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Research paper

# Morphology and molecular phylogeny of *Pauciconfibuloides amazonica* gen. n. sp. n. (Platyhelminthes, Monogenoidea) parasitizing the Amazonian croaker *Plagioscion squamosissimus*

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ABSTRACT

An integrative study was performed to understand the phylogenetic relationships of an undescribed, freshwater species of microcotylid parasitizing *Plagioscion squamosissimus* from the Amazon River Basin. Based on morphological and molecular analysis (18S rDNA and partial 28S rDNA genes), a new genus is proposed to accommodate this new species, *Pauciconfibuloides amazonica* gen. n. sp. n. The new genus is closely related to Protastomicrocotylinae and *Pauciconfibula* by sharing the vagina, male copulatory organ, and genital atrium all unarmed. However, *Pauciconfibuloides* gen. n. can be distinguished from those taxa by the prostatic system and position of the vaginal pore. Molecular phylogenetic inference suggests a sister relationship with species of *Polylabris* (Prostatomicrocotylinae), but to date, there are no available 18S or 28S rDNA sequences of *Pauciconfibula* to be compared. This is the first report of a microcotylid parasitizing a freshwater sciaenid from South America.

## 1. Introduction

A considerable part of the worldwide fish diversity is concentrated in South America, where 27% of known species are reported [1]. The great contributors to this megadiversity are the Amazonian ecosystems, responsible for harbouring the richest fauna of freshwater fishes in the world, with around 2500 known species [2,3]. Such richness is the result of a myriad of diversification processes, characterized by long superposed and complex evolutionary histories [4,5]. These events gave rise to conditions that enabled the emergence of many lineages as the marine-derived lineages (MDL) represented by, between other, the freshwater Sciaenidae Cuvier, 1829 [2,4,6].

Sciaenidae accounts 66 genera and 293 species of demersal and commercially important fishes, most of which are marine with occurrence in the coastal waters of the Atlantic, Indian and Pacific oceans [7–10]. They form a monophyletic group [7,11] whose the most recent common ancestor (MRCA), a euryhaline fish, occurred in the Neotropical Region between the Late Oligocene and Early Miocene (27 Ma) [7]. This ancestor would have adapted to marine environments and, through numerous independent transitions, returned to the estuaries at different times during the Miocene [7]. Moreover, three independent transitions from marine/euryhaline to freshwater environments occurred in South

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America, North America, and Asia [7].

Around the early Miocene (~ 21 Ma), the marine introgression in course in South America led to the divergence of lineages of the freshwater sciaenids [7], which today account for 19 species on the continent [12–15], classified into *Pachypops* Gill 1861, *Pachyurus* Agassiz 1831, *Plagioscion* Gill 1861 and the monotypic *Petilipinnis* Casatti, 2002. More than 40 species of monogenoids have been described in association with these fishes [16], but, these parasitological surveys were unable to reveal associations beyond those involving dactylgyrids and diplectanids [16–19].

Microcotylidae Taschenberg, 1879, represents the largest family of Monogenoidea within Oligonchoinea, and almost all its species are parasites of marine fishes [20], mainly percomorpharians. However, there are rare reports of these parasites in freshwater environments from the USA, Canada, Turkey and Iraq [21–28]. Nevertheless, other reports of undetermined microcotylids in freshwater can be found in India and Japan.

In the South America, 22 microcotylids species were reported parasitizing different groups of hosts, 21 from marine (13 spp. in percomorpharians, 6 spp. in carangimorpharians, one species in gobiomorpharians, and one in scombriomorpharians) and just one from freshwater, *Paranaella luquei* Kohn, Baptista-Farias and Cohen, 2000, associated with siluriformes and characiformes from Brazil [16,29,30]. The results obtained here report for the first time, the evolutionary history of a microcotylid infecting a freshwater sciaenid from South America. Based on morphological study and phylogenetic inferences using 18S rDNA and partial 28S rDNA genes, the monotypic *Pauciconfibuloides* n. gen. was erected to accommodate *Pauciconfibuloides amazonica* gen. n. sp. n. of *Plagioscion squamosissimus* (Heckel, 1840) from the Amazon River Basin.

## 2. Material and methods

### 2.1. Sampling and morphological investigations

The field research was conducted in several different Amazonian hydrographic sub-basins. The host fish were collected from eight capture points, six in the Tapajós River Basin, one in the Xingú River Basin, both in the state of Pará, and one in the Amazon River, in the state of Amazonas (Table 1). The euthanasia method was approved by the Ethics Committee on Animal Research of the State University of Campinas (CEUA No. 3179–1). The sampling and access to genetic heritage was authorized by the Brazilian Ministry of the Environment (authorization SISBIO # 42427–3 and SISGEN # AD28DC2). The host scientific names and classification were validated based on Casatti [31], Betancur-R et al. [32], Queiroz et al. [33] and Eschmeyer et al. [8]. Fish were deposited under number LBP2011092501 in the collection of the Fish Biology and Genetics Laboratory, of the Universidade Estadual Paulista Julio de Mesquita Filho (Julio de Mesquita Filho São Paulo State University) (LBP/UNESP), São Paulo, Brazil.

To retrieve the parasites, the gills were extracted using dissection scissors, shaken in a flask filled with heated water (~65 °C) and then fixed in 4% formalin for morphological study or 100% ethanol for molecular study. In the laboratory, the gills and sediments were examined in a Petri dish under a stereomicroscope, the helminths were recovered using small probes and fixed within 1.5 ml tubes filled with 4% formalin or 100% ethanol.

