

Review Article










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A new species of *Rhabdias* (Nematoda: Rhabdiasidae), a lung parasite of *Pseudopaludicola pocoto* (Anura: Leptodactylidae) from north-eastern Brazil: description and phylogenetic analyses

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Abstract

Rhabdias pocoto n. sp. is herein described from the lungs of the swamp frog *Pseudopaludicola pocoto* Magalhães, Loebmann, Nogueira, Kokubum, Baptista, Haddad & Garda, 2014, from the Caatinga biome in the state of Ceará, in north-eastern Brazil. The new species is characterized by a body that dilates posteriorly, six small lips (protuberances) and two rounded lateral expansions of cuticular inflation on the anterior end, each containing an amorphous gland-like structure inside and a short and conical tail. Additionally, molecular analysis and comparison of the partial mitochondrial cytochrome *c* oxidase I sequence of *R. pocoto* n. sp. revealed genetic divergence between the new species and the sequences of *Rhabdias* spp. previously deposited in GenBank. Phylogenetic analysis grouped the new taxon into the *R. pseudosphaerocephala* species complex + *R. glaurungi* clade. The new discovery represents the 19th species of *Rhabdias* spp. described in the Neotropical region, the ninth in Brazil and the first species of *Rhabdias* found parasitizing South American frogs of the genus *Pseudopaludicola*, as well as the first Caatinga biome species of *Rhabdias*.

Introduction

In the past 20 years, several new species of helminth parasites of amphibians and reptiles have been described in the Neotropics, proving that the diversity of such parasites remains unknown. Among the groups representing this hidden diversity, the genus *Rhabdias* Stiles & Hassal, 1905 is notable for being the most distinct in the Rhabdiasidae and includes 87 species of which 49 have been described in the past two decades (Kuzmin & Tkach, 2020). Furthermore, the genus is represented by lung-dwelling parasites of amphibians and certain reptiles, and occurs in every continent, except Antarctica.

According to Poulin *et al.* (2019), the use of genetic information is fundamental in systematic parasite taxonomy, especially for descriptions of new species, allowing taxonomic problems among some groups to be solved using molecular phylogenies, description of patterns of gene flow among species and populations, and especially for understanding diversity in complex parasite groups such as Rhabdiasidae (Tkach *et al.*, 2014; Müller *et al.*, 2018).

Considering the exceptionally high diversity of amphibians in Brazil (31 families, 1139 species) it is unsurprising that the country also possesses a great diversity of parasites associated with this host group. Nevertheless, the knowledge of parasite species such as representatives of *Rhabdias* is poor, and only eight species have been described in amphibians (four in Bufonidae, two in Leptodactylidae, one in Dendrobatidae and one in Hylidae) (Kuzmin & Tkach, 2020). Among the 18 known species of *Rhabdias* described from the Neotropical region, until now only *Rhabdias brevisensis* Nascimento, Gonçalves, Melo, Giese, Furtado & Santos, 2013 and *Rhabdias glaurungi* Willkens, Rebêlo, Santos, Furtado, Vilela, Tkach, Kuzmin & Melo, 2019

have been described using a combination of morphology and molecular data (Nascimento *et al.*, 2013; Willkens *et al.*, 2019).

The genus *Pseudopaludicola* Miranda-Ribeiro, 1926 (Anura: Leptodactylidae: Leiuperinae) comprises 25 species widely distributed in South America, from northern Argentina to the east of the Andes in Venezuela, and is associated with open formations close to tropical forests, such as the Chaco, Pampas, Cerrado and Caatinga (Segalla *et al.*, 2019; Frost, 2020) biomes. *Pseudopaludicola pocoto* Magalhães, Loebmann, Nogueira, Kokubum, Baptista, Haddad & Garda, 2014 is commonly found in the Caatinga biome in Brazil, in the states of Ceará, Paraíba, Pernambuco, Piauí and Rio Grande do Norte (Magalhães *et al.*, 2014; Silva *et al.*, 2015, 2017), while there is a record of this species in the Atlantic Forest biome in the state of Minas Gerais (Andrade *et al.*, 2015).

During a survey of parasites of *P. pocoto* in the state of Ceará, Brazil, nematodes were found with characteristics that corresponded to the diagnoses of *Rhabdias*; however, due to particular morphological characters, these could not be assigned to any known species of the genus. Thus, we herein describe a new species of *Rhabdias* parasitizing the lungs of *P. pocoto* using both morphological and molecular phylogenetic analyses, based on the mitochondrial gene cytochrome *c* oxidase I (coi).

Material and methods

Host collection and morphological study of parasites

During a helminthological survey performed in February 2014 (rainy period), 115 specimens of *P. pocoto* were collected from the Benguê Reservoir, in the Aiuaba municipality, in the state of Ceará, Brazil (06°35'35"S, 40°08'31"W).

The frogs were anaesthetized with 2% lidocaine hydrochloride (CFMV, 2013) and the internal organs were removed, dissected and analysed under a stereomicroscope. The nematodes found in the lungs were rinsed in saline solution and fixed in pre-heated 70% ethanol. For morphological identification, the specimens were cleared with lactophenol, mounted on temporary slides and observed using a Leica application suite (V3) adapted for a DM5000B light microscope equipped with differential interference contrast optics and a computerized image analysis system (LAS DIC, Leica Microsystems, Wetzlar, Germany).

Some specimens were also analysed using scanning electron microscopy and post-fixed in 1% osmium tetroxide, dehydrated in a graded alcohol series and dried at the critical point of CO₂. The specimens were then mounted on an aluminium stub using conductive double-sided tape, coated with gold-palladium and examined using a Vega3 electron microscope (TESCAN, Brno, Czech Republic) at the Laboratory of Embryology and Histology of the Federal Rural University of Amazonia (Universidade Federal Rural da Amazônia, UFRA).

Morphological measurements are given as the values of the holotype followed by the mean of the paratypes, and range in parentheses (reported in micrometres, unless otherwise indicated) in accordance with the standardization proposed by Willkens *et al.* (2019).

Molecular analyses and phylogenetic study

For molecular analysis, nematodes fixed in heated 70% ethanol were posteriorly transferred to absolute ethanol (Merck®) and stored in a freezer at -20°C for subsequent molecular analysis.

