

Review Article

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

Integrative taxonomy; nematode; Parque Nacional do Itatiaia; rodent

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New species of the genus *Alippistrongylus* (Nematoda: Trichostrongylina) parasitic in *Delomys dorsalis* (Rodentia: Sigmodontinae) in the Atlantic Forest of Rio de Janeiro, Brazil

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Abstract

We describe a new species of the genus *Alippistrongylus* (Nematoda: Trichostrongylina) in the small intestine of *Delomys dorsalis* (Rodentia: Sigmodontinae) found in Itatiaia National Park (Parque Nacional do Itatiaia, PNI), state of Rio de Janeiro, Brazil. The new species can be distinguished between the other two species previously described by the following morphological characteristics: pattern of the rays from the caudal bursa rays in males and the spherical-shaped appendage in the female. Additionally, we provide molecular genetic data from the new species. The discovery of *Alippistrongylus itatiaiaensis* sp. n. expands our understanding of nematode diversity and underscores the importance of parasite studies in biodiversity conservation. Its occurrence in a preserved area like the PNI emphasises the role of such habitats in maintaining unique ecological interactions.

Introduction

The Atlantic Forest is the second largest tropical forest in South America, boasting one of the areas with the greatest biodiversity on the planet in the Brazilian, Argentine, and Paraguayan territories (Marques *et al.* 2021). It is a region where the richest and most threatened plant and animal species on the planet are found (Myers *et al.* 2000), due to deforestation driven by various human activities (MMM 2023).

The Itatiaia National Park (PNI) covers regions of the state of Rio de Janeiro and Minas Gerais, whose main objective is the conservation of the endemic fauna and flora of the Atlantic Forest biome. The park is recognised by UNESCO and located in areas of high priority for biodiversity conservation (ICMBio 2023).

The Atlantic Forest harbors over 270 mammal species (MMA 2023), including 40 species of rodents belonging to the subfamily Sigmodontinae (Bonvicino *et al.* 2008). Sigmodontine rodents are relevant to public health, serving as potential reservoirs for hantaviruses, *Bartonella* spp., *Leptospira* spp., *Trypanosoma cruzi*, and *Schistosoma mansoni* (Maldonado Jr. *et al.* 2006; Orozco *et al.* 2014; Vieira *et al.* 2019; Salvo *et al.* 2020; Bellomo *et al.* 2021).

Among these rodents, *Delomys dorsalis* (Hensel, 1873) is a sigmodontine species with terrestrial habits endemic to the Atlantic Forest (Bonvicino *et al.* 2008). This species thrives at altitudes that can reach up to 2,000 m (Gonçalves and Oliveira 2014) and feeds mainly on plant matter and soil arthropods (Pinotti *et al.* 2011). *Delomys dorsalis* remains relatively understudied in terms of its helminth fauna, with only two helminths reported (Cardoso *et al.* 2018).

This study is part of a broader biodiversity research project investigating various taxa within the Atlantic Forest. Utilising a multidisciplinary approach encompassing taxonomic, evolutionary, ecological, and parasitological perspectives, our primary aim was to describe a new species of nematode parasitising the small intestine of *Delomys dorsalis* found in the PNI using integrative taxonomy.

Material and methods

The research was conducted within the confines of Itatiaia National Park (PNI), in the municipality of Itatiaia, state of Rio de Janeiro, Brazil. The PNI encompasses an area of 28,086 hectares (ha) with altitudes ranging from 600 to 2,791 m (ICMBio 2023). The climate within the park is

characterised as humid, with average temperatures reaching up to 20°C during the summer. The PNI is part of a strategic protection program aimed at conserving the Atlantic Forest, designated as the Atlantic Forest Biosphere Reserve (RBMA 2023).

Rodents were captured within approximate altitude ranges of 800 to 1,500 meters. Six linear transects were established in these areas, comprising 15 capture stations each, and were georeferenced using GPS technology. Live traps of Tomahawk (40.64 cm x 12.70 cm x 12.70 cm) and Sherman (7.62 cm x 9.53 cm x 30.48 cm) models were utilised, baited with a mixture of bananas, oats, peanut butter, and bacon. Traps containing the animals were promptly transported to a field laboratory for processing and sample collection, adhering strictly to biosafety standards. Permits for the collection and handling the animals were issued by the Chico Mendes Institute for Biodiversity Conservation (ICMbio), under authorisation N° 74498-11, and the Ethics Committee on the Use of Animals (CEUA/IOC), with license N° L-036/2018-A1, respectively.

