.6 μm (43.9–45.1) in total length, 18.7 ± 0.9 μm (16.8–19.6) in spore body length, 7.1 ± 0.2 μm (6.6–7.4) in width and 5.5 ± 0.3 μm (4.9–5.6, n=5) in thickness (Figs. 1B and 2). Two elongated polar capsules, equal in size, measuring 9.1 ± 0.1 μm (8.8–9.4) in length and 1.7 ± 0.1 μm (1.6–1.8) in width, occupying half of myxospore body (Figs. 1B-C and 2). Polar tubules had 10 to 11 coils, perpendicular to long axis of polar capsule (Figs. 1C and 2). Non-bifurcate caudal appendage, measuring 25.8 ± 0.6 μm (24.7–26.5) in length (Figs. 1B and 2).

168 **Molecular characterization and phylogenetic analysis**

169 Partial 18S rDNA sequence obtained from mature myxospores of *H. multiradiatus* n. sp.
170 resulted in 1400 bp with a CG content of 49.4% and did not match any other myxozoans
171 available in GenBank. The comparison of the new sequence obtained by BLAST revealed
172 the highest sequence similarity to *Henneguya loretoensis* Mathews, Naldoni and Adriano
173 2017 (GenBank accession number MF434827.1, query coverage 99%, maximum
174 identities 98.2%). The phylogenetic analysis placed *H. multiradiatus* n. sp. in a well-
175 supported subclade of *Henneguya* parasites of callichthyids fish, including *Henneguya*
176 *loretoensis*, and *Henneguya guanduensis* Abdallah, Azevedo, Luque and Bomfim 2007
177 (Fig. 3). This subclade, was close to a lineage formed by *Henneguya* species described
178 from Amazonian cichlids. 18S rDNA pairwise comparisons between *H. multiradiatus* n.
179 sp. described and the closest relatives resulted in 1.8% to *H. loretoensis*, 5.3% to *H.*
180 *guanduensis*, 6.1% to *Henneguya peruvien*sis Mathews, Mertins, Pereira, Maia and
181 Adriano 2018, 7.6% to *Hennguya jariensis*, 9.5% to *Henneguya tapajoensis*, 7.2% to
182 *Henneguya tucunarei* Zatti, Atkinson, Maia, Bartholomew and Adriano 2018 and 8.6%
183 to *Henneguya paraensis* Velasco, Videira, Nascimento, Matos, Gonçalves and Matos
184 2016.

185 **4. Discussion**

186 The Peruvian Amazon is the second largest portion of the Amazon rainforest
187 concentrating a high aquatic biodiversity, with several commercially exploited fish
188 species for human consumption and global aquarium trade [26,27]. However, little is
189 known about the diversity of myxozoans in this region, with only three species described
190 to date [28–30]. In contrast, over 40 myxozoan species have been described in the
191 Brazilian Amazon region [31–33], indicating that there are likely many more myxozoan
192 parasites to be identified in Peruvian Amazon, considering the recognized high diversity.

193 This study reports, for the first time, a myxozoan species infecting a fish from the genus
194 *Brochis* in the Amazon basin.

195 For robust identification and description of new myxosporeans taxa, it has been
196 recommended, that the studies integrate multiple characters, including morphology,
197 biological traits, host ecology factors and molecular data [34]. Following these delineated
198 criteria for classifying myxozoans, we described a novel *Henneguya* species, *Henneguya*
199 *multiradiatus* n. sp. from an important Amazonian ornamental fish. In the morphological
200 comparison, considering the twenty-two *Henneguya* species previously described to
201 infect fishes from Amazon basin, the most morphologically similar species to the new
202 species were *Henneguya torpedo* Azevedo, Casal, Matos, Alves and Matos, 2011 and
203 *Henneguya testicularis* Azevedo, Corral and Matos, 1997. Nevertheless, these differ from
204 *H. multiradiatus* n. sp. in myxospore body length (28.5 ± 0.3 μm in length for *H. torpedo*,
205 14.0 μm for *H. testicularis* and 18.7 ± 0.9 μm to the new species), number of coils of the
206 polar tubule (12 to 13 in *H. torpedo*, six to seven in *H. testicularis* and ten in the new
207 species) and in the length of the caudal appendage, which is substantially larger in the
208 new species (19.6 ± 0.4 μm in *H. torpedo*, 13.5 μm in *H. testicularis*, and 25.8 ± 0.6 μm
209 in *H. multiradiatus* n. sp.). Plasmodia of *H. multiradiatus* n. sp. were located in the serous
210 membrane of the visceral cavity, while *H. torpedo* and *H. testicularis* were described
211 infecting brain, spinal cord, and testis respectively [35,36]. On the same premise, host
212 species and locality of collection are indispensable traits for accurately distinguishing
213 new histozoic platysporines species, since host-specificity and geography may play an
214 important role in speciation [37]. Accordingly, differences were observed in the infected
215 host, *e.g.*, *H. multiradiatus* n. sp. infecting *B. multiradiatus*, a Siluriform fish; *H. torpedo*
216 infecting *Brachyhypopomus pinnicaudatus* (Hopkins 1991), a Gymnotiform fish; and *H.*
217 *testicularis* infecting *Moenkhausia oligolepis* (Günther 1864), a Characiform fish.