Part of the nematode was taken for scanning electron microscopy (anterior part with buccal capsule) and part (posterior or medium part) for DNA extraction. Genomic DNA was extracted using DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol, with a final volume of 30 µl. A Polymerase chain reaction was performed to amplify the partial mitochondrial coi gene using primers and cycling conditions, as well as purification and sequencing procedures in accordance with Müller *et al.* (2018). The sequence identity was verified using the Basic Local Alignment Search Tool (BLAST), and contiguous sequences were assembled using Sequencer v. 5.2.4 (Gene Codes, Ann Arbor, MI) and submitted to GenBank (accession numbers presented in table 1).

For the molecular phylogenetic analyses, we constructed a matrix using sequences of the coi gene of *Rhabdias* spp. obtained from GenBank (table 1). As an outgroup, the taxon chosen was *Serpentirhabdias viperidicus* Morais, Aguiar, Muller, Narciso, Silva & Silva, 2017 (GenBank accession number KX350054). Sequences were aligned using the default parameters of the Muscle algorithm (Edgar, implemented in Geneious 7.1.3 (Kearse *et al.*, 2012). Stop codons and indels were also checked in Geneious 7.1.3 (Kearse *et al.*, 2012), translation frame 2, table 5 invertebrate mitochondrial. The substitution saturation index was estimated in DAMBE 5 (Xia, 2013) to calculate its occurrence. The best-fit model for nucleotide substitution in the resulting matrix was GTR+I+G, determined by the Akaike information criterion (AIC) in a jModelTest (Posada, 2008).

Phylogenetic reconstructions were performed using maximum likelihood (ML) and Bayesian inference (BI). ML inference was carried out using RAxML (Guindon & Gascuel, 2003) with bootstrap support values of 1000 repetitions. BI analyses were run using MrBayes (Ronquist & Huelsenbeck, 2003), with Markov chain Monte Carlo chains run for 10 million generations and sampling tree topologies for every 1000 generations. 'Burn-in' parameters were set to the first 25,000 generations. Both ML and BI phylogenetic analyses were performed on CIPRES (Miller *et al.*, 2010). The trees were visualized and edited using the FigTree v1.3.1 software program (Rambaut, 2009). Genetic divergence was calculated for the coi gene matrix using the Kimura 2-parameter model, with 1000 bootstrap replicates in MEGA7 software (Kimura, 1980; Tamura *et al.*, 2011).

Results

Systematics

Order: Rhabditida Chitwood, 1933

Family: Rhabdiasidae Railliet, 1915

Rhabdias pocoto Morais, Melo & Müller n. sp.

Taxonomic summary

Type locality: Reservoir Benguê, Aiuaba municipality, state of Ceará, Brazil (06°35'35"S, 40°08'31"W).

Type host: *Pseudopaludicola pocoto* Magalhães, Loebmann, Nogueira, Kokubum, Baptista, Haddad & Garda, 2014 (Amphibia: Leptodactylidae: Leiuperinae).

Site of infection: Lung (mainly the right).

Numbers of specimens/hosts, prevalence, mean intensity of infection and range: a total of 125 nematodes were found in 115 frogs, *P* = 100%; Mean infection intensity (MII) = 1.09 (1–2).

Table 1. Sequences used in the phylogenetic and distance analysis with GenBank accession numbers.

Species	Host species	Collection site	GenBank Accession No.	Source
<i>Rhabdias breviensis</i>	<i>Leptodactylus petersii</i>	Eastern Amazon, Brazil	KC512382	Nascimento <i>et al.</i> (2013)
<i>Rhabdias breviensis</i>	<i>Leptodactylus fuscus</i>	Barro - CE, Brazil	MH548260	Müller <i>et al.</i> (2018)
<i>Rhabdias breviensis</i>	<i>Proceratophrys aridus</i>	Aiuaba - CE, Brazil	MH548261	Müller <i>et al.</i> (2018)
<i>Rhabdias breviensis</i>	<i>Rhinella granulosa</i>	Picus - PI, Brazil	MH548262	Müller <i>et al.</i> (2018)
<i>Rhabdias breviensis</i>	<i>Leptodactylus fuscus</i>	Castilho - SP, Brazil	MH548263	Müller <i>et al.</i> (2018)
<i>Rhabdias breviensis</i>	<i>Scinax fuscovarius</i>	Castilho - SP, Brazil	MH548264 MH548265	Müller <i>et al.</i> (2018)
<i>Rhabdias breviensis</i>	<i>Rhinella schneideri</i>	Castilho - SP, Brazil	MH548266	Müller <i>et al.</i> (2018)
<i>Rhabdias breviensis</i>	<i>Leptodactylus podicipinus</i>	Castilho - SP, Brazil	MH548268 MH548269 MH548267	Müller <i>et al.</i> (2018)
<i>Rhabdias breviensis</i>	<i>Leptodactylus fuscus</i>	Castilho - SP, Brazil	MH548270	Müller <i>et al.</i> (2018)
<i>Rhabdias cf. stenocephala</i>	<i>Leptodactylus macrosternum</i>	Barro - CE, Brazil	MH548271	Müller <i>et al.</i> (2018)
<i>Rhabdias cf. stenocephala</i>	<i>Leptodactylus macrosternum</i>	Farias Brito - CE, Brazil	MH548272	Müller <i>et al.</i> (2018)
<i>Rhabdias cf. stenocephala</i>	<i>Leptodactylus vastus</i>	Barro - CE, Brazil	MH548273	Müller <i>et al.</i> (2018)
<i>Rhabdias cf. stenocephala</i>	<i>Leptodactylus vastus</i>	Aiuaba - CE, Brazil	MH548274 MH548275	Müller <i>et al.</i> (2018)
<i>Rhabdias cf. stenocephala</i>	<i>Leptodactylus vastus</i>	Farias Brito - CE, Brazil	MH548276	Müller <i>et al.</i> (2018)
<i>Rhabdias engelbrechti</i>	<i>Phrynomantis bifasciatus</i>	South Africa	MG428410	Kuzmin <i>et al.</i> (2017)
<i>Rhabdias esculentorum</i>	<i>Rana lessonae</i>	Italy	JN580995	Cipriani <i>et al.</i> (2012)
<i>Rhabdias glaurungi</i>	<i>Scinax gr. ruber</i>	Pará, Brazil	MK820652	Willkens <i>et al.</i> (2019)
<i>Rhabdias mariauxi</i>	<i>Rieppeleon brevicaudatus</i>	Tanzania	FN395319	Lhermitte-Vallarino <i>et al.</i> (2009)
<i>Rhabdias okuenses</i>	<i>Chamaeleo weidersheimi weidersheimi</i>	Cameroon	FM179479	Lhermitte-Vallarino <i>et al.</i> (2008)
<i>Rhabdias pocoto n. sp.</i>	<i>Pseudopalodicola pocoto</i>	Aiuaba - CE, Brazil	MW041239	This study
<i>Rhabdias pseudosphaerocephala</i>	<i>Rhinella schneideri</i>	Castilho - SP, Brazil	MH548278	Müller <i>et al.</i> (2018)
<i>Rhabdias pseudosphaerocephala</i>	<i>Hypsiboas raniceps</i>	Porto Rico - PR, Brazil	MH548279	Müller <i>et al.</i> (2018)
<i>Rhabdias pseudosphaerocephala</i>	<i>Rhinella schneideri</i>	Porto Rico - PR, Brazil	MH548280	Müller <i>et al.</i> (2018)
<i>Rhabdias pseudosphaerocephala</i>	<i>Rhinella jimi</i>	Santana do Cariri - CE, Brazil	MH548281 MH548282 MH548283 MH548284	Müller <i>et al.</i> (2018)
<i>Rhabdias pseudosphaerocephala</i>	<i>Rhinella jimi</i>	Farias Brito - CE	MH548285	Müller <i>et al.</i> (2018)
<i>Rhabdias pseudosphaerocephala</i>	<i>Rhinella jimi</i>	Picus - PI, Brazil	MH548286	Müller <i>et al.</i> (2018)
<i>Rhabdias pseudosphaerocephala</i>	<i>Rhinella marina</i>	Manaus - AM, Brazil	MH548287	Müller <i>et al.</i> (2018)
<i>Rhabdias pseudosphaerocephala</i>	<i>Rhinella marina</i>	Manaus - AM, Brazil	MH548288	Müller <i>et al.</i> (2018)
<i>Rhabdias pseudosphaerocephala</i>	<i>Rhinella schneideri</i>	Botucatu -SP, Brazil	MH548289	Müller <i>et al.</i> (2018)
<i>Rhabdias sp. 4</i>	<i>Rhinella schneideri</i>	Botucatu -SP, Brazil	MH548290 MH548291	Müller <i>et al.</i> (2018)
<i>Rhabdias sp. 5</i>	<i>Norops brasiliensis</i>	Crato - CE, Brazil	MH548292 MH548293	Müller <i>et al.</i> (2018)

