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GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

A new species of *Rhabdias* (Nematoda: Rhabdiasidae), a lung parasite of *Pristimantis chiastonotus* (Anura: Strabomantidae) from the Brazilian Amazon: description and phylogenetic analyses

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Abstract

Rhabdias Stiles and Hassal, 1905 comprises approximately 90 species of parasitic lung nematodes of amphibians and reptiles that have a wide distribution, with 21 species occurring in the Neotropics. In the present study, we describe *Rhabdias waiapi* n. sp. found parasitizing the lungs of the anuran species *Pristimantis chiastonotus* from the Amazon Biome in the Amapá State, Northern Brazil. The new species is characterized by having an elongated body, expansions of the cuticular inflation in the anterior end that become more discrete along the body, an anterior end with a slight constriction at the level of the esophageal apex with four rounded subapical elevations of the body wall, six lips, four near the edge of the oral opening and two more distant lateral ones, and a gradually tapering elongated tail. In addition, molecular analyses and phylogenetic reconstructions were made, with sequences from the coding region of the mitochondrial cytochrome c oxidase subunit I gene. Those results strongly support the status of the new taxon, which formed a poorly supported clade with *Rhabdias* sp. 5 from *Anolis brasiliensis* from Northeast Brazil. *Rhabdias waiapi* n. sp. is the 19th species of the genus described in the Neotropics for amphibians, the 10th in Brazil, the second described from hosts of the family Strabomantidae from the Neotropical region, and the first amphibian nematode species described in the Amapá State.

Keywords Taxonomy · Molecular · Nematodes · Amphibians · Amazon

Introduction

The genus *Rhabdias* Stiles and Hassal, 1905 is represented by 90 species of parasitic lung nematodes of amphibians and reptiles, distributed across several biogeographic regions, with the exception of the poles (Kuzmin and Tkach 2021). Currently, only 18 species are known to parasitize amphibians of the Neotropics, which, when compared to the diversity of amphibians known for the region, shows that this group is undersampled and that there are many Neotropical species still to be discovered (Mendola 2003; Kuzmin and Tkach 2021).

Identifications of *Rhabdias* spp. have mainly been based on the morphology of hermaphroditic females, which have high morphological uniformity, which has caused taxonomic incongruities and compromises the diagnosis of species, hindering parasite surveys and phylogenetic reconstructions (Morais et al. 2020). According to Poulin et al. (2019), the use of genetic data is essential in parasite systematics and

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taxonomy, especially in new species descriptions, as they prevent and solve taxonomic problems, in addition to enabling more complete descriptions and favoring the differentiation and diagnosis of numerous species.

In recent years, descriptions of rhabdiasid nematode species have used morphology and data obtained from molecular biology techniques, providing new evolutionary history data and revealing cryptic species; however, studies involving integrative approaches for the genus are still incipient (Tkach et al. 2014; Müller et al. 2018; Morais et al. 2020; Willkens et al. 2020). Among the 21 species described in the Neotropical region, until now, only *Rhabdias breviensis* Nascimento, Gonçalves, Melo, Giese, Furtado, and Santos, 2013; *Rhabdias glaurungi* Willkens, Rebêlo, Santos, Furtado, Vilela, Tkach, Kuzmin, and Melo, 2020; and *Rhabdias pocoto* Morais, Melo, and Müller, 2020 have been described using morphological and molecular data (Nascimento et al. 2013; Willkens et al. 2020; Morais et al. 2020).

Pristimantis Jiménez de la Espada, 1870 is an anuran genus belonging to the family Strabomantidae, subfamily Pristimantinae Pyron and Wiens, 2011 that is widely distributed in the Americas, occurring from eastern Honduras to Colombia, Ecuador, Peru, and northern Argentina, in addition to Trinidad and Tobago, Grenada, Lesser Antilles, Brazil, and the Guianas (Frost 2021). *Pristimantis chiastonotus* (Lynch and Hoogmoed 1977) is a nocturnal species usually found in low-altitude primary forests in northern Brazil, Guyana, French Guiana, and Suriname (Padial et al. 2014).

During the survey of the helminth fauna of *P. chiastonotus* in the Amapá State, Brazil, lung nematodes of the genus *Rhabdias* were found, morphologically similar to a specimen described by Kuzmin et al. (2015), which the authors had indicated to be a species distinct from its congeners and that needed a formal description. Thus, we describe these nematodes as a new species based on morphological, molecular, and phylogenetic analyses using DNA sequences of coding region of the enzyme cytochrome c oxidase subunit I of mitochondrial DNA (CO1).

Materials and methods

Host collection and morphological study of parasites

During a survey of the parasitic fauna of anurans in the Municipal Natural Park of Cancão, in April 2019, 30 specimens of *P. chiastonotus* were manually captured through an active/visual search. After collection, the specimens were anesthetized with 2% lidocaine hydrochloride (CFMV 2013), measured, weighed, and necropsied. The internal organs were removed, dissected, and analyzed under a stereomicroscope. The nematodes found in the lungs were collected, cleaned in saline solution, sacrificed in heated 70% ethanol, and preserved in microtubes containing room-temperature 70% ethanol. For morphological and morphometric analyses, the nematodes were clarified in Amann lactophenol®, mounted on temporary slides, and observed on an Olympus BX41 microscope equipped with a camera lucida to perform drawings and an Olympus BX53 equipped with an image capture system to capture photomicrographs and take measurements. Prevalence and mean intensity were calculated according to Bush et al. (1997). The morphological measurements of the specimens are presented as the values of the holotype followed by the mean of the paratypes and range in parentheses (reported in micrometers, except where indicated) in accordance with the standardization proposed by Willkens et al. (2020).

For the external ultrastructural analyses, some specimens were postfixed in 1% OsO_4 (osmium tetroxide), dehydrated in an increasing ethanol series, and dried in a CO_2 critical point drier. Subsequently, the helminths were mounted on aluminum supports, coated with a thin layer of gold, and analyzed in a Tescan VEGA 3 scanning electron microscope at the Laboratory of Structural Biology, Biological Sciences Institute, Federal University of Pará (Universidade Federal do Pará — UFPA).