All nematodes collected from the small intestine were conserved in 70% ethanol. Ten male and 10 female nematodes were cleared using lactophenol and examined using a Zeiss Standard 20 light microscope. Drawings were produced with the assistance of a camera lucida. The morphological structures were measured using a Zeiss Axio Cam HRC digital imaging system (Zeiss, Germany), with the Axio Vision Rel accessory software, version 4.7. Measurements were provided in micrometers (unless otherwise specified) for holotype or allotype samples, followed by the paratype range within parentheses. The description of the general morphology of nematodes was based on Digiani and Kinsella (2014), the pattern of the bursa was in accordance with Durette-Desset and Digiani (2012), and the orientation of the synlophes was following Durette-Desset and Digiani (2005). The holotype, allotype, and paratypes were deposited in the helminthological collection of the Oswaldo Cruz Institute (CHIOC) in Rio de Janeiro.

For scanning electron microscopy analysis, nematodes were dehydrated in graded ethanol (30–100%) for 2 hours and subjected to critical point drying with CO₂ (CPD 030, Balzers, Switzerland). The samples were mounted on aluminum stubs, coated with a 20-nm layer of gold, and examined using a Jeol JSM 6390LV scanning electron microscope (operating at 15 kV) (JEOL, Akishima, Tokyo, Japan) at the Rudolf Barth Electron Microscopy Platform of the Oswaldo Cruz Institute, Rio de Janeiro.

Genomic DNA was isolated from a pool of 10 nematodes using the QIAamp DNA Mini Kit following the manufacturer's protocol (QIAGEN, Hilden, Germany). Polymerase chain reaction (PCR) amplification of a partial large subunit ribosomal RNA (28S rRNA) gene was conducted using the primers NC28-7 (5'-GAC CCT GTT GAC CTT GAC TC-3') and NC28-BR (5'-GTC TAA ACC CAG CTC ACG TT-3') (Chilton *et al.* 2003). Each PCR reaction consisted of 12.5 µL of PCR Master Mix (Promega Corporation, Madison, USA), 8.5 µL of DNA-free water, 0.5 µL of each forward and reverse primer, and 3 µL of the DNA sample, making up a total reaction volume of 25 µL. The thermal cycling program involved an initial denaturation step at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. Amplicons were visualised on 1.5% agarose gels after electrophoresis, stained with Gel Red nucleic acid gel stains (Biotium, Hayward, California, USA), and examined under a UV transilluminator. Successfully amplified amplicons were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit according to the manufacturer's protocol (GE Healthcare, Little Chalfont, UK). Amplicons were cycle-

sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on both strands using the PCR primers mentioned, resulting in bidirectional sequencing for improved data accuracy. Sequencing was performed using the ABI3730 DNA Analyzer. Both procedures and cycle sequenced products precipitation were conducted at the Platform of the Oswaldo Cruz Foundation for DNA sequencing by capillary electrophoresis (P01-001-RPT/FIOCRUZ). Fragments were assembled into contigs and edited for ambiguities using the Geneious Prime 2023 software platform (Kearse *et al.* 2012), resulting in a consensus sequence. The DNA sequence obtained was compared with those available in the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>) using the BLASTn algorithm (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The 28S sequence data of *Alippistrongylus itatiaiaensis* sp. n. was deposited in the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>).

We aligned the sequence of *Alippistrongylus itatiaiaensis* sp. n. with 11 sequences of other nematode species belonging to the suborder Trichostrongylina (*sensu* Durette-Desset and Chabaud, 1993) retrieved from GenBank. As outgroups we used two sequences of nematode species belonging to the suborder Ancylostomatina (*Ancylostoma caninum* (Ercolani, 1859) and *Necator americanus* (Stiles, 1092)). We aligned the sequences using the ClustalW multiple sequence alignment program (Thompson *et al.* 1994). We trimmed poorly aligned regions using the Mesquite software package, version 3.81 (Maddison and Maddison 2021).

Phylogenetic reconstruction using maximum likelihood (ML), as the optimality criterion, was carried out using the PhyML 3.0 web server (Guindon *et al.* 2010). The best-fit nucleotide evolutionary model was calculated under the Akaike information criterion (AIC), via SMS (Smart Model Selection) (Lefort *et al.* 2017). Branch supports were assessed by the approximate likelihood-ratio test (aLRT) (Anisimova and Gascuel 2006).

Bayesian phylogenetic inference (BI) was carried out using MrBayes version 3.2.7a (Ronquist *et al.* 2012) on XSEDE using the CIPRES Science Gateway (Miller *et al.* 2010). Markov chain Monte Carlo (MCMC) samplings were performed for 10,000,000 generations, with four simultaneous chains, in two runs. Branch supports were assessed by Bayesian posterior probabilities (BPP), calculated from trees sampled every 1,000 generations, after a 25% fraction burn-in removal.