(Continued)

Table 1. (Continued.)

Species	Host species	Collection site	GenBank Accession No.	Source
<i>Rhabdias</i> sp.1	<i>Smilisca baudinii</i>	Colima, México	KC130697	Prosser <i>et al.</i> (2013)
<i>Rhabdias</i> sp.2	<i>Rana</i> sp.	Nayrit, Mexico	KC130737	Prosser <i>et al.</i> (2013)
<i>Rhabdias</i> sp.3	<i>Rhinella marina</i>	Colima, México	KC130736	Prosser <i>et al.</i> (2013)
<i>Rhabdias tarichae</i>	<i>Taricha granulosa</i> , <i>Taricha torosa</i>	California, USA	MH021882	Johnson <i>et al.</i> (2018)
<i>Rhabdias vencesi</i>	<i>Boophis madagascariensis</i>	Madagascar	FN434104	Junker <i>et al.</i> , (2010)
<i>Serpentirhabdias viperidicus</i>	<i>Boothrops moojeni</i>	Castilho – SP, Brazil	KX350054	Morais <i>et al.</i> (2017)

New sequence obtained is expressed in bold.

Type material. Holotype and 22 paratypes were deposited at the CHIOC, under number: Holotype: CHIOC 38948 and paratypes: CHIOC 38942 – CHIOC 38963.

GenBank Accession number: MW041239.

ZooBank registration. The Life Science Identifier for *R. pocoto* n. sp. is 175D3EDE-61DD-4B1E-A5F6-34E13A8CD6B5.

Etymology. The new species is named after the specific epithet of the type-host.

Description

Based on the holotype and 22 paratypes, all gravid hermaphrodites. Body 5.88; 5.62 (3.41–7.43) mm long, curved dorsally, gradually tapering from mid-region to anterior end and expanding gradually from mid-region to posterior part of body, with a gradual narrowing of the caudal end (fig. 1a). Anterior end rounded, posterior end extremely pointed. Outer layers of body cuticle prominently swollen, especially at anterior end, with irregular transverse folds along entire body. Presence of lateral pores and ducts (figs 1a and 2a).

Body width at vulva 287; 256 (135–380), width at esophago-intestinal junction 183; 195 (112–187). At anterior extremity, subapical lateral pores surrounded by rounded cuticular expansions on lateral sides of body. Both expansions filled with amorphous gland-like material (figs 1b, f and 2a, b). Oral opening round. Six small lips similar in shape and size; submedian lips located closer to edge of oral opening, lateral lips some distance away (figs 1b and 2c). Each lip associated with one papilla, amphidial openings located at base of lateral lips. Two external pores situated at the edge of oral region. Each pore connected to an amorphous material, glandular-like internal structure below the cuticular inflation (figs 1b, f and 2b, c). Vestibulum cylindrical, cuticularized, with narrow lumen (fig. 1c). Buccal capsule small, cup-shaped (fig. 1c–f). Anterior part of buccal capsule circular in shape, with smooth internal wall (fig. 1d), posterior part with irregularly folded internal surface (fig. 1e). Maximum diameter of buccal capsule 12; 12 (11–17), total depth of both parts 12; 12 (9–17); depth-to-width ratio 1; 1.1 (0.6–1.1). Oesophagus club shaped, 605; 598 (475–677) long, representing 11.6%; 10.8% (8.9–13.9%) of body length, with posterior bulb. Width of oesophagus 48; 53 (43–64) in anterior part; width of middle part 53; 57 (41–76), and 96; (65–137) in maximum width. Nerve-ring surrounding oesophagus, approximately at its mid-length, 303; 299 (238–339) from anterior end of body (fig. 1f). Excretory pore not observed. Intestine thick walled.

Rectum short, funnel shaped and lined with thin cuticle. Contents of intestine brown throughout length (fig. 1a).