The illustrations were prepared in the CorelDraw 2018 software and processed using the Adobe Photoshop Version 21.0.2 software.

Molecular analyses and phylogenetic study

For molecular analysis, the nematodes were transferred to microtubes containing 100% ethanol and stored in a freezer at −20 °C. The anterior and posterior portions were cut and stored in absolute ethanol to confirm the identity of each sample, and molecular characterization was performed with the medial portion of the nematode body; the hologenophore (Pleijel et al. 2008) was also preserved and deposited in a helminth collection as a voucher. Genomic DNA was extracted in 200 µl of 5% Chelex® Molecular Biology Grade Resin in suspension in deionized water and 2 µl of proteinase K, in accordance with the manufacturer's protocol, and then was incubated at 56 °C for 14 h. The material was then boiled at 90 °C for 8 min and centrifuged at 14,000 rpm for 10 min. Polymerase chain reaction (PCR) was performed to amplify a partial fragment of the cytochrome c oxidase subunit I (COI) gene of mitochondrial DNA using specific primers and cycles in accordance with Müller et al. (2018). The PCR products were analyzed on a 1% agarose gel to determine the yield and size of the amplified fragments and were then purified using a Qiagen® QIAquick PCR Purification kit.

The amplified and purified fragments were subjected to sequencing following the protocol for the Big Dye®

Terminator v.3.1 Cycle Sequencing kit in an ABI 3730 DNA Analyzer at the Human Genome and Stem Cell Research Center, Biosciences Institute, University of São Paulo. The sequences were assembled using Sequencher v. 5.2.4, and then, a search for similar sequences of the same genomic region was performed in BLASTn in NCBI database. For the outgroup, the taxon chosen was *Serpentirhabdias viperidicus* Morais, Aguiar, Müller, Narciso, Silva, and Silva, 2017 (KX350054). For the phylogenetic analyses, the sequences were aligned using the default parameters of the program Muscle (Edgar 2004) implemented in Geneious 7.1.3. The aligment was trimmed at the ends, and the stop codons (translation frame 2, invertebrate mitochondrial) were verified using Geneious 7.1.3 (Kearse et al. 2012). To evaluate substitution saturation, the aligned matrix was tested and the Iss index was estimated using the DAMBE 5 software package (Xia 2013). The number of base substitutions between sequences per site was calculated. Standard error estimates were obtained using a bootstrap procedure with 1000 replicates. Analyses were conducted by the Kimura 2-parameter model using the MEGA5 software package (Kimura 1980, Tamura et al. 2011).

The most appropriate evolutionary nucleotide substitution model was GTR + I + G, determined by the Akaike Information Criterion (AIC) in the jModelTest program (Posada 2008). Phylogenetic reconstructions were performed using the maximum likelihood (ML) and Bayesian inference (BI) methods. ML analysis was performed using RAxML (Guindon and Gascuel 2003). BI analysis was performed using MrBayes (Ronquist and Huelsenbeck 2003). Both analyses were performed in CIPRES Science Gateway (Miller et al. 2010). Bayesian analysis employed the following settings for the dataset: 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. On the burn-in, the first 25% of generations were discarded and the consensus tree (majority rule) was estimated using the remaining topologies; only nodes with posterior probabilities greater than 90% were considered well supported. Maximum likelihood inference (ML) was implemented using bootstrap support values of 1000 repetitions, and only nodes with bootstrap values greater than 70% were considered well supported. The trees were visualized and edited using the FigTree v1.3.1 software (Rambaut 2009).

Results

Systematics

Family: Rhabdiasidae Railliet, 1915

Genus: *Rhabdias* Stiles and Hassall, 1905

Species: *Rhabdias waiapi* Tavares-Costa and Melo n. sp.

Taxonomic summary

Type host: Pristimantis chiastonotus (Lynch and Hoogmoed, 1977) (Amphibia: Strabomantidae: Pristimantinae).

Type locality: Cancão Municipal Natural Park, Serra do Navio municipality, Amapá State, Brazil (0°54′8.68″N, 52° 0′19.62″W)

Site of infection: Lung.

Numbers of specimens/hosts, prevalence, mean intensity of infection, and range: A total of 12 nematodes were found in 10 frogs, *P* = 33.3%; 1.2 (1–2).

