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Morphology and molecular characterization of *Polystoma goeldii* n. sp. (Monogenea, Polystomatidae) parasite from the urinary bladder of *Physalaemus ephippifer* (Steindachner) (Anura, Leptodactylidae)

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ABSTRACT

Polystomatid flatworms of amphibians are represented in the Neotropical realm by species of *Mesopolystoma*, *Nanopolystoma*, *Parapseudopolystoma*, *Polystoma*, *Riojatrema* and *Wetapolystoma* but only species of *Polystoma* are known from Brazil, namely *Polystoma cuvieri, P. knoffi*, *P. lopezromani* and *P. travassosi*. During a survey of monogeneans infecting amphibians in the north-eastern region of Pará State, the Cayenne Caecilian *Typhlonectes compressicauda* was found to be infected with *Nanopolystoma tinsleyi* and the Veined Tree Frog *Trachycephalus typhonius* was found to harbor *Polystoma lopezromani.* A yet unknown species of *Polystoma* was also encountered in the urinary bladder of the Steindachner's Dwarf Frog, *Physalaemus ephippifer*. This new species, which is the second species reported from *Physalaemus* spp., is described herein as *Polystoma goeldii* n. sp. and its life cycle is also illustrated. The new species can be distinguished from *Polystoma* spp. from other neotropical realm by a combination of characteristics, including hamuli morphology, outer/inner hamuli length ratio, haptor/total body length ratio, genital bulb/total body length ratio, genital spine number and COI molecular characters.

1. Introduction

With more than 3500 amphibian species, the Neotropical Realm is one of the world's megadiverse regions [1], having Brazil as the second highest species richness country of the world with 1188 known species [2]. However, the exact number of amphibian species for this country is still approximate, basically because there are still extensive regions not inventoried. Estimates suggest that its number may increase by 15% and that many species are declining, suggesting that unknown species can disappear before we could describe them [3]. Of the known species, less than 10% of Brazilian amphibians have been surveyed for helminth parasites [3]. Currently a total of 167 metazoan parasite species are reported including 91 nematodes, 57 digenetic trematodes, nine acanthocephalans, six cestodes and four monogeneans [4–7]. These worms were recorded from less than 100 host species [4], corroborating, therefore, the fact that the parasite fauna of amphibians in Brazil requires greater attention from the scientific community. For example, while about 130 monogenean species have been reported to infect frogs around the world, only four species are recorded from Brazil.

Polystomatid flatworms of amphibians are represented in the Neotropical realm by *Mesopolystoma* Vaucher, 1981, *Nanopolystoma* Du Preez, Huyse & Wilkinson 2008, *Parapseudopolystoma* Nasir & Fuentes Zambrano, 1983, *Polystoma* Zeder, 1800, *Riojatrema* Lamothe-Argumedo, 1964 and *Wetapolystoma* Gray, 1983. To date, only *Polystoma* has been reported from Brazil with *P. knoffi* Du Preez & Domingues, 2019 from *Trachycephalus nigromaculatus* Tschudi in the southeast, *P. travassosi* Du Preez & Domingues, 2019 from *Trachycephalus mesophaea* (Hensel) in the southeast, *P. lopezromani* Combes & Laurent, 1979 from *Trachycephalus typhonius* (Linnaeus) and *P. cuvieri* Vaucher, 1990 from *Physalaemus cuvieri* Fitzinger in the south. It is important to note that, although the Brazilian Amazon represents a wide range of habitat for amphibian species, encompassing approximately 21% of the diversity of amphibians reported in Brazil, no polystome species has been registered in this ecosystem.

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Following a screening of several amphibian species at a few localities in the northeastern Pará province for monogenean parasites, the Steindachner's Dwarf Frog *Physalaemus ephippifer* (Steindachner), which is a diurnal and nocturnal small leaf-litter anuran species, was found to harbor a new *Polystoma* species. Herein, we provide a formal description of this parasite based on morphological and molecular data and illustrate several features of its life cycle following experimental infestations. We also reported *Nanopolystoma tinsleyi* du Preez, Badets & Verneau, 2014 from the Cayenne Caecilian *Typhlonectes compressicauda* (Duméril & Bibron) and *P. lopezromani* from the Veined Tree Frog *T. typhonius*.

2. Material and methods

2.1. Sample collection

During February and March 2018 and from January to March 2019, amphibians were collected at several localities around the towns of Bragança, Iritua, and Igarapé-Açu in the State of Pará, Brazil. The protocol of screening frogs for polystome eggs follows the methods presented by Landman et al. [8]. Following capture, specimens were individually placed in plastic bags with approximately 50 ml of water. Subsequently, host specimens were relocated individually to 500 ml plastic containers with dechlorinated tap water to a depth of about 50 mm and kept at room temperature. After a period of 24 h, frogs were removed and the water screened for the presence of polystome eggs. The water from the containers in which frogs were housed was poured through two plankton sieves with respective mesh sizes of 500 μm and 100 μm. The first sieve which removed the coarse debris in the water were discarded while the second sieve was washed into a Petri dish and examined for polystome eggs under a dissecting microscope. Frogs that did not release any polystome eggs were screened a second and a third time after 24-h intervals. A subset of 10 frogs from each locality that did not produce polystome eggs were dissected to verify that they did not contain any subadult parasites (see protocol below) while the remainder of the frogs were released where collected.