A number of helminths were stained with Gomori's trichrome and mounted in Canada balsam to study their soft internal structures, while others were prepared with Gray and Wess's medium or Hoyer's medium to examine the sclerotized structures [34]. The measurements were taken in millimeters, except where shown in micrometers. Almost all the helminths and their structures were measured in the dorsoventral view, through digital images processed using ImageJ 1.43 m software [35]. The average measurements are shown, followed by the ranges, and the number of specimens measured (n) is given in parentheses. Some specimens were observed and photographed using differential interference contrast (DIC) and phase-contrast optics through an Axioplan 2 Zeiss microscope. Scanning electron microscopy (SEM) was performed in a Leica/Cambridge Leo Stereoscan S-440 scanning electron microscope. Specimens prepared for SEM were postfixed in 4% formalin during 1 h, and posteriorly in 1% osmium tetroxide for 1–2 h at room temperature, dehydrated in an ethanol series, dried to a critical point and sputter-coated with gold. Illustrations were prepared with the aid of a drawing tube on a Leica DM 2500 microscope with DIC. The quantitative descriptors of the parasitic population used here are those suggested by Bush et al. (1997) [36].

Type specimens and vouchers were deposited in the collection of Platyhelminthes of the Adão José Cardoso Museum of Zoology of the State University of Campinas, São Paulo (ZUEC PLA) and the details of the new taxa were submitted to ZooBank. The following specimens from the US National Parasite Collection of the Smithsonian National Museum of Natural History (USNM) were examined: 2 specimens of *Aspinatrium pogoniae* (USNM 1398080), 1 specimen of *Aspinatrium kahala* (USNM 1359393), 2 specimens of *Microcotyle macroura* (USNM 1335871, 1,337,244), 3 specimens of *Microcotyle scomberomori* (USNM 1351590), 1 specimen of *Heteraxinoides hargisi* (USNM 1338411), 2 specimens of *Heteraxinoides oligoplitis* (USNM 1321912), 2 specimens of *Heteraxinoides xanthophilis* (USNM 1338757), and 6 specimens of *Pauciconfibula subsolana* (USNM 1377233). Six specimens of *Anakohnia brasiliana* (ZUEC PLA 178-183) were also examined and a new record of geographical locality for this species is provided here (Gararu, Sergipe, Brazil, in the São Francisco River, Lat. −9.924444°; Long. −37.122917°).

### 2.2. DNA extraction, amplification, sequencing, alignment and congruence analysis

One helminth was mounted on a slide with glycerin, photographed for identification and posteriorly used for molecular characterization. The total DNA genomic was extracted using the Qiagen Dneasy® Blood and Tissue Kit (animal tissue protocol), according to the manufacturer's

**Table 1**
Summary of the field samplings and infection diagnosis, with the geographic locality of fish capture (longitude and latitude); number of fishes caught (*n*); prevalence (P %), mean intensity (MII); and mean abundance (MAI) of infection.

| Sample area | Long.; Lat. | *n* | Total length (cm) | Weight (g) | Catch dates |
|---|---|---|---|---|---|
| Tapajós River, South of Itaituba, PA | −56.037793°; −4.297224° | 15 | 35 (30–41) | 415 (140–432) | September and October 2011 and July 2012 |
| Tapajós River, Comunidade Vila Rayol, Itaituba, PA | −56.267500°; −4.458194° | 1 | 24 | 190 | |
| Tapajós River, Pimental, Itaituba, PA | −56.264613°; −4.568505° | 2 | 31 (28–34) | 378 (292–464) | |
| Tracuá River, Tapajós River Basin, Itaituba, PA | −56.283917°; −4.486417° | 1 | 36 | 582 | |
| Tapajós River, National Park of Amazonia, Itaituba, PA | −56.299889°; −4.552694° | 8 | 35 (20–48) | 594 (103–1324) | |
| Mouth of Tapajós River, Santarém, PA | −54.813158°; −2.277381° | 22 | 24 (19–31) | 162 (85–335) | April 2018 |
| Amazonas River, Manaus, AM | −59.802940°; −3.085335° | 10 | 22 (19–25) | 180 (110–305) | May 2018 |
| Xingu River, Altamira, PA | 52.196528°; −3.354361° | 1 | 25 | 250 | June 2015 |

protocol, with a final volume of 30 μl. The DNA concentration was verified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) at 260 nm.

The 18S rDNA was amplified using the WormA (forward; 5′ – GCGAATGGCTCATTAAATCAG – 3′) and WormB (reverse; 5′ – CTTGTTACGACTTTTACTTCC – 3′) primers [37]. The 28S rDNA was amplified using the U178 (forward; 5′- GCACCCGCTGAAYTTAAG – 3′) and L1642 (reverse; 5′ – CCAGCGCCATCCATTTTCA – 3′) primers [38]. The Polymerase Chain Reactions (PCRs) were performed in a Mastercycler® nexus (Eppendorf, Hamburg, Germany) with a final volume of 25 μl using DreamTaq Green PCR Master Mix (2×) Thermo Scientific (Wilmington, USA), following the manufacturer's recommendations. A quantity of 0.1 mM of each primer and 3 μl of the extracted DNA was used in the reactions.