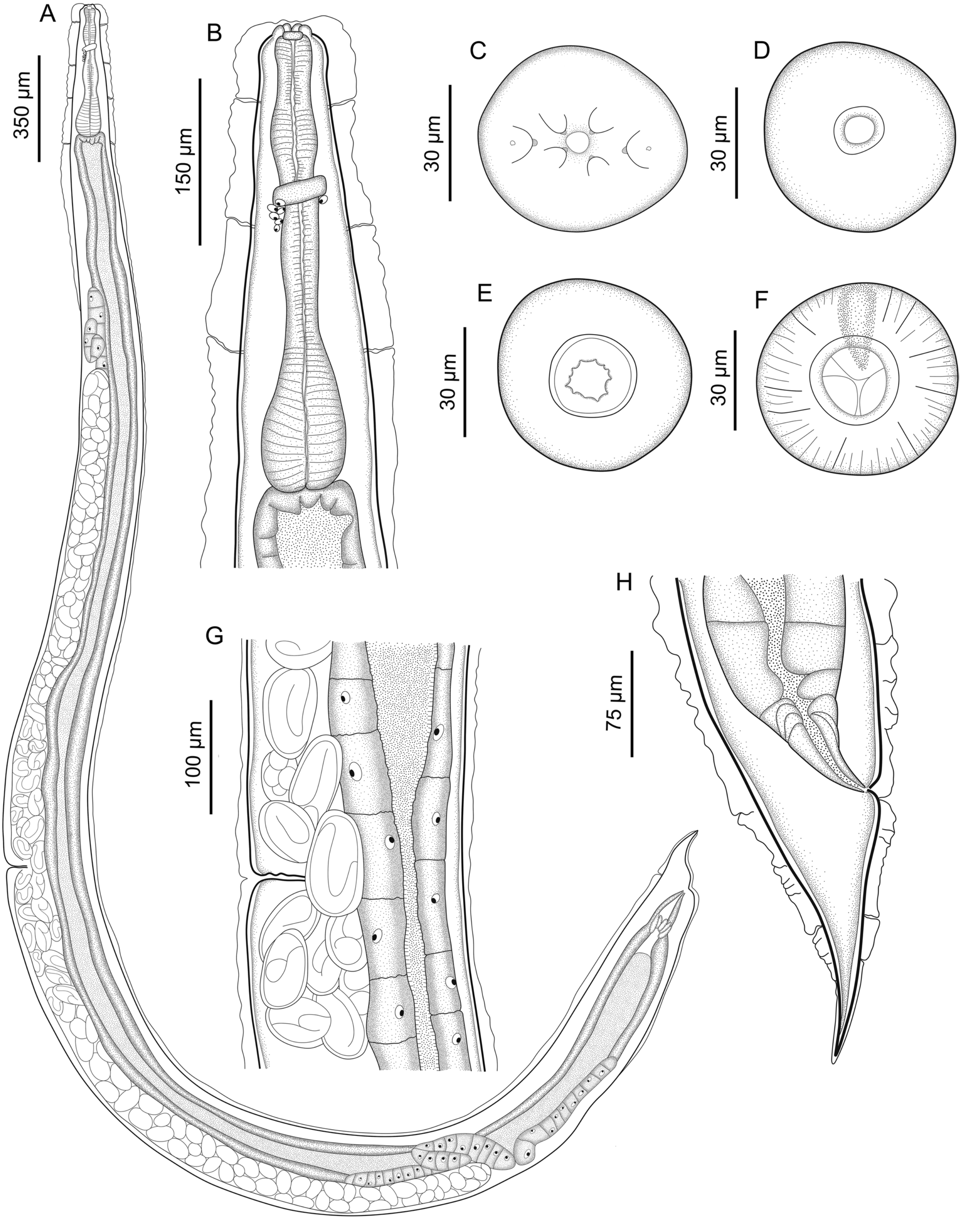
Type material: Holotype and 11 paratypes were deposited at the Museum Emílio Goeldi, under number: Holotype: (MPEG 00266) and paratypes: (MPEG 00267),

GenBank Accession numbers: OL689010, OL689011, OL689012.

ZooBank registration: The Life Science Identifier for *R. waipi* n. sp. is urn:lsid:zoobank.org:act:EEEC35A3-C12C-4E86-927C-F838DF7CD034.

Etymology: The specific epithet is in honor of the "Waiãpi" indigenous group that live in western of Amapá State. This name is a non-Latin nor Greek word and is being established as a noun in apposition

Description (Figs. 1 and 2) (Based on the holotype and 11 paratypes, all gravid hermaphrodites). Body slender, elongated, 5.38; 4.5 (3.36–4.91) mm long (Figs. 1A and 2A). Body surface covered by prominent cuticular inflation, with a dilation at anterior end, that becomes discreet along entire body (Fig. 1B). Body width at vulva 210; 206 (171–267), width at esophagus–intestine junction 130; 144 (123–173). Oral opening surrounded by six lips, four smaller lips close to the edge of the oral opening, lateral lips somewhat larger, situated at some distance from oral opening, each lip has a papilla on its inner edge and amphids located posterior to the lateral lips (Figs. 1C and 2B). Vestibulum has a circular shape in apical view, cylindrical, with narrow lumen (Fig. 1D). Buccal capsule cup-shaped with 8; 8 (8–8) deep and 16; 16 (16–19) wide, 0.50 depth/width ratio; 0.49 (0.43–0.50). Buccal capsule walls consisting of anterior part, with irregular folds in internal surface and posterior part of the capsule with a smooth internal wall (Fig. 1E). Buccal capsule close to the entrance to esophageal lumen has smooth surface. Entrance of esophageal lumen triangular, with rounded edges and esophageal gland, located in the dorsal region of esophagus (Fig. 1F). Esophagus length 423; 452 (405–533), representing 7.84%; 10% (7.84–12.62%) of the body length, claviform with a rounded apex and with distinctly rounded dilation in the anterior muscle region. Width of the anterior end of the esophagus 35; 36 (29–48), width of anterior dilation of the esophagus 44; 48 (42–56), width


A
350 µm
B
150 µm
C
30 µm
D
30 µm
E
30 µm
F
30 µm
G
100 µm
H
75 µm

◂**Fig. 1** Line drawings of *Rhabdias waiapi* n. sp. from *Pristimantis chiastonotus*. (A) Entire body, lateral view; (B) Anterior end of the body, in lateral view; (C) Anterior end of the body, en face view; (D) Optical section through vestibulum; (E) Optical section through anterior part of buccal capsule; (F) Optical section through posterior part of buccal capsule; (G) Mid-body region with vulva, lateral view; (H) Caudal end, lateral view

after dilation 36; 39 (32–43), width of bulb 77; 77 (69–91). Nerve-ring surrounding the esophagus after the previous dilation was located at 162; 164 (145–187) from the anterior end. Excretory pore not observed. Intestine thick-walled. Rectum short, funnel-shaped, and lined with thin cuticle. Contents of intestine brown throughout length. Genital system typical of Rhabdiasidae, amphidelphic with anterior and posterior ovaries, transverse vagina, post-equatorial vulva, located at 2.8; 2.4 (1.9–2.9) mm from the anterior extremity (52%; 55% (51–61%) of the body length) (Figs. 1G and 2C). Lips of the vulva slightly protruding. Thin-walled uterus, with numerous eggs (> 100), embryonated eggs close to the vulva. Egg size 77; 74 (70–80) × 40; 38 (35–43) ($N =$ 10 eggs measured from the uterus of the holotype and from each paratype). Female reproductive system flexed in a "U" shape at 915; 1070 (911–1316) from the anterior extremity (near the esophagus – intestine junction) and 552; 751 (552–963) of the posterior region (close to the rectum). Tail long, gradually tapering 192; 208 (173–274) and 3.6%; 5% (3.6–6.4%) of body length (Figs. 1H and 2D).