Genital system typical of Rhabdiasidae, amphidelphic with anterior and posterior ovaries (fig. 1a). Vulva equatorial, small, vulvar lips distinct, not salient, slightly prominent in some specimens, aperture short and transverse, weakly cuticularized (fig. 1h). Distance from anterior end to vulva 3.0; 2.8 (1.6–4.1) mm, representing 53% (39–64%) of body length. Uteri joined, sac-like, containing numerous eggs (>100 in total); eggs near vulva with fully developed larvae (fig. 1h). Eggs 83–101 × 45–54 (N = 9, measured in uteri close to vulva of holotype). Oviducts long, straight, thick walled, slightly shorter than uterus. Ovaries wide, elongated and reflected in oocyte zone, sometimes with one or two bends closer to the beginning of the oviducts (fig. 1a).

Tail comparatively short, conical (figs 1a, g and 2a, d), length 137; 135 (98–163) and representing 2.3%; 2.8% (2.8–5.8%) of body length. Presence of a pore-shaped phasmid located approximately 26; 27 (17–67) from the tail.

Remarks

The new species have been assigned to the genus *Rhabdias* based on molecular data and the following morphological characters: inflated body cuticle, small buccal capsule, amphidelphic reproductive system with a short transverse vagina and joined uteri; and due to its parasitism in the lungs of amphibian hosts. The number, shape, structure and position of the circumoral lips or pseudolabia, the relative position of the vulva and the shape of the tail are among the essential characters that can be used for specific diagnosis of the genus (Kuzmin *et al.*, 2003; Nascimento *et al.*, 2013).

Rhabdias pocoto n. sp. differs from all congeneric species through the combination of a unique set of morphological characters: (1) body gradually expanding from mid-region to posterior part of the body and gradual narrowing of the caudal end; (2) at anterior end, two lateral pores connected to amorphous material, gland-like internal structure inside the cuticular inflation; (3) relatively short tail.

Therefore, considering the peculiar set of morphological characters in *R. pocoto* n. sp., the new host, record in the Caatinga biome and molecular data, we herein propose a new species, and will compare the new taxon with only those species reported in the Neotropical region which are morphologically similar to *R. pocoto* n. sp. However, as proposed by Willkens *et al.* (2019), we will not use the species *Rhabdias mucronata* Schuurmans-Stekhoven, 1952, and *Rhabdias truncata* Schuurmans-Stekhoven, 1952 for comparison,

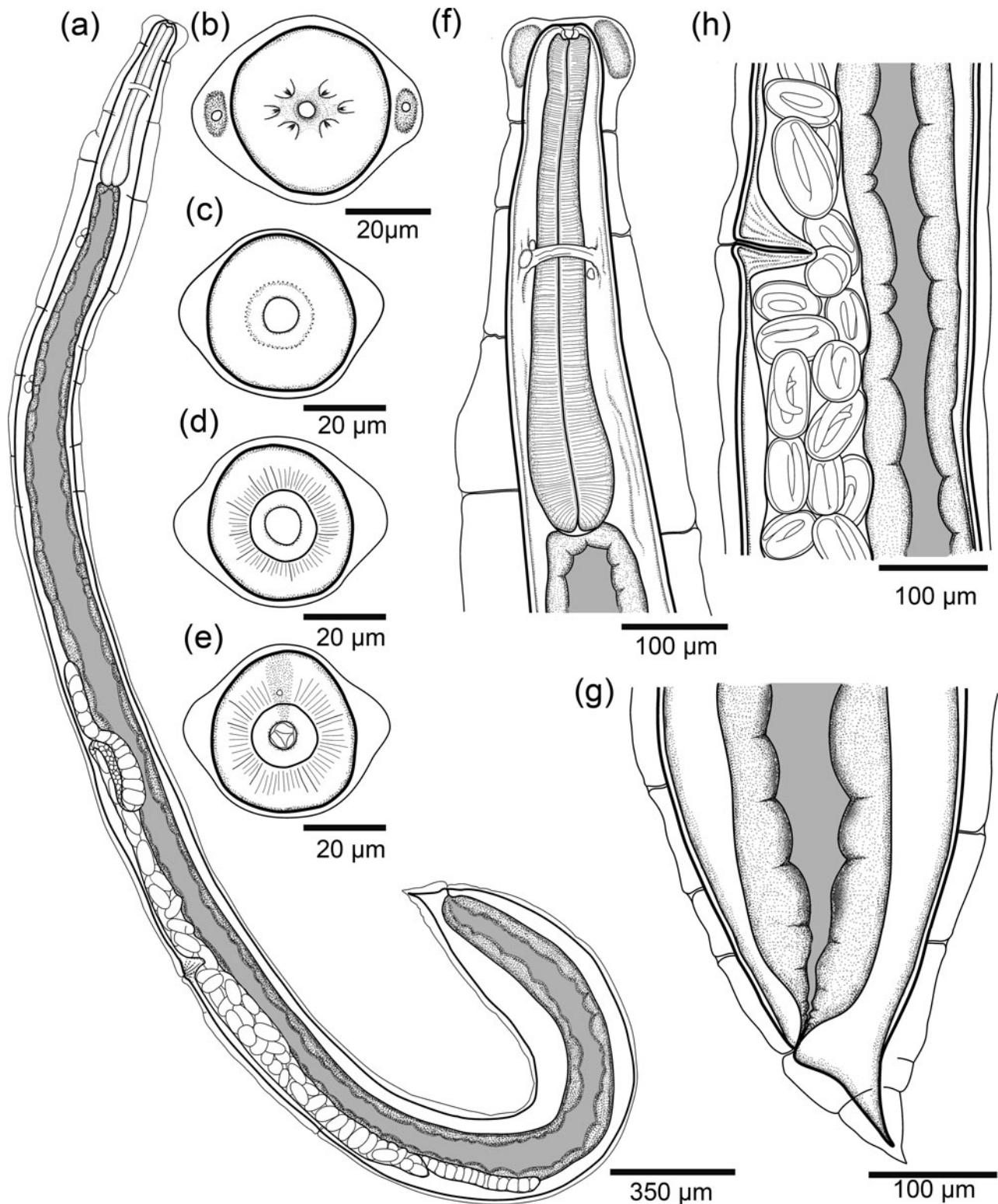


Fig. 1. Line drawings of *Rhabdias pocoto* n. sp. (a) Entire body, lateral view; (b) anterior end of body, en face view; (c) optical section through vestibulum; (d) optical section through anterior part (buccal capsule); (e) optical section through posterior part of buccal capsule; (f) anterior end of body, in lateral view; (g) mid-body region with vulva and (h) caudal end, lateral view.

as the author described those species based only on juvenile forms (Schuurmans-Stekhoven, 1952).