To amplify the 18S rDNA, the PCRs programs were set up for an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 90 s, and a final elongation at 72 °C for 10 min. For the 28S rDNA, initial denaturation was performed at 95 °C for 3 min, followed by 34 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 90 s, and a final elongation at 72 °C for 4 min. Amplicons were electrophoresed on 1.5% agarose gel in a TAE buffer (Tris 40 mM, Acetic Acid 20 mM, EDTA 1 mM) stained with SYBRsafe® (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) alongside a 1 kb Plus DNA Ladder (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) at 90 V for 30 min. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, USA) and sequenced with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™) in a 3500 DNA sequencing analyzer (Applied Biosystems, California, USA) from the Helixxa Company (municipality of Paulinia, state of São Paulo, Brazil). For the sequencing were used the primers used in the amplification plus the additional primers 1270R (reverse; 5′ – CCGTCAATTCCTTTAAGT – 3′) and 930F (forward 5′ – GCATGGAATAATGGAATAGG – 3′) [37] for 18S rDNA and 900F (5′ – CCGTCTTGAAACACGGACCAAG – 3′) and 1200R (5′ – GCATAGTTCACCATCTTTCGG – 3′) [38] for the 28S rDNA.

BioEdit 7.1.3.0 [39] was used to visualize, assemble and edit the sequences, which were submitted to a basic local alignment searches (BLASTn) [40] to verify their similarity with other sequences of monogenoids available in the NCBI BioSystems database [41]. The resulting sequences were aligned against sequences of 39 operational taxonomic units (OTUs) (Table S1). All the sequences used for comparison were downloaded from the NCBI BioSystems database [41], selected according to length (*i.e.*, >1600 bp for 18S and > 700 bp for 28S) and the quality of their fit with the alignments, which was performed by applying the ClustalW algorithm Version 2 [42] implemented in SeaView Version 4 [43]. The OTUs which had their species/genus determined, and had both molecular markers available, were prioritized. However, an exception was made for sequences of *Polylabris* spp. owing to results that inferred phylogeny, separately, for each molecular marker.

The alignments were subjected to the composition of less stringent g-blocks [44]. These alignments were supplied to produce distance matrices through MEGA-X version 10.0.5 using the Maximum Composite Likelihood algorithm [45]. Such matrices were compared by running a Congruence Among Distance Matrices (CADM) analyses, with the algorithm of CADM entered in the 'ape' (Analyses of Phylogenetics and Evolution) package, handling the R Version 3.6.1 [46–48]. The sequence matrices were concatenated with SeaView Version 4 for the phylogenetic inferences [43].

### 2.3. Phylogenetic inferences

Sixteen OTUs were used as the outgroup, two of which were classified into Polyonchoinea Bychowsky, 1937, and 14 of which were classified as members of Heteronchoinea Boeger and Kritsky, 2001, grouped in Polystomatoinea Lebedev, 1986 (Table S1). Three methods were applied to reconstruct the phylogenetic relationships among the taxa investigated. The Maximum Parsimony (MP) was used running the NONA ver. 2.0 implemented in WinClada ver. 1.00.08 [49,50]. In order to achieve the globally optimal trees, thirty heuristic searches were conducted [51], each configurated to reach a maximum of $10^4$ trees within 1000 replications, with the TBR + TBR multiple swapping algorithm and one holding tree. The taxa were randomly added, with characters equally weighted, freely allowed to the reversibility and optimized to accelerate the transformations (ACCTRAN), while gaps were considered missing data. The PhyML with Smart Model Selection [54,55] was employed to apply the Maximum Likelihood (ML) method. For the MP and ML, branch was validated, supported by bootstrapping the analyses with 1000 replicates. Bayesian Inference (BI) was carried out using MrBayes version 3.2 software package [56]. For these inferences, the analyses were set up to run two independent Markov Chain Monte Carlo (MCMC) trials over $10^6$ generations, sufficient to keep the average standard deviation below 0.001. The MCMC were sampled each $100^{th}$ and diagnosed every $1000^{th}$ generation, with the first 25% of the samples discarded in the burn-in phase. To sample across the substitution models and combine a gamma-distributed rate variation across sites with a proportion of invariable sites, the lset nst = mixed rates = invgamma function was used [56].

## 3. Results

### 3.1. Quantitative descriptors of Pauciconfibuloides amazonica gen. n. sp. n.

Overall, 43 specimens of *P. squamosissimus* were examined, with a mean length of 29 cm and a mean weight of 344 g (Tables 1 and 2). Infection by *Pauciconfibuloides amazonica* gen. n. sp. n. was observed in 25 of these fish and a total of 38 parasites were counted, however, many of them were broken and were just considered for the purpose of quantitative description of parasitism.

### 3.2. Taxonomic acts

**Taxonomic summary**

Class: Monogenoidea Bychowsky, 1937.

Subclass: Heteronchoinea Boeger and Kritsky, 2001.

Order: Mazocraeidea Bychowsky, 1937.

Family: Microcotylidae Taschenberg, 1879.

***Pauciconfibuloides* gen. n.**

Type-species: *Pauciconfibuloides amazonica* gen. n. sp. n. (Figs. 1, A.1).

Type host: *P. squamosissimus* (Heckel, 1840), South American silver croaker.

Site of infection: Gills.

Type locality: Tapajós River, National Park of Amazonia (PARNA da Amazônia), Itaituba, PA.

Etymology: an adjective meaning that the new genus resembles the genus *Pauciconfibula*. It is a Latinized form from -o- + Ancient Greek -ειδής (−eidḗs, "-oid, −like").