Remarks

The new species was attributed to the genus *Rhabdias* based on molecular data and the following morphological traits: inflated body cuticle, presence of buccal capsule, amphidelphic reproductive system with a short transverse vagina, number, shape, and arrangement of the lips surrounding the oral opening and tail shape, in addition having been found parasitizing the lungs of anurans (Kuzmin, 2013; Nascimento et al. 2013).

Rhabdias waiapi n. sp. differs from the other species reported for the Neotropical region by the combination of a unique set of morphological traits: position of the nerve ring, size and proportion of the buccal capsule, shape of cuticular inflation, esophagus size, lip position and size, and tail size. The specimens we found are morphologically similar to a specimen found by Kuzmin et al. (2015) obtained from the same host species collected in French Guiana, and the authors highlighted that it was a species not yet known and that it needed a formal description.

Therefore, considering the set of morphological traits observed in *R. waiapi* n. sp., we propose a new species, and we will compare the new taxon with the species reported in Neotropical anurans that morphologically resemble *R. waiapi* n. sp.; however, as proposed by Willkens et al. (2020), the species *Rhabdias mucronata* Schuurmans-Stekhoven, 1952 and *Rhabdias truncata* Schuurmans-Stekhoven, 1952 will not be included in the comparison because data on the hermaphrodite forms of these species are not available, and their descriptions were performed using juvenile forms found in the host's body cavity. In addition to these species, we will compare the new taxon with *R. hermaphrodita* Kloss, 1971, because it is a Neotropical species for which there is no information about the arrangement of the oral structure in their original descriptions (Kloss 1971; Kuzmin et al. 2015).

Based on the arrangement of the oral structure, *Rhabdias waiapi* n. sp. is similar to the group of species that have six lips, four submedial lips, and two lateral lips: *Rhabdias androgyna* Kloss, 1971; *R. breviensis* Nascimento, Gonçalves, Melo, Giese, Furtado, and Santos, 2013; *R. fuelleborni* Travassos, 1926; *R. galactonoti* Kuzmin, Melo, da Silva Filho, and Santos, 2016; *R. glaurungi* Willkens, Rebêlo, Santos, Furtado, Vilela, Tkach, Kuzmin, and Melo, 2020; *R. manantlanensis* Martínez-Salazar, 2008; *R. pocoto* Morais, Melo, and Müller, 2020; *R. stenocephala* Kuzmin, Melo, da Silva Filho, and Santos, 2016; and *R. tobagoensis* Moravec and Kaiser, 1995.

Rhabdias androgyna Kloss, 1971 described in *Rhinella* cf. *margaritifera* (Laurenti, 1768) (Bufonidae) differs from the new species by the total length (7.9–15 mm *R. androgyna* *vs.* 3.36–5.38 mm *R. waiapi* n. sp.) and by having a wider oral capsule.The relative proportions of the esophagus (4.7–8.0% *R. androgyna* *vs.* 7.84–12.62% *R. waiapi* n. sp.) and position of the vulva also differ, as *R. androgyna* has a pre-equatorial vulva located 3.7–7.2 mm from the anterior end (44.5–50.8% of the body length), while the new species has a post-equatorial vulva located 1.9–2.9 mm from the anterior end (51–61% of the body length). In addition, the anterior region of the *R. androgyna* body has a characteristic shape, which consists of a rounded dilation of the inflation with two layers, with the innermost layer connecting to the body wall in a body dilation similar to a "shoulder"; this characteristic is not observed in *R. waiapi* n. sp. (Kloss 1971; Kuzmin et al. 2015).

Rhabdias breviensis Nascimento, Gonçalves, Melo, Giese, Furtado, and Santos, 2013 from *Leptodactylus petersii* (Steindachner, 1864) (Leptodactylidae), in comparison to the new species, has a smaller and wider body (2.63–3.63 mm × 370–543 *R. breviensis* *vs.* 3.36–4.91 mm × 171–267 *R. waiapi* n. sp.) and has a characteristic dorsal curvature. In addition, *R. breviensis* has a smaller oral capsule (7–13 × 4–9 *R. breviensis* *vs.* 8 × 16–19 *R. waiapi* n. sp.), shorter esophageal length (238–410 *R. breviensis* vs. 405–533 *R. waiapi* n. sp.), and a shorter distance from the anterior end to the nerve ring (41–84 *R. breviensis* *vs.* 187–145 *R. waiapi* n. sp.). The post-equatorial vulva in

Fig. 2 Scanning electron micrographs of *Rhabdias waiapi* n. sp. from *Pristimantis chiastonotus*. (A) Entire gravid hermaphrodite; (B) Apical view (arrows; Ll — lateral lips; Sl — submedian lips); (C) Mid-body region with front view of the vulva (arrows; Vu — vulva); (D) Posterior end, front view (arrows; An — anus). Scale bars: (A) 250 µm; (B) 5 µm; (C) 10 µm; (D) 50 µm

R. breviensis is similar to that in the new taxon, but in this species, the distance from the anterior end to the vulva is greater, corresponding to 65–71% of the body length (post-equatorial vulva), and the tail is shorter (139–191 *R. breviensis* *vs*. 173–274 *R. waiapi* n. sp.) (Nascimento et al. 2013).

Rhabdias elegans Gutierrez, 1945 from *Rhinella arenarum* (Hensel, 1867) (= *Bufo arenarum*) (Bufonidae) differs from *R. waiapi* n. sp. by having a larger body (4.55–9.5 mm *R. elegans*, after Kloss 1974 *vs*. 3.36–4.91 mm *R. waiapi* n. sp.), shorter esophagus (314–490 *R. elegans* *vs*. 405–533 *R. waiapi* n. sp.), and longer tail (255–400 *R. elegans* *vs*. 173–274 *R. waiapi* n. sp.) (Gutiérrez 1945; Kloss 1974). Additionally, Ramallo et al. (2020) collected *R. elegans* parasitizing *R. arenarum* from Argentina and did not observe lips at oral opening, while the new species has oral opening surrounded by four submedian small lips and two large lateral lips.