Based on the oral structure arrangement, the new species resembles those from the group are characterized by having six

lips (four submedian and two lateral lips): *Rhabdias androgyna* Kloss, 1971, *Rhabdias brevisensis*, *Rhabdias fueleborni* Travassos, 1926, *Rhabdias galactonoti* Kuzmin, Melo, Filho & Santos, 2016, *R. glaurungi*, *Rhabdias manantlanensis* Martínez-Salazar,

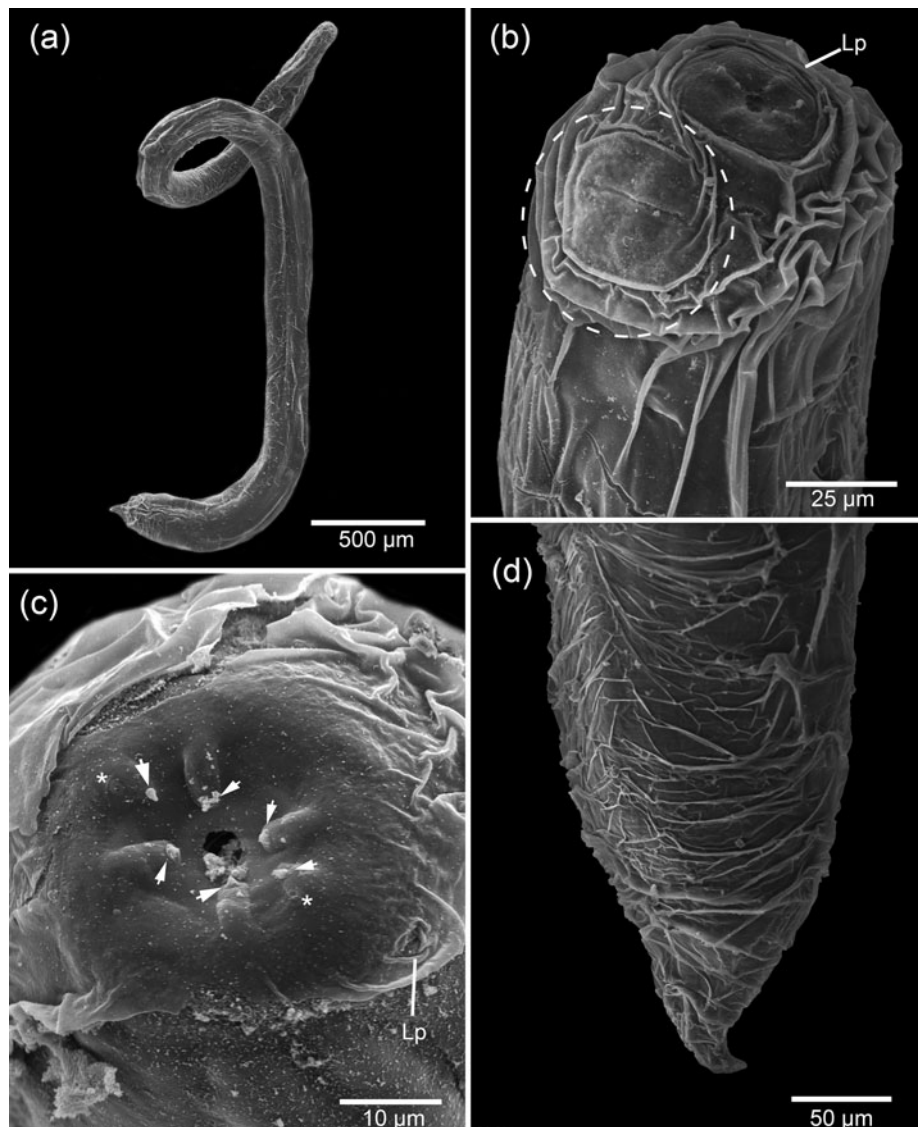


Fig. 2. *Rhabdias pocoto* n. sp. scanning electron micrographs. (a) entire gravid hermaphrodite; (b) anterior end, lateral view (circle dashed lines, firm rounded dilation between inflation and body wall, Lp, lateral pore); (c) apical view, (arrowheads, cephalic papillae; asterisks amphids; Lp, lateral pore); (d) posterior end, lateral view.

2008, and *Rhabdias stenocephala* Kuzmin, Melo, Filho & Santos, 2016 (Martínez-Salazar, 2008; Nascimento *et al.*, 2013; Kuzmin *et al.*, 2015; Kuzmin *et al.*, 2016; Willkens *et al.*, 2019). We will also compare *R. pocoto* n. sp. with the Neotropical species *Rhabdias elegans* Gutiérrez, 1945 and *Rhabdias hermaphrodita* Kloss, 1971 as information about the arrangement of the circumoral structures was not reported by the authors.

The new species can easily be distinguished from *R. androgyna* by body length, as *R. pocoto* n. sp. is much smaller than *R. androgyna* (3.41–7.43 × 9.35–13.4 mm) (Kloss, 1971), smaller buccal capsule (11–17 × 11–17 µm in the new species vs. 19–27 × 7–9 µm wide in *R. androgyna*) and the particular shape of the anterior end of the body, as according to Kloss (1971) and Kuzmin *et al.* (2015), *R. androgyna* has a shoulder-like circular dilatation with an extended cuticular swelling arranged in two layers, while the new taxon has two lateral inflations of cuticle containing an amorphous gland-like structure around the sub-apical lateral pores.

Rhabdias breviensis parasitizing *Leptodactylus petersii* Steindachner, 1864 and *Leptodactylus macrosternum* Miranda-Ribeiro, 1926 resembles the new species by its dorsally curved

body and parasitism of the hosts of the same family (Nascimento *et al.*, 2013). However, *R. breviensis* has a shorter and significantly wider body (2.63–3.63 mm long and 370–543 µm wide at vulva level). Additionally, *R. breviensis* has a smaller buccal capsule (7–13 width × 4–9 µm depth), a nerve-ring situated closer to the anterior end, and a post-equatorial vulvar opening. Although *R. pocoto* n. sp. and *R. breviensis* (139–191 µm) share a short and conical tail, the new taxon has a shorter tail (98–163 µm).