Some foam nests produced by *P. ephippifer* were also collected and maintained in plastic bags with water. Subsequently, foam nests were placed in aquarium filled with water from the locality where the specimens were collected and kept at room temperature. Tadpoles that hatched from the foam nests were maintained in an aerated aquarium, fed on frozen lettuce, and monitored daily.

2.2. Eggs development and experimental infestation

Eggs development and experimental infestation follows the methods described by Kok & Du Preez [9]. Eggs found were isolated from the debris in Petri dish, transferred to another glass Petri dish containing filtered pond water and incubated at room temperature ($\pm$ 26°C). Eggs hatched after 13 days. Freshly hatched oncomiracidia were collected and mounted semi-permanently using ammonium picrate [10] or Hoyer's medium [11] as mounting medium to clear the parasites and reveal the marginal hooklets. The remaining oncomiracidia were used to infect naïve tadpoles of *P. ephippifer* hatched in the laboratory. For experimental infestations, three to five oncomiracidia were then placed into a Petri dish containing a single tadpole ranging between 13 and 20 days of age and still in pre-metamorphic stage.

At intervals of three days, 10 representative tadpoles were euthanized using a 1% ethyl-4-aminobenzoate (MS 222) solution in order to recover the parasites. Tadpoles were pinned upside down in a dissecting tray. The skin covering the gills was loosen by inserting the tip of a pair of fine scissors in the sinistral spiracle and cutting around the gill chamber. Using a stereomicroscope, gills were then examined for the presence of polystomes. Parasites aged sixteen, twenty and twenty-six days were carefully removed using camel-hair brush and mounted in Hoyer's medium to view sclerotized structures.

2.3. Polystome recovery

Infected adult frogs were euthanized using a 3% ethyl-4-aminobenzoate (MS 222) solution for approximately 3 min before they were dissected. The bladder was carefully removed and placed into a Petri dish containing a 0.6% amphibian saline solution to be examined for the presence of parasites. The dark-colored intestine of the hematophagous parasites facilitated their detection within the transparent bladder. The majority of the parasite specimens that were collected were fixed in 10% formalin under light coverslip pressure for light microscopy while others were fixed under light coverslip pressure in 96% ethanol for molecular studies.

2.4. Morphological study

Parasites were rinsed several times in tap water for 1 h, stained overnight in a weak solution of acetocarmine, dehydrated, cleared in xylene and mounted using Dammar gum according to Georgiev et al. [12]. The dimensions of organs and other structures characterizing worms were measured in ventral view. The length of curved or bent structures were measured by the straight-line distances between extreme ends. Illustrations were prepared with the aid of a drawing tube on a Leica DM 2500 microscope using differential interference contrast and phase contrast optics and LEICA M205A stereomicroscope. Marginal hooklet pairs were numbered one to eight with pair one being the posterior-most pair closest to the median line of the haptor [13]. All measurements are given in micrometers with the range followed by the mean in parentheses. Type specimens of new species were deposited in Museu Paraense Emilio Goeldi (MPEG), Belém, Pará State, Brazil as well as in the Parasitic Worm Collection, National Museum (NMBP), Bloemfontein, South Africa.

2.5. Molecular, phylogenetic and distance analyses

Two polystome specimens (Field numbers: PL171029C1 and PL171029C2) were processed according to the molecular procedure described in Verneau et al. [14] for DNA extraction and amplification. The portion of the COI gene was amplified with the primers forward L-CO1p and reverse H-Cox1p2 [15] for both specimens, yielding a PCR product of about 400 bp that was subsequently sequenced with PCR primers at the Genoscreen Company (Lille, France). Because the same COI haplotype was obtained for both polystomes, a single individual (PL171029C2) was selected to investigate 18S and 28S sequences. The complete 18S rRNA gene was amplified in two rounds with the respective combinations of primers F18/18Rg and 18F3/IR5 [14], yielding two overlapping PCR products of about 1000 bp each, while the partial 28S rRNA gene was amplified in two rounds with the respective combinations of primers LSU5'/IR16 and IF15/LSU3' [14,16] yielding two overlapping PCR products of about 1000 bp and 500 bp, respectively. All nuclear PCR products were sequenced with their respective amplification primers at the Genoscreen Company. Sequences were read and edited with the DNA sequencing software Chromas, version 2.6.2 (South Brisbane, Queensland, Australia) to check chromatograms before use for phylogenetic and distance analyses.