Besides, differences in the locality of these species were noted, with *H. multiradiatus* n. sp. found in the Napo River, a tributary of the upper Amazon River, near the village of Cabo Pantoja in Department of Loreto, Peru, and *H. torpedo* and *H. testicularis* found in the lower Amazon River, both near Belém city in Pará state, Brazil. In addition to the large geographic distance between these species (2, 965 km in straight line), the nonmigratory behavior of their hosts is an important ecological character for the separation of these species, taking in account that host behavior may drive both parasite endemism and the radiation within the Amazon basin [32]. Unfortunately, there is no molecular data available for *H. torpedo* and *H. testicularis* in GenBank, so it is not possible to compare them with *H. multiradiatus* n. sp. However, differences observed in the integrative comparison, including morphology, geography, biological traits in conjunction with the ecological characters of their hosts, are sufficient arguments to conclude that *H. multiradiatus* n. sp. is a separate species.

In our phylogenetic analysis, *Henneguya* spp. showed tendency to cluster, largely based on vertebrate host fish family, and this corroborates the results pointed out by other authors who evidenced that host group is a strong evolutionary signal within the Myxobolidae [32,38,39]. *Henneguya multiradiatus* n. sp. was placed in a well-supported subclade of parasites of callichthyids fishes, with *H. loretoensis* as the closet related species (Fig. 3). This close phylogenetic relationship was corroborated by the pairwise analysis, which showed a slight genetic divergence of 1.8% on their 18S rDNA sequences. Nevertheless, there is no exact value for determining the level of genetic variation in 18S rDNA that equates to species differentiation within this enigmatic group of parasites [38,40]. In this context, species differentiation should be assessed for each individual case and always with the aid of biology and/or ecology traits of the organisms, such as their morphology, tissue and/or organ tropism, host species, geography, and host

ecological aspects such as endemic character, migratory behavior and distribution [16,40]. In our study, remarkable morphometrical differences can be observed between these two corydoradines *Henneguya* parasites, with myxospores substantially larger in total length and width in the newly identified species (44.5 ± 0.6 μm × 7.1 ± 0.2 μm *vs* 36.2 μm × 5.1 μm for *H. loretoensis*), larger body length (18.7 ± 0.9 μm *vs* 14.3 ± 0.1 μm for *H. loretoensis*), longer polar capsules (9.1 ± 0.1 μm *vs* 5.1 ± 0.1 μm in *H. loretoensis*), higher number of coils in the polar tubule (ten to eleven coils *vs* five coils in *H. loretoensis*) and the caudal appendage which is substantially longer (25.8 ± 0.6 μm *vs* 21.9 ± 0.1 in *H. loretoensis*). Furthermore, *H. multiradiatus* n. sp. the caudal appendage is not bifurcated, as occurs in *H. loretoensis*. Differences can also be outlined concerning the host-, tissue- and organ infected, with the new species proposed found in the serosa layer of the visceral cavity of *B. multiradiatus* n. sp. whereas *H. loretoensis* is found in gill filaments of *Corydoras leucomelas* Eigenmann and Allen, 1942, a host of a different genus. While these two corydoradines parasites inhabit the Amazon biome of Peru (*H. multiradiatus* n. sp. in Napo River and *H. loretoensis* Nanay River), they are separated by 381 km from each other. Another important point to consider in establishing *H. multiradiatus* n. sp. as a new species, is in regards to the endemic character and the absence of migratory reproduction, characteristics which are highly recognized to corydoradines fish [33].