The description of *R. elegans* found parasitizing *Rhinella arenarum* Hensel, 1867 toads from Argentina, is not complete in Gutiérrez (1945). Furthermore, while Kloss (1974) provided additional characters and measurements for the species, the author did not describe the structure of the oral aperture and lips. *Rhabdias elegans* and the new species possess an approximately similar body length and position of the vulva, though *R. pocoto* n. sp. has larger buccal capsule (11–17 µm in *R. pocoto* n. sp. vs. 7 × 9 µm in *R. elegans*; after Kloss, 1974) and longer oesophagus (475–677 µm) than *R. elegans* (314–490 µm) (Kloss, 1971). Additionally, according to Gutiérrez (1945) and Kloss (1974), *R. elegans* do not exhibit cuticular inflation at the anterior end, which was observed in the new species.

Rhabdias fuelleborni parasitic in bufonid hosts (Kuzmin *et al.*, 2015) is distinguished by being larger than *R. pocoto* n. sp. (10–12 mm), and despite the two species sharing a morphology of the internal surface of the anterior and posterior segments of the buccal capsule, *R. fuelleborni* has a larger buccal capsule (18–19 µm, based on the description of Kuzmin *et al.*, 2015). Furthermore, according to the description of Kuzmin *et al.* (2015), *R. fuelleborni* has a slightly narrower portion of the body at the level of the oesophageal apex, which was not observed in the new species.

Rhabdias galactonoti resembles the new species by its total length and dimensions of the buccal capsule. However, *R. galactonoti* differs in size and in the morphology of the internal surface (Kuzmin *et al.*, 2016) of the buccal capsule; in this species, the anterior part is transparent, circular in the apical view, with a regularly folded inner surface, while in *R. pocoto* n. sp. the anterior part is smooth. The position of the vulva in *R. galactonoti* is slightly pre-equatorial vs. the equatorial vulva of *R. pocoto* n. sp., and in the caudal region the cuticular inflation does not reach the tip of the tail in *R. galactonoti* (Kuzmin *et al.*, 2016), whereas in the new species it does. The former species is also more elongated and larger in size (229–333 vs. 98–163 µm) (Kuzmin *et al.*, 2016).

Rhabdias glaurungi found parasitizing the lungs of *Scinax* gr. *ruber* Laurenti, 1768 is similar to the new species in body length. The two species can easily be distinguished, however, because *R. pocoto* n. sp. has a larger buccal capsule (width × depth: 11–17 × 9–17 µm vs. 10–16 × 6–9 µm), larger relative proportions of the oesophagus, a different vulva position (equatorial in the new species vs. post-equatorial in *R. glaurungi*), and a short tail (98–163 µm (8.9–13.9%) vs. 151–216 µm (4.9–8.2%)) (Willkens *et al.*, 2019).

As discussed by Willkens *et al.* (2019), the description of *R. hermaphrodita* is superficial and incomplete, with no measurement of the buccal capsule or the structure of the apical region. The species described by Kloss (1971) is similar in its equatorial vulva position (49% and 54% of the body length vs. 51% in *R. pocoto* n. sp.). However, *R. hermaphrodita* is larger than *R. pocoto* n. sp. (up to 12 mm total length), with a larger tail length (226–524 µm).

When compared with *R. manantlanensis*, the new species has a smaller body size (up to 6.48–9.64 mm long in *R. manantlanensis*), the cuticular swelling is more prominent at the anterior part of the body and present along the entire body, while according to the description of Martínez-Salazar (2008), the body cuticle of *R. manantlanensis* is not swollen or only slightly swollen, thin, and smooth. Furthermore, the two species differ in the buccal capsule (11–17 µm in *R. pocoto* n. sp. vs. 19–27 µm wide in *R. manantlanensis*), and while both have a similar, comparatively short tail, the shape of the tail in the new species is remarkably different.

Rhabdias stenocephala also described from leptodactylid frogs (*Leptodactylus pentadactylus* Laurenti, 1768 and *Leptodactylus paraensis* Heyer, 2005) is easily distinguished from the new species by the morphology of its anterior end (Kuzmin *et al.*, 2016). In this species, the anterior end has a narrow anterior part separated from the remainder of the body by a distinct constriction, which was not observed in *R. pocoto* n. sp. Additionally, they have almost identical buccal capsule dimensions (11–17 × 9–17 µm, in *R. pocoto* n. sp. vs. 9–11 × 15–18 µm, wide in *R. stenocephala*), and morphologies of the internal surface of the buccal capsule; the anterior part of the buccal capsule has a smooth internal wall in the new species, while the inner surface of the

anterior part is irregularly folded in *R. stenocephala* (Kuzmin *et al.*, 2016).

Molecular analyses and phylogenetic study

We obtained the partial coi sequence (376 bp fragment) of *R. pocoto* n. sp., which for phylogenetic analysis was aligned with 44 other sequences retrieved from the GenBank database. From table 1 it can be seen that just 15 species were included in the ingroup identified as *Rhabdias*, and also in the outgroup *Serpentirhabdias viperidicus* (KX350054). All the sequences used for phylogenetic analyses are presented in table 1.

DAMBE revealed lower Iss values than Iss.cAsym and Iss.cSym values, which mean the lack of a saturation signal in the matrix. The final trimmed alignment comprised 298 characters with 46 sequences, and both ML and BI converged, with similar results clustering *Rhabdias pocoto* n. sp. with low support (0.85 for BI and 49 for ML) in the nodes within the *Rhabdias* clade A2'' (0.85 for BI and 18 for ML), closely related to the *Rhabdias pseudosphaerocephala* Kuzmin, Tkach & Brooks, 2007 species complex clade, grouped with *R. glaurungi* (MK820652) from *Scinax* gr. *ruber*, *Rhabdias* sp. (KC130736) from *Rhinella marina* Linnaeus, 1758 from Colima, Mexico, and *Rhabdias* sp. 5 (MH548293, MH548292) from *Rhinella schneideri* (= *Rhinella diptycha* Cope, 1862) (Clade A2'') (fig. 3).

The genetic divergence using the coi gene of *R. pocoto* n. sp. compared to the *Rhabdias* spp. sequences used in the present study varied from 2% to 16%, with the lowest divergence observed within species of the *R. pseudosphaerocephala* complex (2%), and 16% within the outgroup (supplementary table S1).