A single COI sequence was subsequently aligned using the software Clustal W [17] implemented in Mega 7 [18] with 13 other sequences characterizing *Metapolystoma*, *Polystoma* and *Wetapolystoma* spp. that were extracted from GenBank. Six of these polystomes were from North and South America, while all others were from Eurasia and Africa. At last, two species of *Eupolystoma* were used as an outgroup for tree rooting (see [19]). Following the Akaike Information Criterion (AIC) implemented in Modeltest 3.06 [20], a GTR model (nst = 6; rates = invgamma; ngammacat = 4) was selected for the final COI dataset that comprised 395 characters. A Bayesian analysis was conducted using MrBayes 3.04 [21], with four chains running for ten million generations and sampled every 100 cycles. Convergence was assessed with the

program Tracer v1.7.1 (http://beast.community/tracer). A consensus tree was then reconstructed after removing the first 10,000 trees (10%) as the burn-in phase. Finally, COI p-distances as well as total differences were computed with Mega 7 for species delimitation following threshold designed in Du Preez et al. [22] for anuran polystomes.

3. Results

3.1. Host species diversity and levels of infection

Following fieldwork sampling, 320 amphibians were collected covering one caecilian and 20 frog species (Table 1). One specimen of the two *T. compressicauda* was infected with a single worm of *N. tinsleyi* (collection of LdP) and one specimen of the two *T. typhonius* was infected with six worms of *P. lopezromani* (CHIOC 38412–38,414, MPEG 0080) (Table 1). Of the 192 specimens of *P. ephippifer*, two from municipality of Irituia, Pará, Brazil (-186382 S, −47,41287 W) and one from municipality of Igarapé-Açu, Pará, Brazil (−1,1315S, −47,6825 W) released polystome eggs. Following dissections, only the three ones that released eggs were found being infected with respectively 3, 3 and 6 worms. The Prevalence and Mean intensity were 4.0% and 4.0, respectively, for the frog sample of Irituia and 1.3% and 2.0 for the frog sample of Igarapé-Açu.

3.1.1. Systematic

Class Monogenea Carus 1863
Subclass Polyopisthocotylea Odhner, 1912
Order Polystomatidea Lebedev, 1988
Family Polystomatidae Gamble, 1896
Polystoma Zeder, 1800

3.1.2. Polystoma

3.1.2.1. Differencial diagnosis. Polystoma is characterized by the presence of one pair of vaginae, a single diffuse post-ovarian testis, an opisthaptor with a single pair of hamuli, a short, preovarial uterus containing several eggs, an ovary situated anteriorly and a diverticulated digestive tract with or without prehaptorial anastomoses (See [23,24]).

3.1.3. Polystoma goeldii n. sp.

3.1.3.1. Specimens studied. Morphological description based on nine sexually mature worms. The holotype (MPEG 00355) and six paratypes (MEPG MEPG 00356–00358) are hosted in Museu Paraense Emilio Goeldi, Belém, Pará State, Brazil; two other paratypes NMBP 906–907) are housed in the Parasitic Worm Collection, National Museum, Bloemfontein, South Africa.

Type host: *Physalaemus ephippifer* (Steindachner 1864) sexually mature male.

Type locality: Ramal do Maneta, Itabocal Village, municipality of Irituia, Pará, Brazil (-186382 S, 47,41287 W).

Other localities: Municipality of Igarapé-Açu, Pará, Brazil (−1,1315 S, −47,6825 W).

Site: Urinary bladder.

Description: Description and measurements based on seven adults, egg-producing parasites. Marginal hooklets measurements are also given for oncomiracidia.

Adult: Body elongate and pyriform, total length 2371–4893 (3327), greatest width 786–2403 (1356), width at vagina 515–940 (661), haptor length 747–1706 (1027); haptor width 1237–2852 (1712); haptor length to body length ratio 0,23–0,39 (0,31). Mouth sub-terminal, ventral. Oral sucker 199–277 (242); pharynx length 150–189 (173); pharynx width 115–171 (147). Intestine forms a reticulated network of

Table 1
Amphibians collected and screened for polystomes.