In the present study, an integrative taxonomy approach was employed in the description of an unknown myxosporean. Given that these criteria are compelling evidence for the characterization of new species [16,40], we confidently considered that this isolate is a new species, *H. multiradiatus* n. sp. Hence, this new data contributes to increase the knowledge of the myxosporean diversity from the Amazon biome, as well to clarify the

267 relationships of myxozoan parasites of corydoradines, an economic important assemblage
268 of catfishes in the lucrative global aquarium industry.

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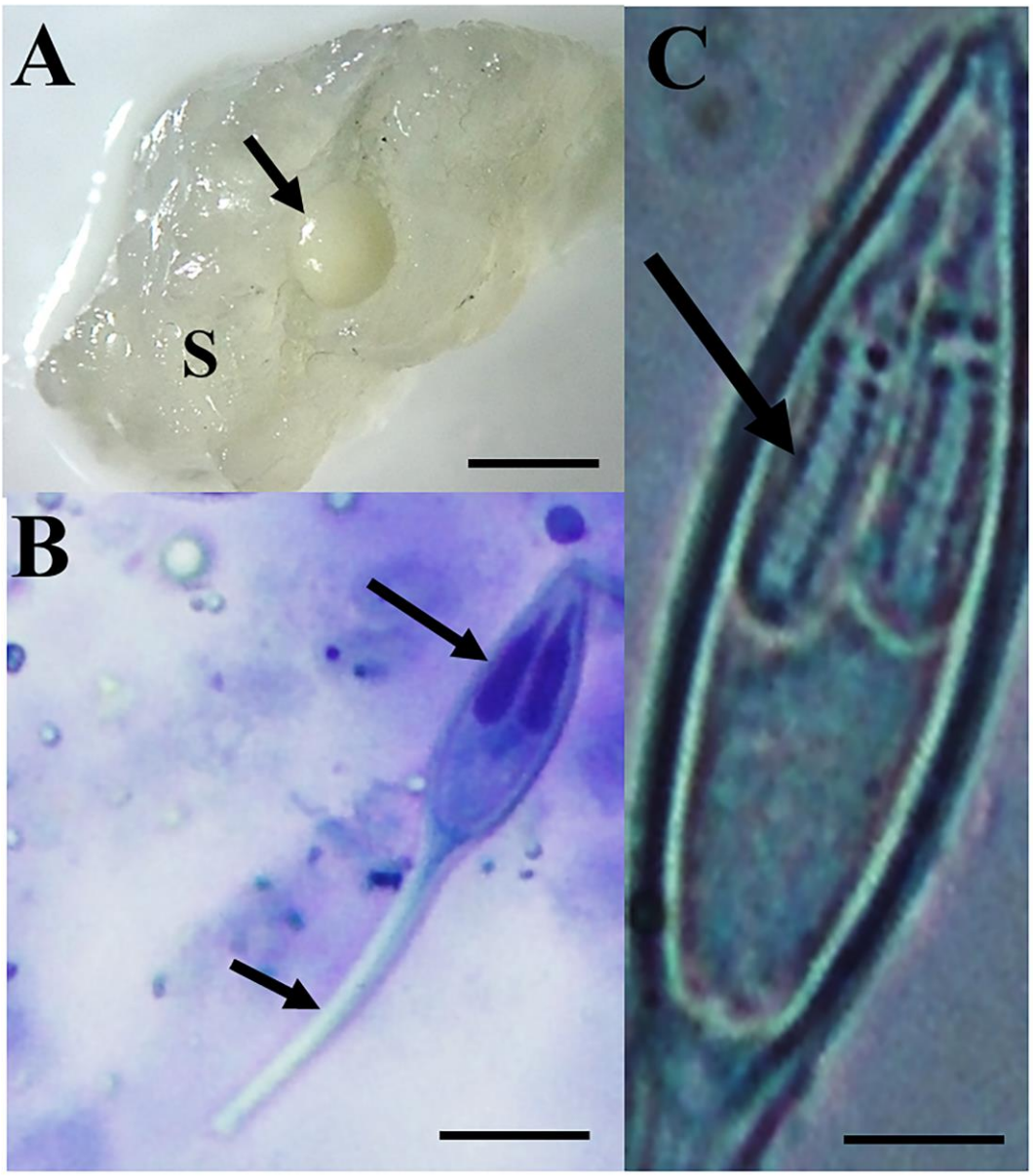
Legends

Table 1. Primers used in the amplification and sequencing of the 18S rDNA gene of *Henneguya multiradiatus* n. sp.