Discussion

The morphological and biological features of the new species correspond to the diagnosis of *Rhabdias* proposed by Tkach *et al.* (2014). Our morphological data identified several characters specific to the new taxon, mainly body shape, the structure of the apical end, and the length of the tail, which differentiate the species of our study from its congeners. Additionally, our molecular data grouped the new species with the *R. pseudosphaerocephala* species complex and identified divergence from the sequences previously deposited in GenBank.

In the first molecular phylogenetic study of Rhabdiasidae, Tkach *et al.* (2014) inferred that the oral arrangement (presence or absence of various structures [e.g. lips, pseudolabia and submedian lips], variations in shape and position) represents one of the best characters for the differentiation of *Rhabdias* species, despite its limited usefulness for phylogenetic inferences.

Based on the set of morphological and molecular data produced (Tkach *et al.*, 2014; Müller *et al.*, 2018; Willkens *et al.*, 2019) so far, we can observe three patterns of oral arrangements in species in the Neotropical region parasitizing amphibians and for which information about the oral arrangement is available: (1) four submedian lips and two lateral pseudolabia (five spp. *R. manantlanensis*, *R. savage*, *R. kuzmini*, *R. pseudosphaerocephala* and *R. elegans*); (2) six equal lips (seven spp. [*R. androgyna*, *R. fuelleborni*, *R. breviensis*, *R. galactonoti*, *R. glaurungi*, *R. stenocephala* and *R. tobagoensis*]); and (3) absence of lips (two spp. *R. alabialis* and *R. paraensis*). Using molecular tools, in a recent study of *Rhabdias* populations of amphibians and lizards in Brazil, including those also from the Caatinga biome, Müller *et al.* (2018) reported that all studied species (five species, 26

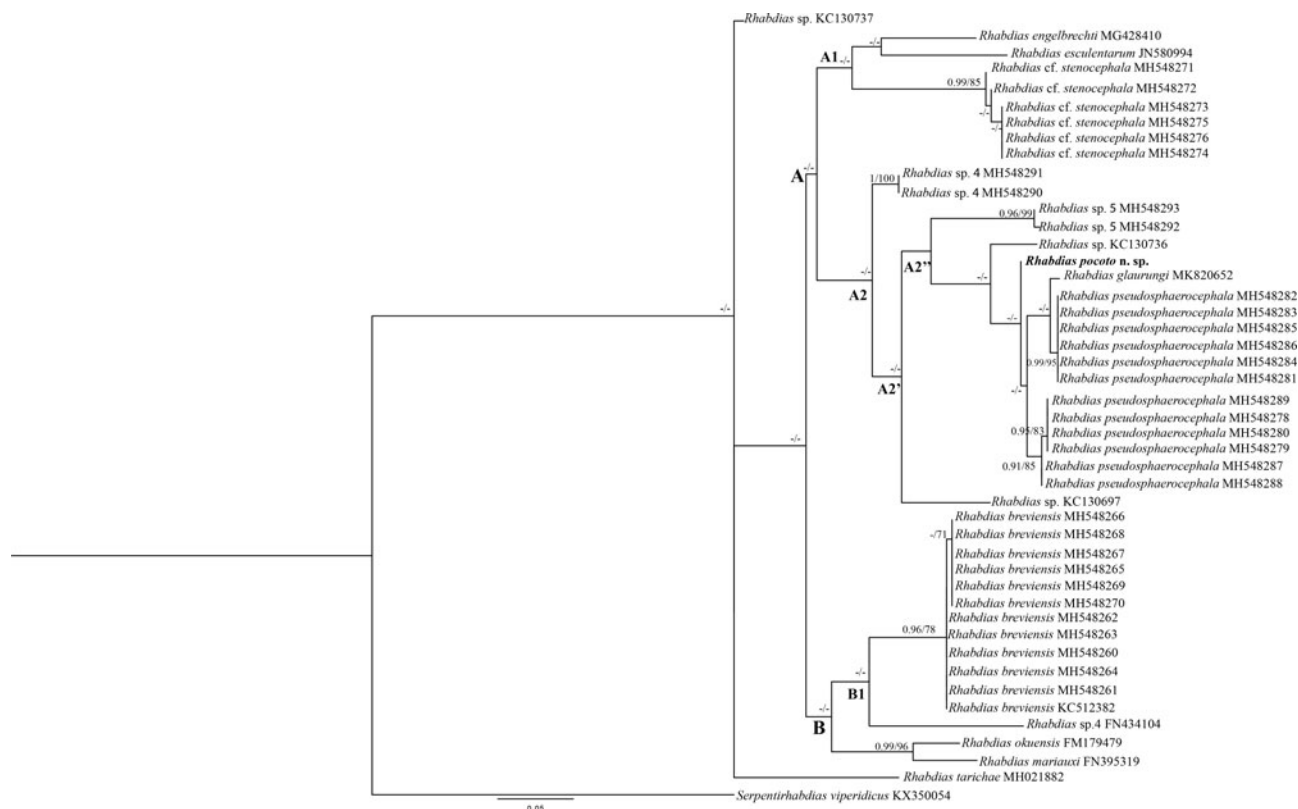


Fig. 3. Maximum Likelihood phylogenetic topology of *Rhabdias* spp. of *coi* gene using *Serpenterhabdias viperidicus* as outgroup. Support values are above or below nodes: posterior probabilities <0.90 and bootstrap scores <70 are not shown or are represented by a dash. Branch-length scale bar indicates number of substitutions per site.

specimens) exhibited an oral arrangement with four submedian lips and two lateral pseudolabia (pattern 1). However, this pattern was not observed in the morphology of species with no molecular data; although pattern 1 includes most known species. Kuzmin (2013) produced a schematic figure and the position of the circumoral structures and suggested that the transformation of the lateral lips into lateral pseudolabia is due to an evolutionary pattern.

Rhabdias pocoto n. sp. is the 19th species from the Neotropical region, the ninth from Brazil, the eighth species exhibiting pattern two (six lips) in the Neotropical region, and the first species with a unique feature that has not previously been observed in any of the known species of amphibian *Rhabdias* (two lateral cuticular inflations containing an amorphous gland-like structure around the sub-apical lateral pores). Lhermitte-Vallarino *et al.* (2009) described a similar structure in *Rhabdias brevicorne* Lhermitte-Vallarino, Junker & Bain, 2009, found in a lizard from Madagascar, reported by the authors as a ‘dense internal circular tissue surrounding the mouth’ and called ‘cephalic pad’; however, they did not mention the presence of sub-apical lateral pores.