Amphibian	GPS coordinates	Number of amphibians collected	Number of amphibians dissected	Polystome recovery
Typhlonectes compressicauda	−186382 S; −47,41287 W	2	2	1 worm
Dendrosophus leucophyllatus	−107981 S; −46,73839 W	5	5	–
Dendrosophus leucophyllatus	−118581 S; 46,67014 W	1	1	–
Hypsiboas boans	−118581 S; 46,67014 W	1	1	–
Hypsiboas calcaratus	−118581 S; 46,67014 W	4	4	–
Hypsiboas cinerascens	−107829 S; −46,73974 W	1	1	–
Hypsiboas fasciatus	−118581 S; 46,67 014 W	7	4	–
Hypsiboas geographicus	−118581 S; 46,67 014 W	2	2	–
Hypsiboas grandulosus	−118581 S; 46,67 014 W	7	4	–
Leptodactylus longicollis	−107829 S; −46,73 974 W	1	1	–
Leptodactylus longicollis	−107921 S; 46,73 854 W	2	2	–
Leptodactylus pentadactylus	−187017 S; 47,39 576 W	3	3	–
Leptodactylus sp.	−186382 S; −47,41 287 W	2	2	–
Leptodactylus sp.	−118581 S; 46,67 014 W	7	4	–
Osteocephalus sp.	−107981 S; −46,73 839 W	8	4	–
Osteocephalus taurinis	−107829 S; −46,73 974 W	1	1	–
Phylomedusa tomopterna	−107981 S; −46,73 839 W	17	6	–
Physalaemus ephippifer	−186382 S; −47,41 287 W	75	19	12 worms
Physalaemus ephippifer	−1,0746 S; −46,7384 W	42	8	–
Physalaemus ephippifer	−1,1315 S; −47,6825 W	75	11	2 worms
Pipa pipa	−187007 S; −47,39445 W	3	3	–
Pipa pipa	−186382 S; −47,41287 W	1	1	–
Pipa pipa	−107829 S; −46,73974 W	1	1	–
Rhinella margaritifera	−107921 S; 46,73854 W	1	1	–
Rhinella margaritifera	−118581 S; 46,67014 W	5	5	–
Rhinella marina	−187007 S; −47,39445 W	3	3	–
Rhinella marina	−186382 S; −47,41287 W	7	7	–
Rhinella marina	−118581 S; 46,67014 W	3	3	–
Schinax boesemani	−187007 S; −47,39445 W	4	4	–
Schinax boesemani	−107981 S; −46,73839 W	6	6	–
Schinax sp.	−118581 S; 46,67014 W	21	21	–
Trachycephalus typhonius	−186932S; 47,39309 W	1	1	6 worms
Trachycephalus typhonius	−107940 S; −46,73871 W	1	1	–

anastomoses (Fig. 1A). Testes and vas deferens obscured by intestinal caeca; seminal vesicle a dilatation of vas deferens, sigmoid, crossing midline, dorsal to ootype and uterus. Vas deferens widens slightly anteriorly forming a seminal vesicle, narrows to open at common genital bulb. Genital pore posterior to intestinal caeca bifurcation; genital atrium muscular; genital bulb relatively small in relation to the body size, armed with nine genital spines; genital spines length 31–35 (33) (Fig. 1B). Ovary sinistral pear-shaped, anterior in body, curved; ovary length 296–564 (429), ovary width 155–278 (242). No intra-uterine development, operculated egg. Two vaginae, on lateral margins just anterior to the level of the ovary, vaginal vestibule cup-shape with soft tissue; vaginal canal elongate with soft tissue. Haptor with three pairs of suckers and a pair of hamuli posteriorly between posterior most sucker pair. Haptoral suckers mean diameter 247–361 (306). Hamulus without a cut between handle and guard, solid and the outer length (Y) representing 67% of the inner length (X), hamulus length to tip of the handle 339–424 (331), hamulus length to tip of the guard 253–312 (284), handle longer than guard and mean X/Y ratio 1,33–1,49 (1,45) (Figs. 1C–D), hamulus hook length 50–66 (58) (Figs. 1C–D). 16 marginal hooklets (Figs. 1E–L; marginal hooklet pairs 1 and 2 located along the periphery between the posterior most pair of suckers, marginal hooklet pairs 6–8 located anteriorly in the haptor between sucker pair 3, marginal hooklet pairs 3–5 imbedded in the suckers.

Oncomiracidia: The ciliated body of the oncomiracidia is elongated and cylindrical, total length 186–188 (186). Placement of marginal hooklets as for other polystomes; pairs 1 and 2 posterior most between suckers 1; pairs 3, 4 and 5 at bases of suckers and pairs 6–8 anterior in haptor between suckers 3. Hooklet I, longest and largest with length 27–29 (28), hooklets II–VII of equal length 20–21 (20) and hooklet VIII with length 25 (Figs. 1 E–L).

Neotenic: As oncomiracidia, hooklet I, longest and largest with length 28, hooklets II–VII of equal length 20–21 (20) and hooklet VIII with length 25.

Etymology: The species is named after Emílio Augusto Goeldi (1859–1917) in recognition of his immense contribution to the knowledge of the Amazonian wildlife.