Zoobank Life Science Identifier: urn:lsid:zoobank.org:pub:12A373BF-0B1C-4FA3-BAAE-C610BABC7077.

**Genus description**

Body lanceolate, flattened dorsoventrally. Opisthohaptor symmetrical or asymmetrical, armed with two approximately parallel (sometimes convergent, other divergent) rows of clamps. Clamps composed by five sclerites. Mouth ventral, subterminal. A paired prehaptoral organ C-shaped, septated, within the mouth, bearing sclerotized toothlike papillae surrounding the anterior and posterior margin. Intestinal caeca branched, extending into haptor, dissimilar and not confluent posteriorly. Common genital pore midventral; common genital atrium midventral, unarmed. Vagina mediodorsal, unarmed. Vaginal duct arising from the meet of the transverse vitelline ducts in the median line of the body. Ovary question mark-shaped, tubular, pretesticular, intercaecal,

**Table 2**
Comparative measurements (μm) and quantitative descriptors of the specimens ($n$ = number of specimens) of *Pauciconfibuloides amazonica* gen. n. sp. n. parasite of *Plagioscion squamosissimus* from five localities in the Amazon River Basin, Brazil.

| | Tapajós River, PARNA da Amazônia, PA ($n = 6$) | Tapajós River, South of Itaituba, PA ($n = 1$) | Mouth of Tapajós River, Santarém, PA ($n = 2$) | Amazonas River, Manaus, AM ($n = 2$) | Xingu River, Altamira, PA ($n = 1$) |
|---|---|---|---|---|---|
| Total length | 4757 (2709–6681) | – | 4775 (4550–5000) | 5675 (3850–7500) | 3050 |
| Maximum body width | 815 (573–1052) | 315 | 600 (400–800) | 650 (450–850) | 400 |
| Haptor length | 2339 (1206–3346) | – | 2075 (1650–2500) | 2850 (1700–4000) | 1250 |
| Haptor width | 493 (321–611) | – | 550 (500–600) | 260 (250–270) | 550 |
| Number of left clamps | 54 (33–96) | – | 55 (45–65) | 72 (49–95) | 47 |
| Number of right clamps | 57 (29–101) | – | 22 (12−32) | 25 (19–32) | 19 |
| Anterior clamps long | 52 (29–79) | – | 80 (75–85) | 82 (80–85) | 100 |
| Medial clamps long | 70 (40–105) | – | 107 (100–115) | 125 | 100 |
| Posterior clamps long | 64 (41–83, $n = 5$) | – | 137 (125–150) | 122 (100–145) | 200 |
| Anterior clamps width | 88 (62–124) | – | 115 (80–150) | 85 (75–95) | 125 |
| Medial clamps width | 91 (35–145) | – | 200 | 137 (125–150) | 200 |
| Posterior clamps width | 100 (56–133, $n = 5$) | – | 110 (100−120) | 110 (95–125) | 110 |
| Common genital pore location | 409 (248–548, $n = 5$) | 454 | 475 (450–500) | 576 (502–650) | 350 |
| Testes number | 22 (19–23) | – | 21; 22 | 22; 23 | – |
| Prevalence of infection (%) | 50 | 33 | 41 | 60 | 100 |
| Mean abundance of infection | 1.5 | 0.4 | 0.5 | 0.8 | 1 |
| Mean intensity of infection | 3 | 1.2 | 1.2 | 1.3 | 1 |

dorsal to vitelline ducts and uterus. Uterus delicate, opening in the genital atrium. Eggs fusiform, with filament at one pole. Genitointestinal canal short. Testes post-ovarian, intercaecal. Vas deferens extending along body midline to male copulatory organ (MCO), long, convoluted. MCO a not sclerotized tube, not armed, opening into genital atrium, with a soft tissue base. Ejaculatory bulb absent. Prostatic system a sac jointing to the base of the MCO. Vitellaria extending along the intestinal caeca.

*Pauciconfibuloides amazonica* gen. n. sp. n. (Figs. 1, 2, A.1).

Type host: *P. squamosissimus* (Heckel, 1840), South American silver croaker.

Site of infection: Gills.

Type locality: Tapajós River, National Park of Amazonia (PARNA da Amazônia), Itaituba, PA.

Other localities: see Table 1.

Etymology: the specific name is an adjective, means that something is relative to or belonging to the Biome Amazônia, in allusion to the geographic locality in which the parasites were found.

Prevalence of infection: 50% (Table 2).

Mean intensity of infection: 1.5.

Mean abundance of infection: 3.

Specimens deposited: Holotype ZUEC PLA 162. Paratypes ZUEC PLA 163-169; Voucher ZUEC PLA 170-177.

GenBank accession number: MT645074 (18S rDNA) and MT645075 (28S rDNA), obtained from one single specimen and MT645076 (28S rDNA) obtained from another single specimen, all from Mouth of Tapajós, in Santarém, state of Pará.

Description (based on 12 specimens: 5 stained with Gomori's trichrome, 4 mounted in Gray and Wess's medium and 3 mounted in Hoyer's medium).