Rhabdias fuelleborni Travassos, 1926, originally described in *Rhinella diptycha* (= *Bufo marinus*) (Schneider, 1799) (Bufonidae), is larger than the new taxon (10–12 mm *R. fuelleborni* vs. 3.36–4.91 mm *R. waiapi* n. sp.), has smaller relative esophageal proportions (6.2–7.0% *R. fuelleborni* vs. 12.62–7.84% *R. waiapi* n. sp.), a vulva located 3.1–3.4 mm from the anterior end (49.4–49.9% of the body length in *R. fuelleborni* vs. 51–61% of the body length in *R. waiapi*), and a longer tail (313–345 *R. fuelleborni* vs. 173–274 *R. waiapi* n. sp.) (Travassos 1926; Kuzmin et al. 2015).

Rhabdias galactonoti Kuzmin, Melo, da Silva Filho, and Santos, 2016 from *Adelphobates galactonotus* (Steindachner, 1864) (Dendrobatidae) has prominent and uniform cuticular inflation throughout the body, while in *R. waiapi* n. sp., the inflation becomes more discrete along the body. Additionally, compared to the new taxon, is larger (5.60–6.04 mm *R. galactonoti* vs. 3.36–5.38 mm *R. waiapi* n. sp.), has a deeper buccal capsule (10–12 *R. galactonoti* vs. 8 *R. waiapi* n. sp.), and the relative proportions of the esophagus (7.3–8.1% *R. galactonoti* *vs*. 7.84–12.62% *R. waiapi* n. sp.) and position of the vulva also differ, as *R. galactonoti* has a pre-equatorial vulva located 2.5–2.8 mm from the anterior end, while the new species has a post-equatorial vulva located 1.9–2.9 mm from the anterior end (43–50% *R. galactonoti* *vs*. 51–61% *R. waiapi* n. sp.) (Kuzmin et al. 2016).

Rhabdias glaurungi Willkens, Rebêlo, Santos, Furtado, Vilela, Tkach, Kuzmin, and Melo, 2020 found in *Scinax* gr. *ruber* (Laurenti, 1768) (Hylidae) has six lips positioned close to the oral opening, while in *R. waiapi* n. sp., the six lips are arranged differently (four lips near the edge of the oral opening and two more distant lateral ones). In addition, the new species has a wider buccal capsule (16–19 *R. waiapi* n. sp. *vs*. 10–16 *R. glaurungi*) and greater proportions for the esophagus (12.62–7.4% *R. waiapi* n. sp. *vs*. 4.9–8.2% *R. glaurungi*) and tail (3.6–6.4% *R. waiapi* n. sp. *vs*. 3.2–4.3% *R. glaurungi*) (Willkens et al. 2020).

The description of Rhabdias hermaphrodita Kloss, 1971 from Rhinella crucifer (Wied-Neuwied, 1821) (= Bufo crucifer) (Bufonidae) is superficial and incomplete, and the author does not provide any morphometric or morphometric information on the buccal capsule or apical structures, as discussed by Willkens et al.(2020). However, it is possible to differentiate the two species by body length, as R. hermaphrodita measures up to 12 mm in total length, while the new species has 3.36–4.91 mm in total length and by the position of the vulva (49–54% of the body length in R. hermaphrodita *vs*. 51–61% of the body length in R. waiapi n. sp.). In addition, R. hermaphrodita does not present dilation of the cuticular inflation in the anterior region (Kloss 1971*;* Willkens et al.2020*).*

Rhabdias manantlanensis Martinez-Salazar, 2008 of *Craugastor occidentalis* (Taylor, 1941) (Craugastoridae) compared to the new taxon has a larger body (6.48–9.64 mm *R. manantlanensis* *vs*. 3.36–4.91 mm *R. waiapi* n. sp.), larger oral capsule (19–27 *R. manantlanensis* *vs*. 16–19 *R. waiapi* n. sp.), greater distance from the anterior end to the nerve ring (193–244 *R. manantlanensis* *vs*. 145–187 *R. waiapi* n. sp.), slightly pre-equatorial vulva (41.66–51.59% of the body length in *R. manantlanensis* *vs*. 51–61% of the body length in *R. waiapi* n. sp.), and shorter tail (143–232 or 1.5–3.3% of the body length in *R. manantlanensis* *vs*. 173–274 or 3.6–6.4% of the body length in *R. waiapi* n. sp.) (Martinez-Salazar 2008).

Rhabdias pocoto Morais, Melo, and Müller, 2020 described in *Pseudopaludicola pocoto* (Magalhães, Loebmann, Kokubum, Haddad, and Garda, 2014) (Leptodactylidae) is larger (3.41–7.43 mm *R. pocoto* *vs*. 3.36–4.91 mm *R. waiapi* n. sp.), has a deeper buccal capsule (9–17 *R. pocoto* *vs*. 8 *R. waiapi* n. sp.), longer esophagus (475–677 or 8.9–13.9% of the body length in *R. pocoto* *vs*. 405–533 or 7.84–12.62% of the body length in *R. waiapi* n. sp.), equatorial vulva (39–64% of the body length in *R. pocoto* *vs*. 51–61% of the body length in *R. waiapi* n. sp.), and shorter tail (98–163 *R. pocoto* *vs*. 173–274 *R. waiapi* n. sp.). In addition, the anterior region of the *R. pocoto* has two subapical lateral pores connected to an amorphous structure that resembles a gland and this characteristic is not present in *R. waiapi* n. sp. (Morais et al. 2020).