Remarks: The newly proposed species of *Polystoma* shares various morphological characteristics with several other species of the genus (Table 2). *Polystoma goeldii* n. sp. is similar to *P. cuvieri* in terms of body shape (i.e., elongate, pyriform) and body length (2371–4893 in *P. goeldii* n. sp., 2400–4230 in *P. cuvieri*). Nevertheless, it differs from this species by the morphology of the hamuli. The hamuli of *P. goeldii* n. sp. are solid and the outer length (Y) represents 67% of the inner length (X) with not cut between them (Figs. 1 C-D) while the hamuli of *P. cuvieri* are unnotched to notched root. In addition, both species differ from each other in the haptor/total body length ratio (0.27 for *P. goeldii* n. sp. and 0.36 for *P. cuvieri*). The newly described species also differs from *P. cuvieri* and other species of *Polystoma* from Neotropical Realms by having the genital bulb relatively small in relation to the body size and the number of genital spines associated to genital bulb: nine in *P. goeldii* n. sp. and eight in the others Neotropical polystomes species.

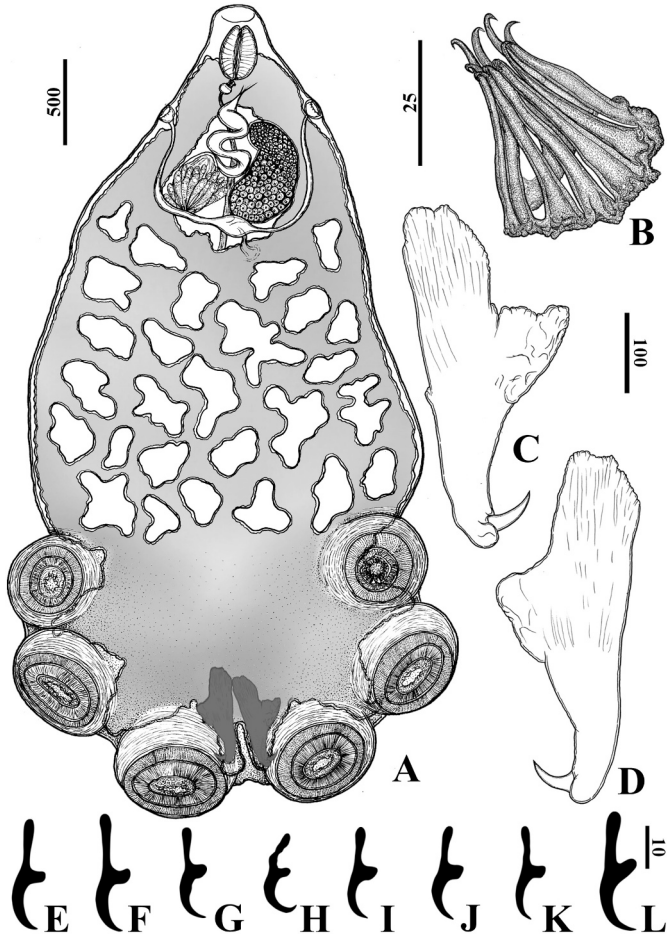

Fig. 1. General morphology of *Polystoma goeldii* n. sp. **A.** Holotype whole-mount, ventral; **B.** Crown of genital spines; **C-D.** Large hamuli from the holotype; **E–L.** Marginal hooklets 1–8, from left to right. Scale bars Fig. 1A (500 μm), Fig. 1B (25 μm), Figs. 1C–1D (100 μm), Figs. 1E–1L (10 μm).

3.2. Life cycle

During the host breeding period, bladder worms lay eggs that develop into aquatic swimming larvae (oncomiracidia) infesting the branchial cavities of tadpoles. The developing embryo was noticeable around day nine and eggs hatched around day 13. It was observed that the larvae undergo an accelerated development of their reproductive organs, becoming neotenic between sixteen and twenty-six days after tadpoles infection. The new species of *Polystoma* from the type locality from host # 1 produced at the peak about 325 eggs /24 h (day 1, 325 eggs; day 2, 21 eggs; day 3, 9 eggs), while specimens from host # 2 produced at the peak about 18 eggs /24 h (day 1, 18 eggs; day 2, 3 eggs; day 3, 0 egg; day 4, 0 egg; day 5, 0 egg). The specimen from Igarapé-Açu produced at the peak 58 eggs/24 h (day 1, 21 eggs; day 2, 58 eggs; day 3, 4 eggs; day 4, 1 egg; day 5, 1 egg).

3.3. Phylogenetic and distance analyses

Bayesian reconstructions with COI (Fig. 2) suggested that *P. goeldii* n. sp. is sister species to *P. cuvieri*, a polystome that was collected from *Physalaemus cuvieri* in Paraguay [22]. The COI genetic p-distance that was estimated between these two species was 0.025, which corresponds to 9 differences, while it varied from 0.025 to 0.199 between species of the distinct genera *Metapolystoma*, *Polystoma* and *Wetapolystoma* (Table 3). Because that distance is above the threshold defined within anuran polystomes, which was fixed to about 0.02 (i.e. 2.0%) in the COI [22], it supports the proposals of a new species of *Polystoma* eventhough no character difference was reported in the 18S and 28S genes between *P. goeldii* n. sp. and *P. cuvieri*. Du Preez et al. [22] indeed noticed that the 28S genetic differentiation observed between two different valid species could be in some cases similar to that observed between conspecifics. All new sequences were deposited under GenBank accession numbers OP537251, OP537252, OP538666 and OP538667.