**Species description**

Total length 4.8 (2.7–6.7; $n = 6$), maximum body width 0.8 (0.6–1; $n = 6$) at level of ootype, with geographical variability (Table 2). Anterior extremity slightly rounded, with a central head organ opening anteriorly. Absence of haptoral peduncle. Haptor arising posteriorly to testes, 2.3 (1.2–3.3; $n = 6$) long, equivalent to 31%–57% of the total length (Figs. 1, 2, A.1) and 0.5 (0.3–0.6; $n = 6$) maximum width. Clamps pedunculated, composed by five sclerites unequal in length but similar in structure; one single median, sometimes curved, which bears, at the anterior and posterior ends, two diverging projections; a paired anterolateral sclerites, twisted at its one third to the middle, bending posteromedially; and a paired curved posterolateral sclerites. At left side of the haptor there are about 54 clamps (76 μm long x 84 μm width) and at right side, 57 clamps (58 μm long x 91 μm width); the largest generally posteriorly distributed (Table 2). Paired prehaptoral organ 59 μm (50–74; $n = 6$) in maximum length; septum laterally dislocated, extending longitudinally, from its posterior to anterior border. Muscular pharynx ovoid, 128 μm (97–181; $n = 6$) long, 85 μm (60–111; $n = 6$) width. Oesophagus short, without diverticula. Intestinal bifurcation immediately anterior to common genital pore; branched intestine, partially obscured by vitelline follicules, extending from $^1/_3$ to less than 85% into the haptor without posterior confluence; intestinal caeca subequal in length, left caecum being slightly longer. Common genital pore located at 409 μm (248–548, $n = 6$) from anterior extremity; common genital atrium 83 μm (67–101, $n = 6$) in diameter, with a strong circular muscle surrounding it. Vagina opening through a single mediodorsal, slightly left pore located at 726 μm (641–857, $n = 3$) from anterior extremity; vaginal canal muscular, short, connecting with vitelline ducts. Paired vitelline ducts directing posteriorly to form a common vitelline duct or vitellovaginal reservoir. Vitellaria laterally scattered, coextensive with intestinal caeca, extending from prostatic organ to near posterior portion of body, not entering the haptor. Ovary long, originating on left side of body, extending anteriorly, then traversing intercaecal region to right side, aftermore ventral and posteriorly, back to left side, until it snaps between the common vitelline duct and the anterior testes. A S-shaped oviduct arises at the end of ovary, extends anteriorly, dorsal to the common vitelline duct or vitellovaginal reservoir, opening near the end of the genitointestinal canal, which comes from left vitellaria. Ootype smooth walled, dorsal to vitellovaginal reservoir. Mehlis' glands not observed. Uterus, a delicate tube, ventral to ovary and vas deferens, relatively straight with some loopings when unpregnant, arising at level of end of vitellovaginal reservoir, extending anteriorly, nearly at body midline, until reaching the genital atrium. Testes 22 (19–23, $n = 6$) in number, post-ovarian, intercaecal, occupying $^1/_3$ of body. Prostatic system a sac with two