Results

Suborder **Trichostrongylina** Durette-Desset and Chabaud, 1993
Superfamily **Heligmosomoidea** Durette-Desset and Chabaud, 1993

Family **Heligmonellidae** Skrjabin and Schikhobalova, 1952

Subfamily **Nippostrongylinae** Durette-Desset, 1971

Alippistrongylus itatiaiaensis sp. n. Lemes, Andrade, Maldonado Jr. & Simões

General: Nematodes of small length, with the posterior region irregularly curled. Cephalic vesicle conspicuous with transverse striations (Figure 1b), 2 amphids, 4 external labial papillae, and 4 submedian cephalic papillae (Figure 1a). Nerve ring anterior to the excretory pore and deirid (Figure 1b). Excretory pore and deirids at about 84% and 67% of oesophagus length in female and 77% and 63% in male, respectively. Oesophagus claviform (Figure 1b).

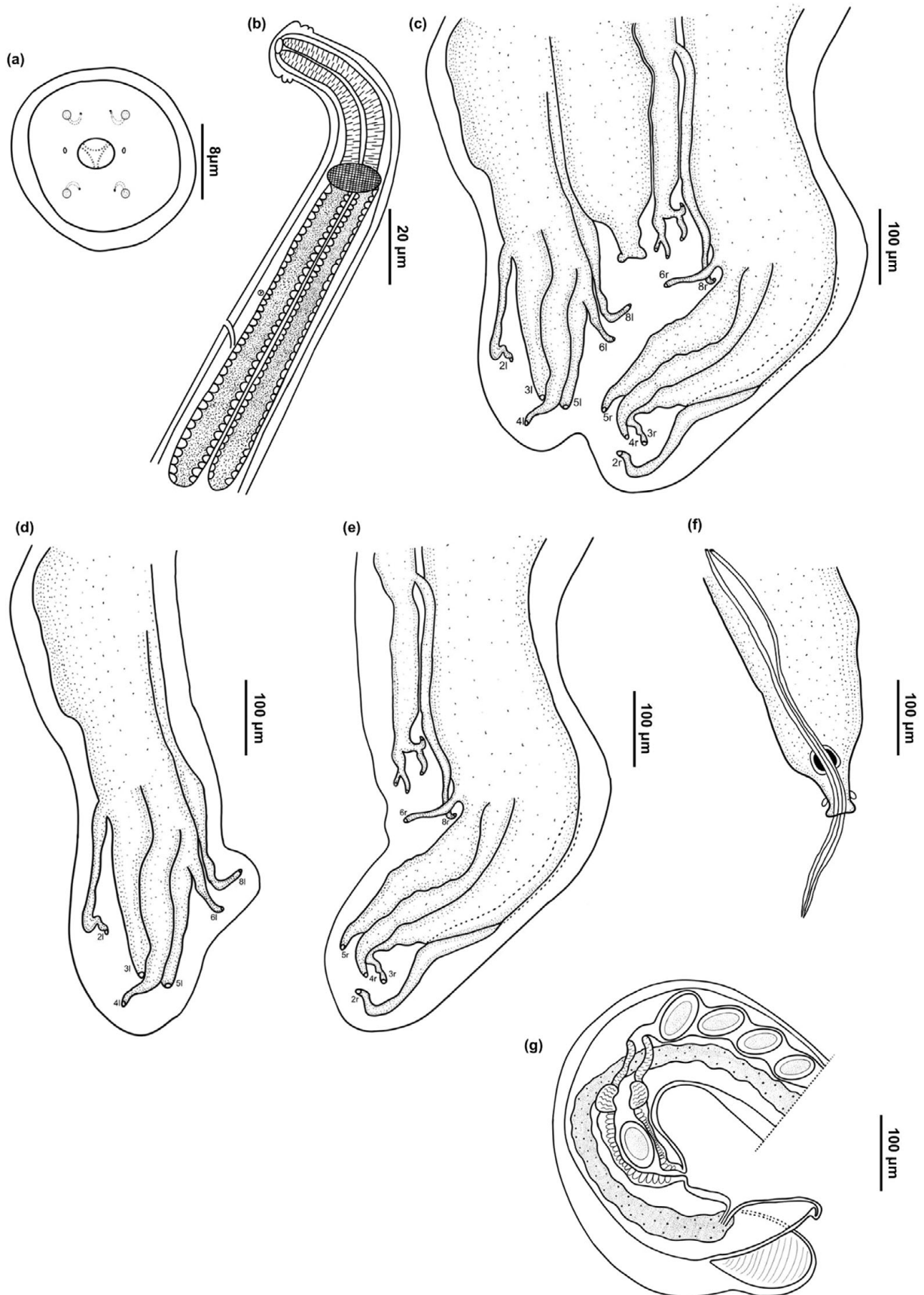


Figure 1. *Alippistrongylus itatiaiaensis* sp. n. (a) Head, apical view. (b) Left lateral view from anterior extremity, showing nerve ring, excretory pore, deirids, and oesophagus. (c) Ventral view from male, caudal bursa. (d) Lateral view from left lobe. (e) Lateral view from right lobe. (f) Ventral view from genital cone, gubernaculum, and spicules. (g) Female, posterior extremity, showing distal uterus, vestibule, sphincter, infundibulum, and eggs. Abbreviations: 2l–8l – left rays 2–8; 2r–8r – right rays 2–8.

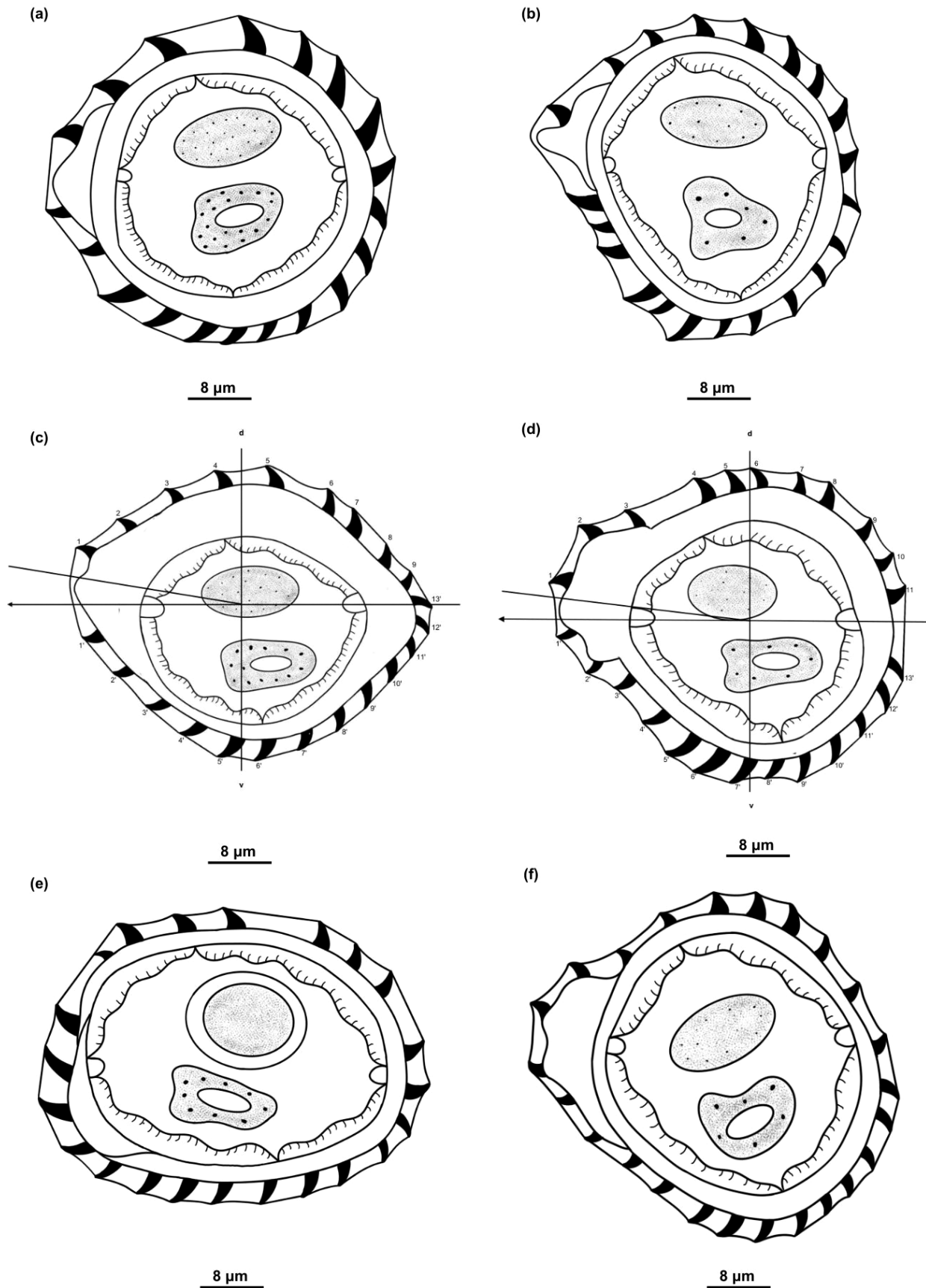


Figure 2. Synopse in transverse sections of the body: **(a)** between deirid and oesophagus female and **(b)** male; at mid-body **(c)** female and **(d)** male; **(e)** anterior to vulva; **(f)** anterior to caudal bursa.