Rhabdias stenocephala Kuzmin, Melo, da Silva Filho, and Santos, 2016 from *Leptodactylus pentadactylus* (Laurenti, 1768) and *Leptodactylus paraensis* (Heyer, 2005) (Leptodactylidae) has, in its anterior end, a distinct constriction of the body and, posteriorly to this constriction, an abrupt widening of the body wall. These morphological characteristics are not observed in the new taxon. Furthermore, *R. stenocephala* is larger (6.9–8.1 mm *R. stenocephala* *vs*. 3.36–4.91 mm *R. waiapi* n. sp.) and has a deeper buccal capsule (8–11 *R. stenocephala* *vs*. 8 *R. waiapi* n. sp.) (Kuzmin et al. 2016).

Rhabdias tobagoensis Moravec and Kaiser, 1995 from *Pristimantis incertus* (Lutz, 1927) (= *Eleutherodactylus terraebolivaris*) (Strabomantidae) differs from *R. waiapi* n. sp. because it is larger (7.34–7.56 mm *R. tobagoensis* vs. 3.36–4.91 mm *R. waiapi* n. sp.), has a deeper and wider buccal capsule (6–9 × 18–21 *R. tobagoensis* vs. 8 × 16–19 *R. waiapi* n. sp.), vulva located near the middle of the body, slightly pre- or post-equatorial (3.33–3.69 mm from the anterior region in *R. tobagoensis* vs. 1.9–2.9 mm in *R. waiapi* n. sp.), and an elongated tail (210–276 *R. tobagoensis* vs. 173–274 *R. waiapi* n. sp.). In addition, the new taxon has a slight constriction at the level of the esophageal apex at the anterior end, with four rounded subapical elevations of the body wall, a characteristic not observed by the authors in *R. tobagoensis* (Moravec and Kaiser 1995).

Molecular analyses and phylogenetic study

Mitochondrial COI sequencing of *R. waiapi* n. sp. resulted in three sequences of 410bp (haplotype 1), 411bp (haplotype 2), and 411bp (haplotype 3), while a BLASTn search revealed no identical match with any other rhabdiasid available in the NCBI database. The alignment of our sequences with those available in GenBank generated a matrix of 357 base pairs. The Iss index indicated no saturation in the transitions or transversions; Iss.c values were greater than the Iss values. Pairwise genetic divergence comparison, considering *Rhabdias* spp. and *Serpentirhabdias viperidicus* (KX350054) as the outgroup, revealed *R. waiapi* n. sp. to be the closest species to *Rhabdias* sp. 5, with a genetic difference of 5.0% (see Supplementary table 1).

Maximum likelihood and Bayesian inference phylogenetic analysis based on 45 taxa revealed similar topologies with two main clades through bootstrap (B) and posterior probability (PP). The sequences of the new species clustered with two sequences of *Rhabdias* sp. 5 (MH548293, MH548292) of *Anolis brasiliensis* Vanzolini and Williams, 1970 (Squamata: Dactyloidae) not supported (0.61 posterior probability and 53 bootstrap) (Fig. 3). The clade *R. waiapi* n. sp. + *Rhabdias* sp. 5 was related as sister of a clade that was subdivided into a branch with a sequence of *Rhabdias* sp. 1 parasitic of *Smilisca* sp. (Hylidae) (KC130697) from Mexico and a large clade that groups two sequences of *Rhabdias* sp. 4 (which is *R. fuelleborni*, Müller et al. in preparation) from *Rhinella diptycha* (Cope, 1862) (= *Rhinella schneideri*) (Bufonidae) (MH548291, MH548290) with *Rhabdias pseudosphaerocephala* from Nicaragua (MK860758), which is a sister group of *Rhabdias pocoto* from *Pseudopaludicola pocoto* (Leuperidae) + clade of the *R. pseudosphaerocephala* species complex from Brazil + *R. glaurungi* from *Scinax* gr. *ruber* (Hylidae) (MK820652) (Fig. 3). The sequences of *Rhabdias breviensis* were divided into two supported clades and formed a not supported clade with a sequence of *Rhabdias vencesi* Junker, Lhermitte-Vallarino, Barbuto, Ineich, Wanji, and Bain, 2010 from *Boophis madagascariensis* (Peters, 1874) (Mantellidae) (FN434104) from Madagascar. The sequences of *Rhabdias mariauxi* Lhermitte-Vallarino, Barbuto, Junker, Boistel, Ineich, Wanji, and Bain, 2009 from *Rieppeleon brevicaudatus* (Matschie, 1892) (Chamaeleonidae) (FN395319) from Africa and *Rhabdias okuensis* Lhermitte-Vallarino, Barbuto, Ineich, Wanji, Lebreton, Chirio, and Bain, 2008 from *Trioceros quadricornis gracilior* (Böhme and Klaver, 1981) (= *Chamaeleo quadricornis gracilior*) (Chamaeleonidae) (FM179479) from Africa formed a well-supported clade, and this clade was related, with no support,