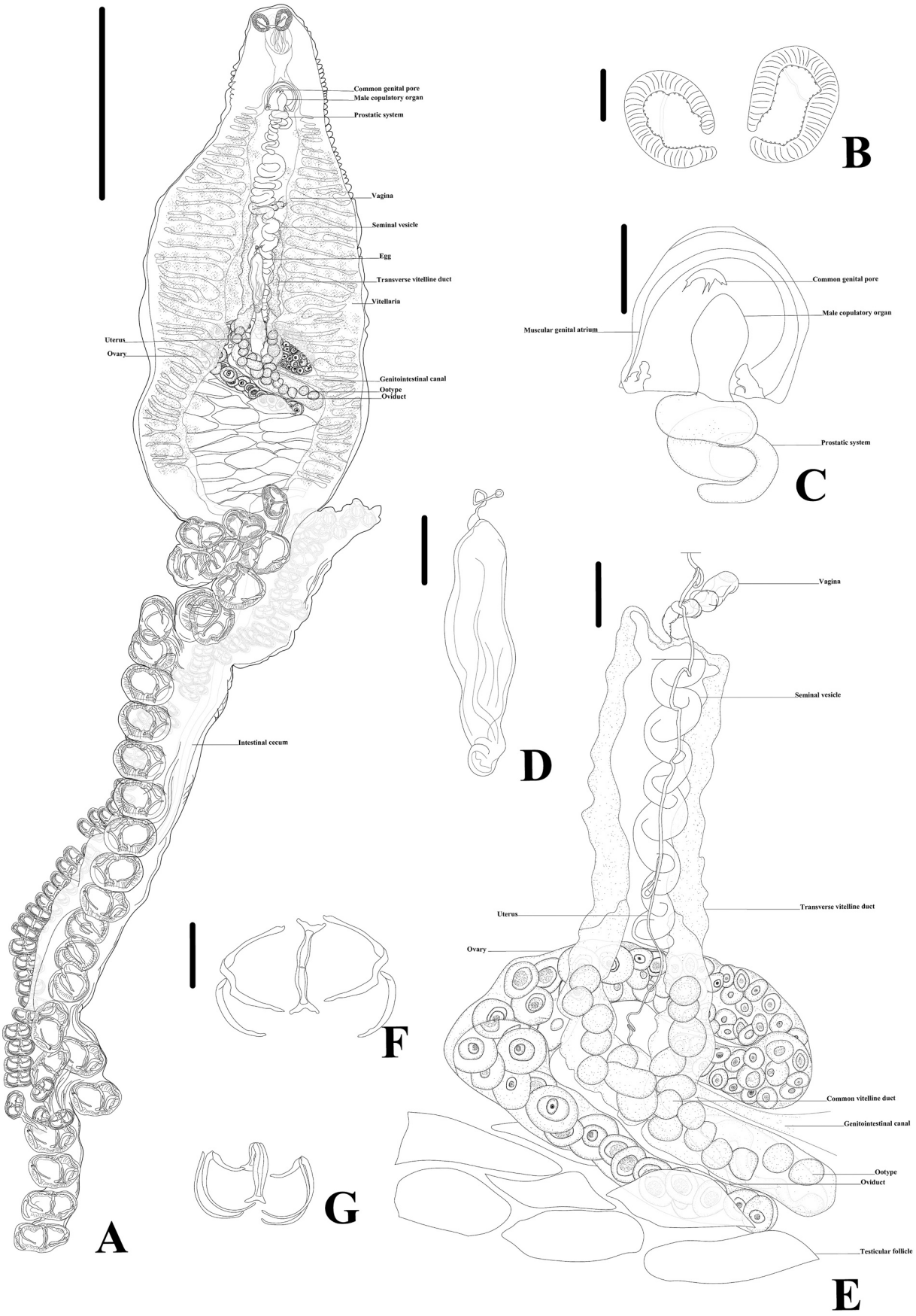


**Fig. 1.** *Pauciconfibuloides* gen. n. *Amazoniacotyle amazonica* gen. n. sp. nov. A: whole composite drawn, ventral view. B: prehaptor. C: male reproductive system. D: egg. E: female reproductive system. F and G: opened and closed clamps. Scale bar = 1000 μm (A), 100 μm (E), 50 μm (C, D, F, G), 25 μm (B).

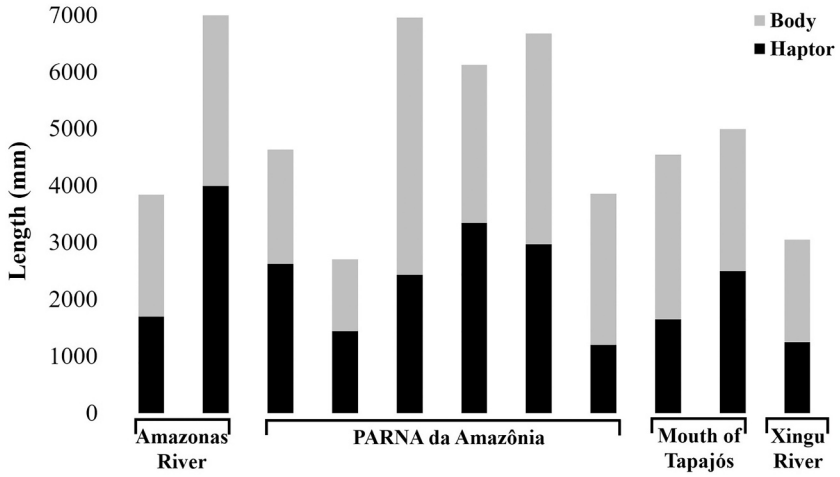


**Fig. 2.** Barplot of the relationship between body and haptor length of *Pauciconfibuloides* gen. n. *amazonica* sp. nov.

chambers, arise as a pouch from the proximal portion of the male copulatory organ (MCO). Egg operculated, 217 × 55 μm (in uterus) ($n$ = 1), 130 × 25 μm (in ootype) ($n$ = 1), with one short polar filament.

### 3.3. Molecular phylogenetic inference

The 18S sequencing of *Pauciconfibuloides amazonica* sp. n. generated a 1724 bp sequence, which in the BLASTn search revealed closest similarity with *Polylabris acanthopagri* Mamaev and Parukhin, 1976, and *Microcotyle sebastis* Goto, 1894 (96.3% and 96.2% respectively). The 28S sequencing recovered 1547 bp, while the BLASTn search evidenced closest similarity with *Microcotyle isyebi* Bouguerche, Gey, Justine and Tazerouti, 2019, and *Microcotyle erythrini* Van Beneden and Hesse, 1863, both with 92.1%. After alignment and g-block selection procedures, 18S and 28S matrices with 1698 and 810 characters, respectively, were reached. The congruence test performed revealed a concordance