According to Langford & Janovy (2013), host specificity varies greatly among species of *Rhabdias*, and the same authors suggest that ecological fitting plays a role in shaping such host specificity. *Rhabdias pocoto* n. sp. is the first *Rhabdias* found in frogs of the genus *Pseudopaludicola* and has a unique set of morphological characters. These features may indicate specific specialization in the host-parasite lineage and suggest a strict host specificity in the *Rhabdias pocoto* n. sp. system.

Moreover, these relationships can be observed at another taxonomic level, such as the *R. pseudosphaerocephala* species complex,

which is commonly found associated with bufonids (Müller *et al.*, 2018). Müller *et al.* (2018) suggested the evidence for host-parasite cophylogeny between the *R. pseudosphaerocephala* complex and toads (Bufonidae). However, these authors also found a host spillover of this parasite in *Boana raniceps* Cope, 1862 (Hylidae) in southern Brazil. On the other hand, the *R. breviensis* species complex, found by Müller *et al.* (2018), appears to be more generalist in terms of host family (associated with the Leptodactylidae, Bufonidae, Odontophrynidae and Hylidae species), suggesting that ecological fitting may be related to the host specificity of the *Rhabdias* species.

Tkach *et al.* (2014) suggested that the colonization of reptiles has occurred more than once in the evolutionary history of *Rhabdias* and presume that host spillover and ecological fitting were more evolutionarily important than the association with particular host taxa. Thus, these findings suggest that host switching appears to be a hallmark of *Rhabdias*, which included numerous exchanges between different lineages of anurans.

The geographic distribution of the species of *Rhabdias* seems to follow a pattern where the species are restricted to their zoogeographic region (Tkach *et al.*, 2014). Two lineages of *Rhabdias* (species complexes of *R. pseudosphaerocephala* and *R. breviensis*) are distributed across the south, southeast, and north-east of Brazil, and according to Müller *et al.* (2018) and Willkens *et al.* (2019), there are cryptic species in the Caatinga biome, in the north-east of Brazil, such as *R. cf. stenocephala* (in *L. macrosternum* Miranda-Ribeiro, 1926 and *L. vastus* Lutz, 1930) and *Rhabdias* sp. (in the lizard *Norops brasiliensis* Vanzolini & Williams, 1970).

Nevertheless, considering the distribution records of the *P. pocoto* frog, which is mainly found in the Caatinga domain (Silva *et al.*, 2017) and the results of helminth records associated with amphibians of other species in the biome (Teles *et al.*, 2014, 2015; 2018a, b; Araujo-Filho *et al.*, 2015; Lins *et al.*, 2017; Alcantara *et al.*, 2018; Müller *et al.*, 2018; Silva *et al.*, 2018; Amorim *et al.*, 2019; Oliveira *et al.*, 2019; Madelaire *et al.*, 2020), we suggest the potential endemism of *Rhabdias pocoto* n. sp. in this biome, and considering the unique morphological character and specificity for the right lung of the host, suggest that the new taxon may have strict specificity for this species of host or host family.

Studies on the ecological aspects of the *Rhabdias* genus in Brazil are scarce, especially in the Caatinga biome. However, Silva *et al.* (2018) studied the ecology of *Rhabdias* sp., and this species is finally described herein as *Rhabdias pocoto* n. sp. It was observed that the environmental characteristics of the type locality support the occurrence of larvae throughout the year, and parasitized frogs were collected in the dry and rainy seasons; additionally, it was noted that the host species can be exposed and act as a potential host for this helminth. The authors also observed that temperature does not influence the parasitological descriptors of this parasite, while high precipitation rates positively influence the infection rates of the *Rhabdias* population.

A priori, regions with high diversity are the best areas for studying a new parasite species and should be key targets of parasite discovery efforts (Jorge & Poulin, 2018). The Caatinga biome has approximately 53 anuran species, approximately 12% of which are endemic (Garda *et al.*, 2017), and most of which have unknown helminth fauna (Camião *et al.*, 2014). We suggest that additional species of *Rhabdias* will be discovered in this region because of this large number of anuran species and the potential hosts for lungworms.

According to Poulin (2007), host phylogeny is one determinant for host spillover and/or host switching. Therefore, it was expected that *Rhabdias pocoto* n. sp. would be a sibling group of the *R. breviensis* species complex, where both nematodes parasitize Leptodactylidae host specimens (see Nascimento *et al.*, 2013). However, we observed that the phylogenetic analysis using the *coi* gene positioned the new species as a sister group of the *R. pseudosphaerocephala* species complex + *R. glaurungi* (from *Scinax* gr. *ruber*) and *Rhabdias* sp. (KC130736) (from *Rhinella marina* from Colima, Mexico) (Clade A2'').

These findings suggest that the new species represent a distinct phylogenetic lineage that evolved among the *R. pseudosphaerocephala* species complex. However, nodal support was low and considered not supported (below 0.9 posterior probability for Bayesian inference and 70% bootstrap for ML inference), and this phylogenetic position might change in future studies when more taxa will be added to the phylogeny. The main clades A and B, despite the general low support in the nodes (apparently a common characteristic of the *coi* gene for *Rhabdias* spp.), corroborate the results demonstrated in recent studies (Müller *et al.*, 2018; Willkens *et al.*, 2019).

This record represents the first of *Rhabdias* described in frogs of the genus *Pseudopaludicola*, which is the basal taxon of Leptodactylidae (Pyrón & Wiens, 2011) and first Caatinga biome species. The evidence of the low diversity of *Rhabdias* (nine species) compared with the high diversity of amphibians from Brazil (1038 species) reveals the limited attempts to map global parasite diversity in Brazilian biomes, especially in the Caatinga biome.

Supplementary material. To view supplementary material for this article, please visit <https://doi.org/10.1017/S0022149X20000929>.

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work complied with all applicable institutional, national and international guidelines for the care and use of animals. Specimens were collected under license number ICMBio/SISBIO 29613-1; 55467-1) and authorized by the Ethics Committee of the Universidad Regional do Cariri (Cariri Regional University) (URCA n° 00260/2016.1).

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