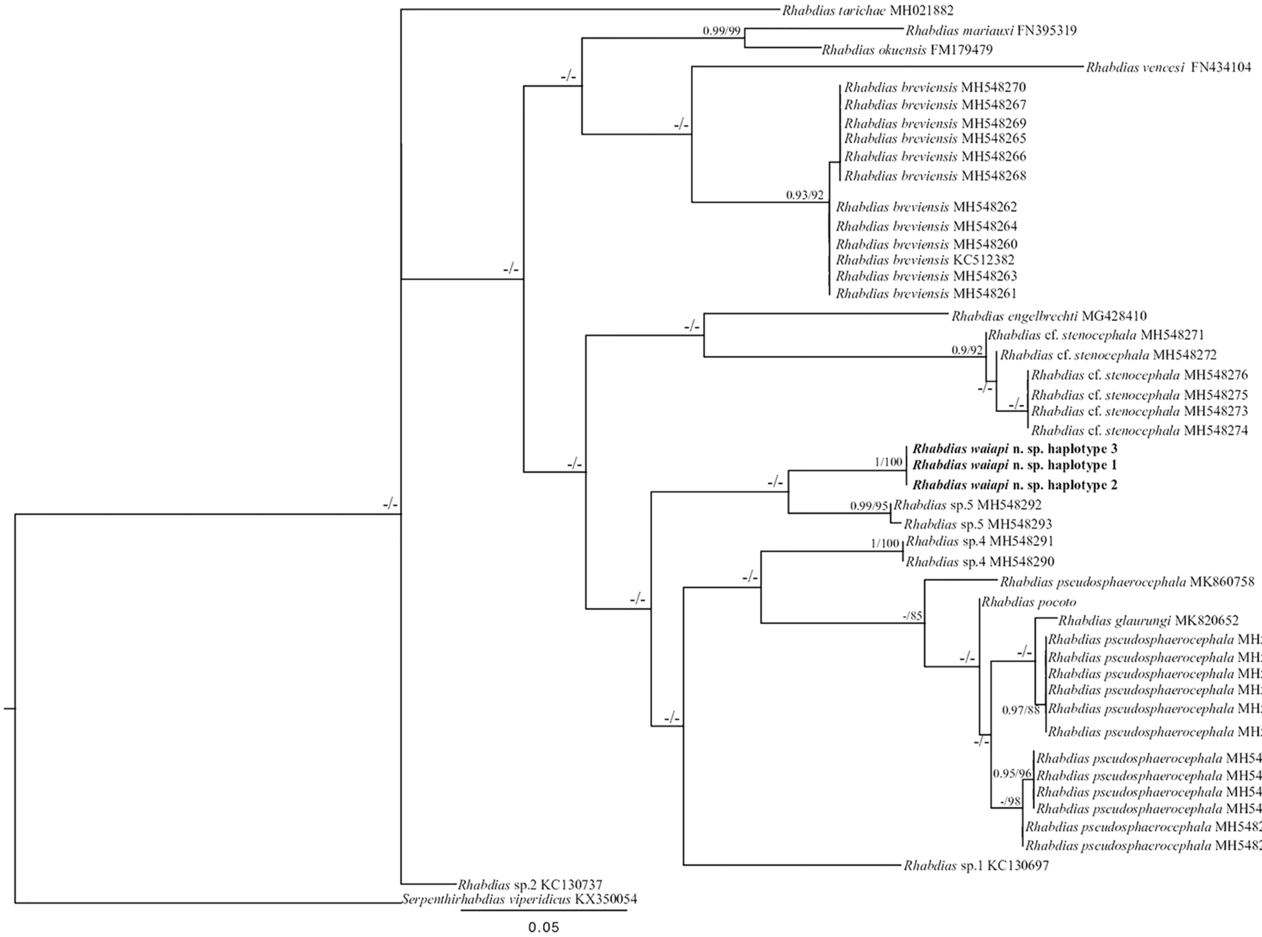


Fig. 3 Maximum likelihood phylogenetic topology of *Rhabdias* spp. of COI gene using *Serpentirhabdias viperidicus* as outgroup indicating the position of *Rhabdias waiapi* n. sp. (represented in bold italics). GenBank accession numbers follow each taxon. Support values are above or below nodes: posterior probabilities <0.90 and bootstrap scores <70 are not shown or are represented by a dash. Branch-length scale bar indicates number of substitutions per site

to the *R. brevienses* + *R. vencesi* species complex. Sequences of *Rhabdias* cf. *stenocephala* parasitic of Leptodactylidae anurans clustered into a clade with multiple lineages, and this clade formed a monophyletic group with *Rhabdias engelbrechti* Kuzmin, Halajian, Tavakol, Luus-Powell, and Tkach, 2017 parasitic of *Phrynomantis bifasciatus* (Smith, 1847) (Microhylidae) from South Africa (Fig. 3).

Discussion

Rhabdias waiapi n. sp. is the 22rd species from the Neotropical region, the 10th from Brazil, and the first described for the Amapá State. Among the morphological traits important to differentiate the new taxon from its congeners, we highlight the shape of the cuticular inflation, arrangement and number of lips, buccal capsule size, esophagus size, and tail length.

The arrangement, organization, and number of circumoral structures are among the main morphological traits used to differentiate *Rhabdias* species. In the Neotropics, the oral structure arrangement is known for 15 species, distributed in three groups: (1) four submedial lips and two lateral pseudolabia (*R. manantlanensis*, *R. savagei*, *R. kuzmini*, and *R. pseudosphaerocephala*); (2) six equal lips (*R. androgyna*, *R. fuelleborni*, *R. breviensis*, *R. galactonoti*, *R. glaurungi*, *R. pocoto*, *R. stenocephala*, and *R. tobagoensis*); and (3) absence of lips (*R. alabialis*, *R. elegans*, and *R. paraensis*) (Tkach et al. 2014; Müller et al. 2018; Morais et al. 2020; Ramallo et al. 2020; Willkens et al. 2020). Three species, for which there is no information about the arrangement of the oral structure in their original descriptions, have not been considered (*R. mucronata*, *R. truncata*, and *R. hermaphrodita*) (Willkens et al. 2020). The new species coincide in the number of lips of the second group of species (six lips). However, it differentiates from most of them by having submedian smaller lips and two lateral larger.

Rhabdias sp. 5 was reported from a molecular study of *Rhabdias* spp. parasites of amphibians and lizards from Brazil. The authors provided the pattern and organization of circumoral structures for this species (four submedial lips and two lateral pseudolabia) (Müller et al. 2018). In the new taxon, we did not observe the presence of pseudolabia; the species has four submedial smaller lips closer to the oral opening and two more distant lateral larger lips. The arrangement of the circumoral structures of these nematodes has been considered one of the best taxonomic traits for species differentiation, but evidence has shown that they may have limited usefulness for phylogenetic inferences (Tkach et al. 2014). These authors observed that after mapping the morphology of the apical structures on the obtained cladogram, the distribution pattern of these morphological traits had no association with different clades. Our results corroborate the hypotheses proposed by these authors that *Rhabdias* species that belong to the same clade may have different apical structure patterns, while phylogenetically distant species may have the same apical pattern. Therefore, similar structures may have evolved independently in several lineages within this genus.