Fig. 1. Plasmodium and myxospores of *Henneguya multiradiatus* n. sp. from *Brochis multiradiatus*. A- Whitish plasmodium macroscopically visible (black arrow) in the serosa of the visceral cavity (S). Scale bar= 3 mm. B- Mature myxospore stained with Ziehl-Neelsen showing two elongated equal polar capsules (black large arrow) and caudal appendage not bifurcated (black small arrow). Scale bar= 10 µm. C: Myxospore showing polar coiled tubules (black arrow) perpendicular to the long axis of the polar capsule. Scale bar= 3 µm.

Fig. 2. Schematic representation of myxospore of *Henneguya multiradiatus* n. sp. from *Brochis multiradiatus*. Scale bar= 7 µm.

Fig. 3. Maximum likelihood phylogenetic tree based on partial 18S rDNA sequences containing *Henneguya multiradiatus* n. sp. and closely related myxozoans based on BLAST. GenBank accession numbers and host family are given in front of species. Bootstrap values above 50 are indicated at the nodes.



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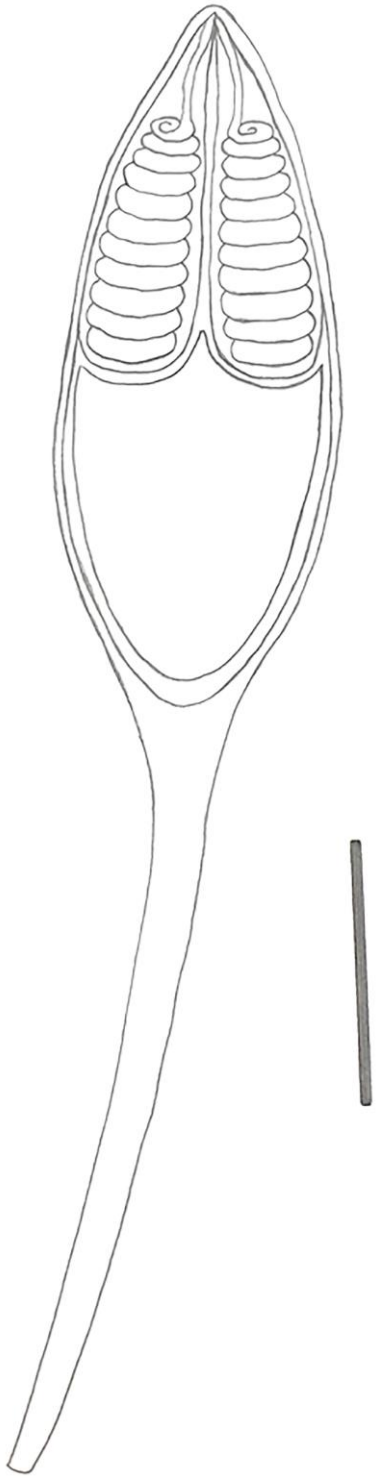