4. Discussion

This study is the first to report the formal description of a new polystome species infecting the urinary bladder of a frog species in the Brazilian Amazon. It is based on morphology and complemented by genetics in order to solve problems with the limited interspecific

Table 2
Morphometrical data for Neotropical *Polystoma* spp.

Species	*Polystoma goeldii* n. sp.	*P. andinum*	*P. borelli*	*P. cuvieri*	*P. diptychi*	*P. guevarai*
Reference	Present study	[42]	[42]	[43]	[43]	[44]
Body length (BL)	2371–4893 (3327)	4900–8000(6100)	4200–5600 (5100)	2400–4230 (3600)	8300	6790–7880
Body maximum width	786–2403 (1356)	1500–2400 (1900)	2000–3200 (2500)	900–1700 (1400)	2600	2050–2390
Haptor length (HL)	747–1706 (1027)	1300–2300 (1600)	1300–1700 (1500)	900–1400 (1300)	2900	960–1330
Haptor width	1237–2852 (1712)	1400–3300 (2500)	2300–3200 (2600)	1200–2100 (1800)	3400	1940–2180
HL/BL ratio	0,23–0,39 (0,31)	0,26	0,29	0,36	0,35	0,10
Sucker diameter	247–361 (306)	380–585 (490)	510–550	295–434	755–836	316–401
Hamulus length	339–424 (331)	–	350–530 (430)	278–413	970–980	298–348
Hamulus hook length	50–66 (58)	–	–	48–68 (59)	–	–
Hamulus shape	Solid	Solid to shallow cut	Solid to deep cut	Solid	Shallow cut	Solid
Pharynx length	150–189 (173)	200–305 (243)	229–274 (250)	164–245 (214)	–	286–342
Pharynx width	115–171 (147)	195–270 (223)	200–285 (240)	131–205 (183)	330	230–274
Anastomoses	Network	Network	Network	Network	Network	1–2
Ovary length	296–564 (429)	570–940 (725)	–	–	–	–
Ovary width	155–278 (242)	340–600 (430)	–	–	–	–
Egg length	158–177 (166)	230–283 (246)	230	165	–	–
Egg width	112–115 (113)	125–135 (133)	120	90–106	–	–
No. of genital spines	9	8	8	8	–	–
Genital spine length	31–35 (33)	54	–	13–28 (18)	–	–
Marginal hooklet 1 length	27–29 (28)	–	–	–	–	–

Species	*P. knoffi*	*P. lopezromani*	*P. naevius*	*P. napoensis*	*P. praecox*	*P. stellai*	*P. touzeti*	*P. travassosi*
Reference	[7]	[44]	[45]	[46]	[42]	[47]	[46]	[7]
Body length (BL)	5198–10,625 (7386)	6990–8160	3864–5876	3120–3470	3000–6400 (4600)	7100	4180	4980–7820 (5869)
Body maximum width	1590–3409 (2494)	2220–2730	1225–1625	1290–1490	700–1900 (1200)	2100–2600	755	1500–2360 (1852)
Haptor length (HL)	1191–1818 (1473)	1160–1430	805–982	1000–1220	900–1200 (1000)	1400	815	800–1373 (969)
Haptor width	1654–2840 (2207)	1600–2110	1062–1685	1200–1410	1000–1900 (1300)	2100	1020	1640–2000 (1741)
HL/BL ratio	0,21	0,18	0,18	0,34	0,22–0,27	0,20	0,20	0,15
Sucker diameter	320–470 (398)	316–401	273–370	286–403	260–410 (330)	350–380	270–311	315–360 (338
Hamulus length	420–571 (509)	544–606	–	286–368	350–377	480	315–319	390–505 (436)
Hamulus hook length	84–100 (94)	–	–	–	–	–	–	75–98 (84)
Hamulus shape	Deep cut	Deep cut	–	–	Deep cut	–	Moderate cut	Deep cut
Pharynx length	240–430 (318)	292–330	161–402	186–209	110–220 (200)	–	213	280–325 (303)
Pharynx width	210–325 (266)	201–241	128–300	139–153	140–210 (170)	–	176	212–270 (237)
Anastomoses	Network	Network	Network	Network	0	Network	0	Network
Ovary length	235–1095 (577)	–	–	–	–	–	–	710–1360 (915)
Ovary width	132–670 (378)	–	–	–	320–620 (425)	530	–	310–580 (396)
Egg length	–	–	–	–	–	–	–	–
Egg width	–	–	–	–	320–620 (425)	530	–	–
No. of genital spines	8	–	–	–	–	21	–	8
Genital spine length	31–42 (34)	–	–	–	–	–	–	41–45 (44)
Marginal hooklet 1 length	31–34 (32)	–	–	–	–	–	–	23–27 (25)