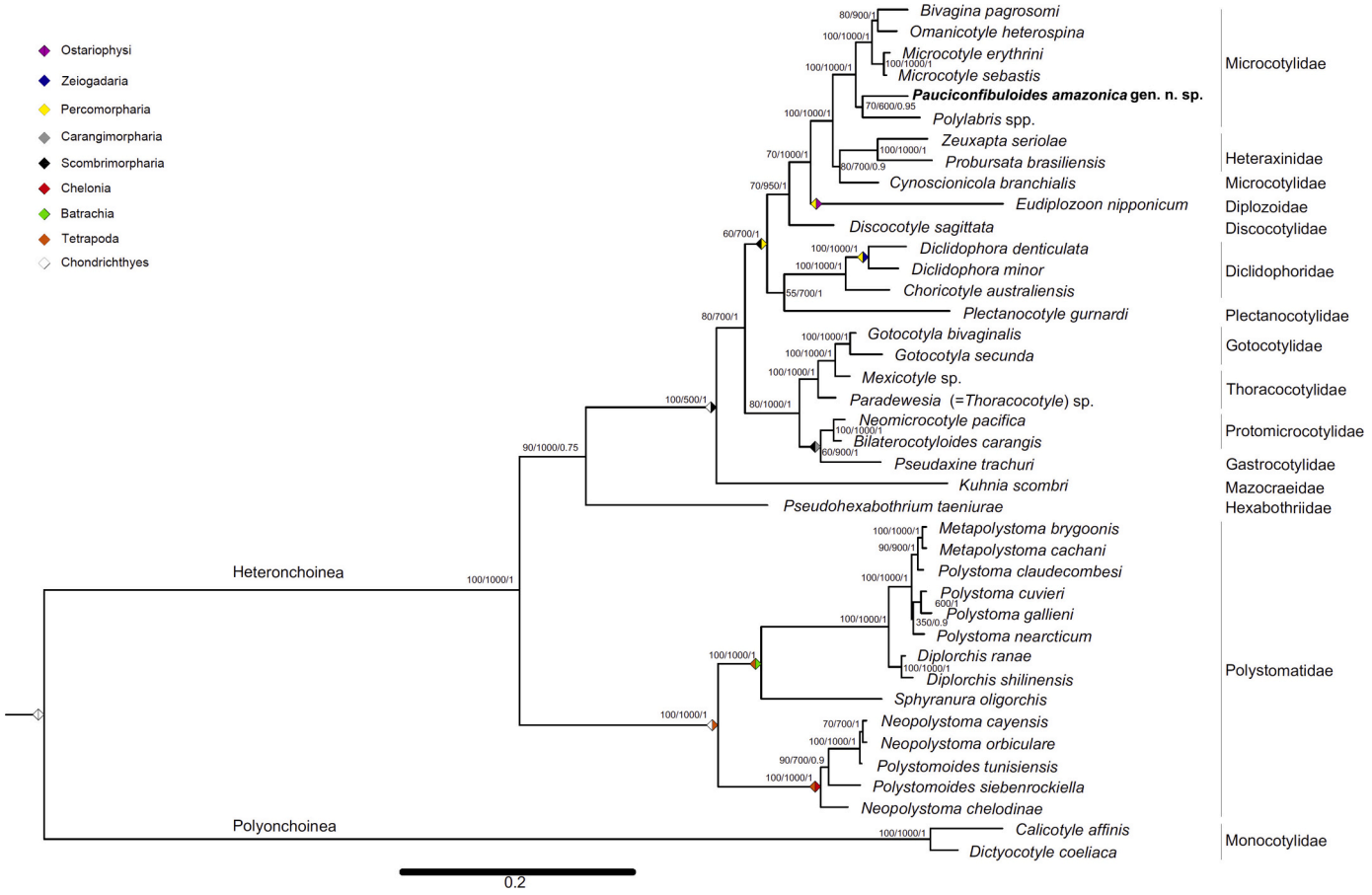


**Fig. 3.** A 50% majority-rule consensus tree based on a concatenated matrix of 18S rDNA and partial 28S rDNA gene sequence. Numbers above the branches are the supports of Maximum Parsimony, Maximum Likelihood and Posterior Probability from Bayesian Inference. The hosts were manually optimized optimized on the tree according to Modified Fitch Optimization [69].

between the distance matrices with a Mantel mean of 0.92 and a Kendall's W range of 0.96. Subsequent to concatenation, a matrix with 2509 characters was reached.

A single most parsimonious MP tree with length of 4703, CI = 46, RI = 75 revealed total congruence with the ML and BI trees, though the values of support can vary for each algorithm. The Generalized Time-Reversible was the selected model for both ML and BI, with submodels GTR + G + I for ML and GTR M177 for BI. The BI read 20,002 trees and sampled 15,002.

*Pauciconfibuloides amazonica* sp. n. emerged within Microcotylidae as a sister species to *Polylabris* spp. and this clade arose in a sister relationship with *Bivagina pagrosomi* (Murray, 1931) + *Omanicotyle heterospina* (Mamaev and Parukhin, 1974) and *M. erythrini* + *M. sebastis* Goto, 1894. *Cynoscionicola branchialis* (*taxon inquirendum*) arose as a sister taxon for the heteraxinids, composing a sister group to Microcotylidae (Fig. 3).

## 4. Discussion

*Pauciconfibuloides* gen. n. is characterized by the question mark-shaped ovary; a genitointestinal canal; a symmetric or asymmetric haptor; numerous clamps without accessory sclerites; and absence of muscular pads in the genital atrium. The genus is also characterized by: (1) the presence of sclerotized toothlike papillae in the prohaptor; (2) intestinal caecum extending into the haptor; (3) the egg with one filament; (4) clamps with five sclerites; (5) the vagina dorsal and unarmed; (6) the genital atrium unarmed; (7) the MCO unarmed; (8) the prostatic system like a sac with two chambers. These characters fit with the diagnosis of Microcotylidae [20,57,58].

The new genus shares the genital atrium, vaginal pore and MCO all unarmed, with members of Prostatomicrocotylinae Yamaguti, 1968 and *Pauciconfibula* Dillon and Hargis, 1965 (Microcotylinae Monticelli, 1892) [20,25,59–62]. These characters neither are shared with members of other lineages reported in Brazil, as the subfamily Anakohninae Bravo-Hollis, 1986, once its only species is represented by specimens bearing a MCO with two terminal spines [63], or *Paranaella* Kohn, Baptista-Farias and Cohen, 2000, whose unique species described has a genital atrium armed with spines [30]. However, *Pauciconfibuloides* gen. n. can be differentiated from members of Protastomicrocotylinae and *Pauciconfibula* by the morphology of the saccular prostatic system, that supports the erection of the new genus. *Pauciconfibula* lacks a prostatic system, while genera included in Protastomicrocotylinae have a symmetric, paired and cylindrical prostatic reservoirs, perpendicular to the MCO [20,60–62]. The mediodorsal vaginal pore of *Pauciconfibuloides* gen. n. also differs the new genus from the genera compounding Protastomicrocotylinae, that have a single medioventral vagina or two mediolateral vagina [20,61,62].