Sequences of the new taxon were grouped with sequences of *Rhabdias* sp. 5 parasitic of *A. brasiliensis* from Chapada do Araripe, forming a poorly supported clade. *A. brasiliensis* and *P. chiastonotus* do not have the same area of occurrence, while the distribution of *P. chiastonotus* is limited to low-altitude forests of North Brazil and the Guiana Shield (Frost 2021). *A. brasiliensis* occurs in the states of Pará, Tocantins, Piauí, Maranhão, Goiás, Mato Grosso, Minas Gerais, São Paulo, Distrito Federal, and Ceará (Ribeiro-Júnior and Amaral 2016). Although they are currently disconnected, the biomes in which both species occur (Amazon and Caatinga) were continuous and formed a single forested region during the Paleogene (Sobral-Souza and Lima-Ribeiro 2017).

The Amazon covered a large geographic area that extended to northern Paraná state (South Brazil) in an area called Pan-Amazonia, but this region has slowly undergone changes. Starting in the Pliocene, geological events resulted in the uplift of the Andes and climate changes in the region. However, it was only at the beginning of the Pleistocene that the Chaco, Cerrado, and Caatinga regions were fully formed, fragmenting Pan-Amazonia (Sobral-Souza et al. 2015). Thus, the clustering observed in the present study of these *Rhabdias* lineages from different hosts and biomes/localities (*Rhabdias waiapi* n. sp. and *Rhabdias* sp. 5) may be related to these complex geological events, which allowed for the encounter of host species and the sharing of parasites.

According to Poulin (2007), the phylogeny of the host is a determining factor for the exchange and/or sharing of parasite species; therefore, in parasitic communities, the evolutionary history of the host acts as one of the determining factors of the community structure. Other factors that influence the structure of the parasite community are biological, physiological, and phylogenetic parameters of the host that may result in an ecological opportunity for adaptation, thus allowing the parasite to colonize one or more species of a given area (D'Bastiani et al. 2020, Rezende et al. 2009, Dormann et al. 2017). Therefore, this sharing of phylogenetically similar parasites observed in host species of different orders (Anura and Squamata) in the present study may be related to phylogenetic attributes and ecological opportunities that modulate interspecific barriers to parasite colonization between hosts.

In addition, Tkach et al. (2014) suggested that reptile colonization occurred more than once in the evolutionary history of *Rhabdias*, and the authors presumed that host exchange and ecological adaptation provided more evolutionary advantages to *Rhabdias* spp. than the association with one host taxon. However, due to the poor support values, the phylogenetic position of *Rhabdias waiapi* n. sp. may change in future studies when more taxa are added to the phylogeny.

During a phylogenetic and biogeographic study with species of the genus, Müller et al. (2018) observed that Neotropical *Rhabdias* have cryptic diversity, identifying two species complexes that have great genetic diversity between the lineages and that were found parasitizing different anuran families: *Rhabdias breviensis* and *R. pseudosphaerocephala*. In our phylogenetic analyses, adding new sequences of the new taxon, we also recovered the clades of the *R. breviensis* and *R. pseudosphaerocephala* species complexes proposed by those authors, further supporting the hypothesis proposed by them.

In our phylogenetic reconstructions, the *Rhabdias breviensis* complex formed a clade with a sequence of *Rhabdias vencesi* from *Boophis madagascariensis* from Madagascar, and this clade was grouped with sequences of *Rhabdias mariauxi* from *Rieppeleon brevicaudatus* and *Rhabdias okuensis* from *Trioceros quadricornis gracilior* both from South Africa. Sequences of *Rhabdias* cf. *stenocephala* clustered into a clade and formed a monophyletic group with *Rhabdias engelbrechti* from *Phrynomantis bifasciatus* from South Africa. Tkach et al. (2014) proposed that the diversification of *Rhabdias* species from the Nearctic and Palearctic occurred before the separation of Laurasia in the Paleocene and early Eocene. In addition, the authors raise the hypothesis that Australian *Rhabdias* may have diverged from rhabdiasid ancestors of Asian origin. Regarding Neotropical and African species, Müller et al. (2018) presume that diversification may also have occurred before the separation of Gondwana in the Paleocene and Eocene, when the South American and African continents were connected. Therefore, this diversification may explain the phylogenetic relationships between the African and Neotropical *Rhabdias* species. However, due to the poor support values, to confirm the evolutionary hypotheses, further biogeographic and genetic data on *Rhabdias* spp. and their hosts are needed.

Kuzmin et al. (2015), during a survey of the anuran helminth fauna of French Guiana, found that *Rhabdias* sp. parasitized *Pristimantis chiastonotus*, and the authors highlighted that it was a species not yet known; however, due to the small sample size, they did not perform a formal description of the species. The border between French Guiana and Brazil is located in the Amapá State. Therefore, based on the geographic location, the host in which it was found and the morphological similarity, we conclude that *Rhabdias waiapi* n. sp. is the species previously found in *P. chiastonotus* from French Guiana

Biodiverse regions are areas with great potential for phylogenetic and taxonomic studies of parasites associated with amphibians. According to Jorge and Poulin (2018), regions with high diversity are areas that should be key targets for efforts to discover new parasite species. The Amazon biome concentrates a large part of the Neotropical herpetofauna, with approximately 355 amphibian species, including 329 anuran species (Hoogmoed and Galatti 2019). The new taxon is the seventh species of *Rhabdias* described in the Amazon biome and the second described for species of the genus *Pristimantis* from the Neotropical region. Therefore, due to the great potential of the Amazon biome, further related studies are needed to unravel the hidden parasite diversity of Amazonian amphibians.

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Data availability Not applicable

Code availability Not applicable

Declarations

Ethics approval The authors assert that all procedures contributing to this work comply with all applicable institutional, national, and international guidelines for the care and use of animals. Specimens were collected under license provided by Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio permit # 48102-2 and #48102-3. The project was approved by the Animal Ethics Committee from Universidade Federal of Amapá, Brazil, process number 0027/18.

Consent to participate Not applicable

Consent for publication Not applicable

Conflict of interest The authors declare no competing interests.

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