morphological variation [25,26]. Haptoral and reproductive structures have proved to be especially useful as taxonomic characteristics to distinguish polystome species [27]. The hamulus shape of the species proposed here is solid (Figs. 1 C-D), similarly to *P. cuvieri*, *Polystoma guevarai* Combes & Laurent, 1979 and *Polystoma andinum* Combes & Laurent, 1978. On the other hand, the newly described species differs from these species by having the length of the outer representing two-thirds of the inner length. Additionally, it has nine genital spines compared to the eight genital spines reported in *P. andinum*, *Polystoma borelli* Combes & Laurent, 1978, *P. cuvieri*, *P. knoffi* and *P. travassosi* and to the 21 reported in *Polystoma stellai* Perez-Vigueras, 1955 (Table 2). Finally, *P. goeldii* n. sp. is characterized by having the genital bulb relatively small in relation to the body size compared to that of other anuran polystomes [28].

The diversity of polystomatids in Brazil is now represented by six species, five of which belong to *Polystoma* and one to *Nanopolystoma*. Of these, *P. goeldii* n. sp., *P. lopezromani* and *N. tinsleyi* are the first species to be documented from the Brazilian Amazon. With the great diversity of frogs in this ecosystem, it is likely that a vast number of polystomes are still undiscovered, though frog species are not equally susceptible to these worms. Indeed, whereas the anuran genus *Ptychadena* Boulenger, appears to be a very susceptible host for polystomes in Africa (see [29,30]), like *Boophis* Tschudi in Madagascar [31], polystomatid infections tend to be overdispersed. To understand polystomatid host preferences and distribution patterns, it is equally important to know which hosts do not harbor parasites than those, which are infected by polystomatids. We thus suggest that negative findings should be also reported (see Table 1) as in Du Preez et al. [32] following an intensive frog survey in French Guiana. Therefore, frogs from Amazonia need further investigation in order to increase the knowledge about frogs' polystomes in the Neotropical Realm.

Like many other parasites, there is a complexity in the biological life cycle of the polystomes. In the present study, infection of *P. ephippifer* with oncomiracidium of *P. goeldii* n. sp. corroborates the life cycle of *Polystoma* as already reported [9]. As observed in this study, the oncomiracidium does not infect directly the bladder of the frog or tadpole, but enters the branchial chamber via the sinistrally positioned spiracle. When it makes contact with a young tadpole in pro-metamorphosis, it establishes on the gills and gives rise to a branchial form that develops rapidly and starts producing eggs within two weeks. If a tadpole in pro-metamorphosis is infected, the oncomiracidium establishes on the gills and develops slowly into a bladder-destined parasite that migrates to the bladder when the front legs of the developing tadpole break through [9]. Kok & Du Preez [9] also stated that the bladder parasite grows into the adult parasite whereas the neotenic parasite dies during tadpole metamorphosis. In order for this to happen, the lifecycle of the parasite must be synchronized with that of the host [33,34].

It is well documented that *P. ephippifer* reproduces in isolated

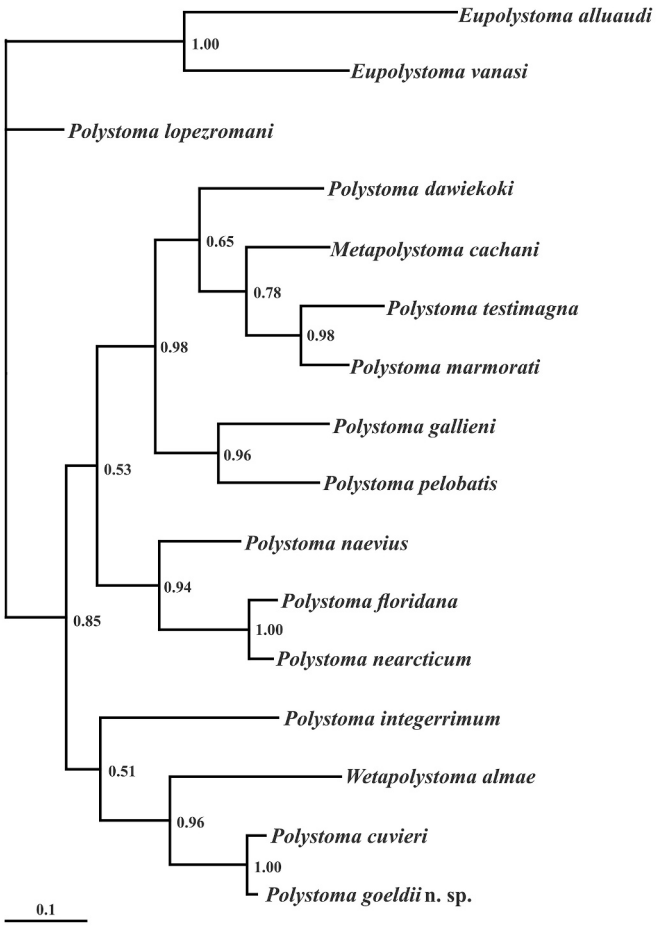

Fig. 2. Bayesian tree for Anuran polystomes inferred from COI analysis.