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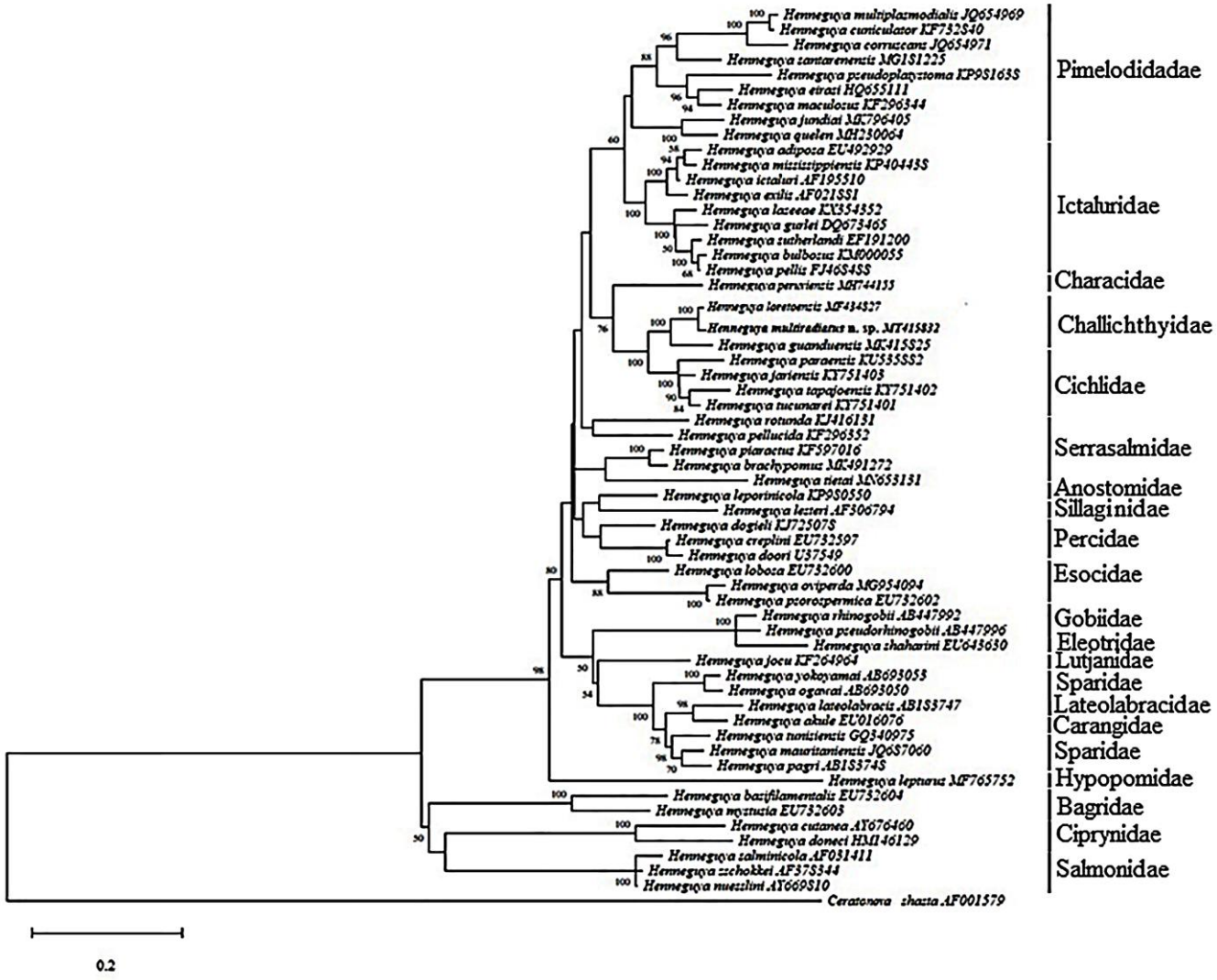
Hemibagrus multiplasmodialis JQ654969
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Hemibagrus tantarenensis MG181225
Hemibagrus pseudoplayztoma KP981658
Hemibagrus etrati HQ655111
Hemibagrus maculosus KF296544
Hemibagrus jundiai MK796405
Hemibagrus quelen MH250064
Hemibagrus adipoza EU492929
Hemibagrus mizzizippiensis KP404458
Hemibagrus ictaluri AF195510
Hemibagrus exilis AF021881
Hemibagrus laceeae KX554352
Hemibagrus guriet DQ673465
Hemibagrus sutherlandi EF191200
Hemibagrus bulbosus KM000055
Hemibagrus pellis FJ465458
Hemexipa peruviensis MH744133
Hemigrammus loretoensis MF434827
Hemigrammus multiradiatus n. sp. MT415832
Hemigrammus guanduensis MG415825
Hemigrammus paraensis KU555582
Hemigrammus jariensis KY751403
Hemigrammus tapajoensis KY751402
Hemigrammus tucunarei KY751401
Hemigrammus rotunda KJ416131
Hemigrammus pellucida KF296552
Hemigrammus piaractus KF597016
Hemigrammus brachypomus MK491272
Hemigrammus tietai MN655131
Hemigrammus leporinicola KP980550
Hemigrammus lezteri AF306794
Hemigrammus dogieli KJ725078
Hemigrammus creplini EU732597
Hemigrammus doori U37549
Hemigrammus loboza EU732600
Hemigrammus oviperda MG954094
Hemigrammus psorospermica EU732602
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Ictaluridae
Characidae
Challichthyidae
Cichlidae
Serrasalmidae
Anostomidae
Sillaginidae
Percidae
Esocidae
Gobiidae
Eleotridae
Lutjanidae
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