In the meantime, *Pauciconfibuloides amazonica* sp. n. is characterized by: (1) the prostatic system like a sac with two chambers; (2) the presence of sclerotized toothlike papillae in the prohaptor; (3) intestinal caecum extending into a maximum of 85% of the haptor; (4) 56 clamps on each side of the haptor; (5) 22 testes (19–23, 5) an egg filament; (6) a dorsal vagina; (7) a genitointestinal canal opening to the left vitellaria. These characters distinguish the new species from all species of *Pauciconfibula* and Prostatomicrocotylinae.

In proposing the systematic reorganization of Microcotylidae, Mamaev [20] brought together *Polylabris* Euzet and Cauwet, 1967 and other genera into the subfamily Prostatomicrocotylinae, by sharing, among other characteristics, a prostatic system. The hypothesis raised in the tree BI tree (Fig. 3), when presenting the microcotylines as a sister group of a clade whose species, *Pauciconfibuloides amazonica* sp. n. and *Polylabris* spp., have prostatic systems, seems to reflect Mamaev's overview. This would be an interesting hypothesis, even more, considering that the species compounding this clade present a gondwanic distribution. *Polylabris bengalensis* Sailaja and Madhavi, 2011, is a parasite of siganids from India, and *Polylabris sillaginae* (Woolcock, 1936), was found infecting a sillaginid from Australia and, in a summarized view, based on inferences by Betancur-R et al. [32], the siganids form a sister group with sillaginids, composing a sister clade to sciaenids. However, it is possible that the putative sister relationship evidenced for *Pauciconfibuloides amazonica* sp. n., is not real and it is a result of the scarcity of sequences of microcotylids, especially those of *Pauciconfibula* spp. which have only mitochondrial sequences available [64]. In this case, it should be assumed that the prostatic system arose independently in these parasites.

The possibility for the existence of another and yet unknown lineage that could fill the gap of a real sister group for *Pauciconfibuloides amazonica* sp. n. sp. n. from Neotropics is not enigmatic, once the branch support of this clade is not strong (70/600/0.95), moreover, there are precedents to sustain such a hypothesis. A similar biogeographical pattern has been observed by Trevisan et al. [65] and Boeger and Kritsky [66] for different host-parasite systems in the Neotropics. This pattern, if summarized, result in an area cladogram (Southeastern Pacific + Southwestern Atlantic (South American Freshwater)) also observed for other marine-derived lineages, arising from a single paleogeographic event, the Miocene marine introgressions [67]. All these studies suggest that the South American MDL has a sister group formed by taxa with occurrence in the Caribbean Sea. Nonetheless, it should be assumed that *Pauciconfibuloides amazonica* sp. n. is also an MDL.

Some microcotylids seems to exhibit resistance to changes in salinity. The report of *Anakohnia brasiliana* Bravo-Hollis, 1986 (see the Sampling and morphological investigations section) in the São Francisco River, more than 120 km off the coast, supports this assertion. This species was described in Barra de São João, the coastal region of Rio de Janeiro, Brazil, in association with *Centropomus parallelus* Poey, 1860 [63]. Later, it was found in the Guandu River, Seropédica, Rio de Janeiro, just over 20 km from the mouth, parasitizing *Centropomus undecimalis* (Bloch 1792) [68]. These two host species are amphidromous estuarine centropomids. The report of *Pauciconfibula subsolana* Chisholm, Beverly-Burton and McAlpine, 1991 was carried out 136 km from the coast [25], and its host, *Morone americana* (Gmelin, 1789), is an anadromous and estuarine moronid. Different from these parasites, *Pauciconfibuloides amazonica* sp. n. was recovered on several occasions, in 2011, 2012, 2015 and 2018, in some of them, such as in the type locality, more than 1000 km from the coastal region. These are strong indications that *Pauciconfibuloides amazonica* sp. n. is a freshwater species, and as such, it may have experienced the same evolutionary processes as its host, *P. squamosissimus*, a freshwater sciaenid whose ancestral is derived from marine lineages. Nevertheless, this cannot be stated with absolute certainty, but future studies on freshwater microcotylids from Neotropics will can highlight this hypothesis.

## 5. Conclusions

Our study reports for the first time the occurrence of a microcotylid parasitizing a freshwater sciaenid from South America. Supported by morphological and molecular characterization, a new genus was erected to accommodate *Pauciconfibuloides amazonica* sp. n. The phylogenetic inference put the new species as sister to *Polylabris* spp. (Prostatomicrocotylinae), suggesting that the prostatic system present in these taxa is homologous. However, the inexistence of sequences of 18S and 28S rDNA for the *Pauciconfibula* spp., which are the morphologically closest species of *Pauciconfibuloides amazonica* sp. n., can be the cause of this result. The occurrence of *Pauciconfibuloides amazonica* sp. n. more than 1000 km from the coastal region strongly suggest that this species is a freshwater one and, as such, it could have experienced the same evolutionary events of its host, *P. squamosissimus*, a freshwater sciaenid that arose from the divergence of marine ancestral that invade the South America freshwater environments. Nonetheless, additional studies on Neotropical microcotylids will can highlight this hypothesis.

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