temporary water pools, making foam nests [35,36]. Although this is seen as a safety measure against predation, both host and parasite are in constant risk, mainly, because they are susceptible to desiccation. As stated by Badets et al. [34], tadpoles have to complete metamorphosis before the ponds dry up, as well as the neotenic parasites must develop rapidly and produce eggs that need to incubate for around 20 days in order to give rise to a second generation of free swimming infective larvae, which themselves have to find a suitable host tadpole. Even though the population of *P. ephippifer* is commonly found, Hödl [36] asserted that calling and breeding activities is associated with increasing rainfall at the beginning of the rainy season, unique period when they can be seen and heard. Then, it is noteworthy to mention that polystomes must utilize the rainy season to infect new hosts. Du Preez and Kok [37] reported that low rainfall would seriously affect anuran reproduction and, as a consequence, parasite transmission. To increase transmission efficiency during the short reproduction period, some polystomatids adopted ovoviviparity and large egg production in few days [38] as reported in *Polystoma integerrimum* (Fröhlich, 1798). This exceptional reproductive output of *P. integerrimum* is short-lived, 90% of the total annual egg production takes place in four days [39]. The low prevalence of *P. goeldii* n. sp. (about 1%) may be associated with the reproductive behavior of its host, as previously observed in other studies [40,41].

5. Conclusions

Our study reports for the first time the occurrence of members of *Polystoma* in amphibians from Brazilian Amazon. From this diversity, a new species of the genus is recovered from the urinary bladder of the Steindachner's Dwarf Frog, *Physalaemus ephippifer*. Morphological and molecular data support the proposal of the new species to accommodate *Polystoma goeldii* n. sp. and indicate the closer relationship of this species to *P. cuvieri*, a polystome that was collected from *Physalaemus cuvieri* in Paraguay.

Table 3
COI p-distances (lower left) and total differences (upper right) inferred from pairwise comparisons in MEGA7.

	P. c.	P. g.	P. t.	P. m.	P. g.	P. p.	P. d.	P. l.	P. n.	P. f.	P. n.	P. i.	W. a.	M. c.	E. a.	E. v.
Polystoma cuvieri (P. c.)		9	49	49	53	53	57	51	55	53	56	53	50	48	80	71
Polystoma goeldii n. sp. (P. g.)	0,025		52	50	58	56	57	51	54	48	51	50	46	50	74	71
Polystoma testimagna (P. t.)	0,131	0,146		30	61	51	49	51	52	56	60	63	56	31	78	69
Polystoma marmorati P. m.)	0,133	0,142	0,081		51	48	45	43	49	52	54	64	58	29	72	70
Polystoma gallieni (P. g.)	0,142	0,156	0,168	0,142		42	47	56	64	46	59	62	60	49	78	80
Polystoma pelobatis (P. p.)	0,152	0,160	0,146	0,136	0,120		46	54	51	51	57	70	65	40	75	73
Polystoma dawiekoki (P. d.)	0,154	0,162	0,133	0,122	0,131	0,132		50	49	56	58	66	61	35	71	62
Polystoma lopezromani (P. l.)	0,137	0,144	0,137	0,117	0,155	0,155	0,136		49	49	57	56	55	40	71	61
Polystoma naevius (P. n.)	0,143	0,146	0,139	0,131	0,169	0,145	0,133	0,132		38	41	61	61	47	73	70
Polystoma floridana (P. f.)	0,162	0,155	0,171	0,156	0,146	0,165	0,171	0,150	0,114		14	56	62	35	69	59
Polystoma nearcticum (P. n.)	0,152	0,143	0,163	0,149	0,162	0,163	0,160	0,156	0,111	0,044		53	67	43	74	68
Polystoma integerrimum (P. i.)	0,144	0,142	0,171	0,171	0,173	0,199	0,179	0,152	0,163	0,168	0,146		60	49	79	77
Wetapolystoma almae (W. a.)	0,133	0,129	0,149	0,155	0,165	0,185	0,165	0,148	0,160	0,186	0,182	0,160		47	75	80
Metapolystoma cachani (M. c.)	0,145	0,148	0,096	0,091	0,145	0,126	0,110	0,125	0,139	0,127	0,133	0,155	0,146		60	54
Eupolystoma alluaudi (E. a.)	0,222	0,216	0,216	0,199	0,223	0,220	0,197	0,197	0,202	0,211	0,208	0,219	0,208	0,194		64
Eupolystoma vanasi (E. v.)	0,190	0,191	0,190	0,196	0,212	0,209	0,173	0,169	0,185	0,187	0,187	0,215	0,220	0,160	0,183	

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Data availability

No data was used for the research described in the article.

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