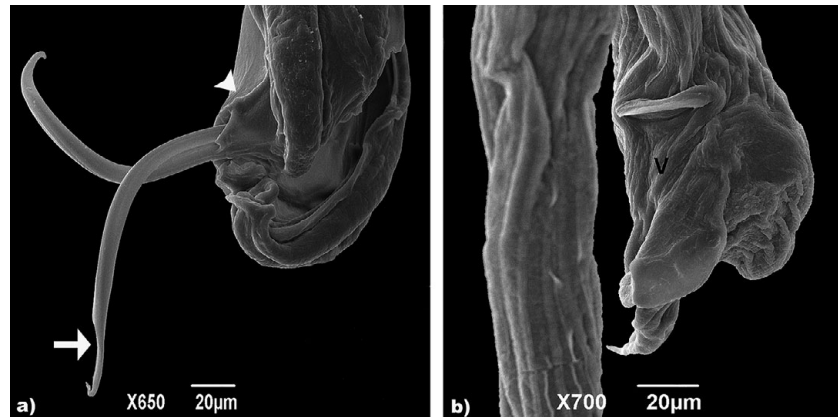


Figure 3. *Allipiststrongylus itatiaiaensis* sp. n. (a) spicules passing through the genital cone, with papilla apparent in lateral view, SEM. (b) posterior region of female from ventral view of vulva, SEM. (v) vulva.

Table 1. Morphological measurements from *Allipiststrongylus*

	<i>Allipiststrongylus bicaudatus</i> Digiani and Kinsella, 2014		<i>Allipiststrongylus marki</i> Drabik, Vivar and Jiménez, 2022		<i>Allipiststrongylus itatiaiaensis</i> n. sp. present study	
	Male	Female	Male	Female	Male	Female
Long	2.75–3.70	2.95–3.75	2.10–3.40	2.13–3.88	2.33–3.51	2.56–3.39
Wide	70	60–90	66–95	82–140	44–78	47–82
Cephalic vesicle	40–45 (L), 30 (W)	40–50 (L), 30–40 (W)	20–62 (L), 25–46 (W)	32–60 (L), 26–43 (W)	23–44 (L), 15–27 (W)	30–46 (L), 19–31 (W)
Nerve ring	165–180	120–190	77–130	81–113	88–171	105–279
Excretory pore	245	210–240	46–198	128–144	145–259	135–292
Deirid	220–270	210–240	-	-	133–242	116–347
Oesophagus	305–320	260–340	146–278 (L), 11–22 (W)	201–318 (L), 15–52 (W)	196–368	160–434
Bursa	Dissymmetrical	-	Symmetrical	-	Dissymmetrical	-
Genital cone	60 (L), 45–50 (W)	-	-	-	55–82 (L), 32–53 (W)	-
Spicules	295	-	1.00–1.46	-	228–361	-
Gubernaculum	-	-	27–39 (L), 13–20 (W)	-	8–16 (L), 24–44 (W)	-
Vulva	-	120–210	-	60–116	-	75–184
Vagina vera	-	10–15	-	33–67	-	18–27
Vestibule	-	50–110	-	92–594 (L), 34–57 (W)	-	32–77 (L), 24–50 (W)
Sphincter	-	22–35 (L), 30–40 (W)	-	31–84	-	42–76 (L), 22–68 (W)
Infundibulum	-	90–100	-	78–250	-	113–218
Uterus	-	500–850	-	-	-	711–1.31
Eggs	-	40–60 (L), 30–45 (W)	-	55–82 (L), 25–46 (W)	-	32–62 (L), 24–51 (W)
Number of eggs	-	12–23	-	3	-	1–6
Appendage	-	85–140 (L), 35–80 (W)	-	53–125 (L), 30–39 (W)	-	82–133 (L), 74–116 (W)
Ventral portion	-	113–185	-	-	-	61–76
Tail	-	50–75	-	-	-	39–59
Mucron distal	-	12–25	-	-	-	9–12
Anus	-	-	-	33–66	-	24–36
Host	<i>Delomys dorsalis</i>		<i>Euryoryzomys nitidus</i>		<i>Delomys dorsalis</i>	
Locality	Argentina		Peru		Brazil	

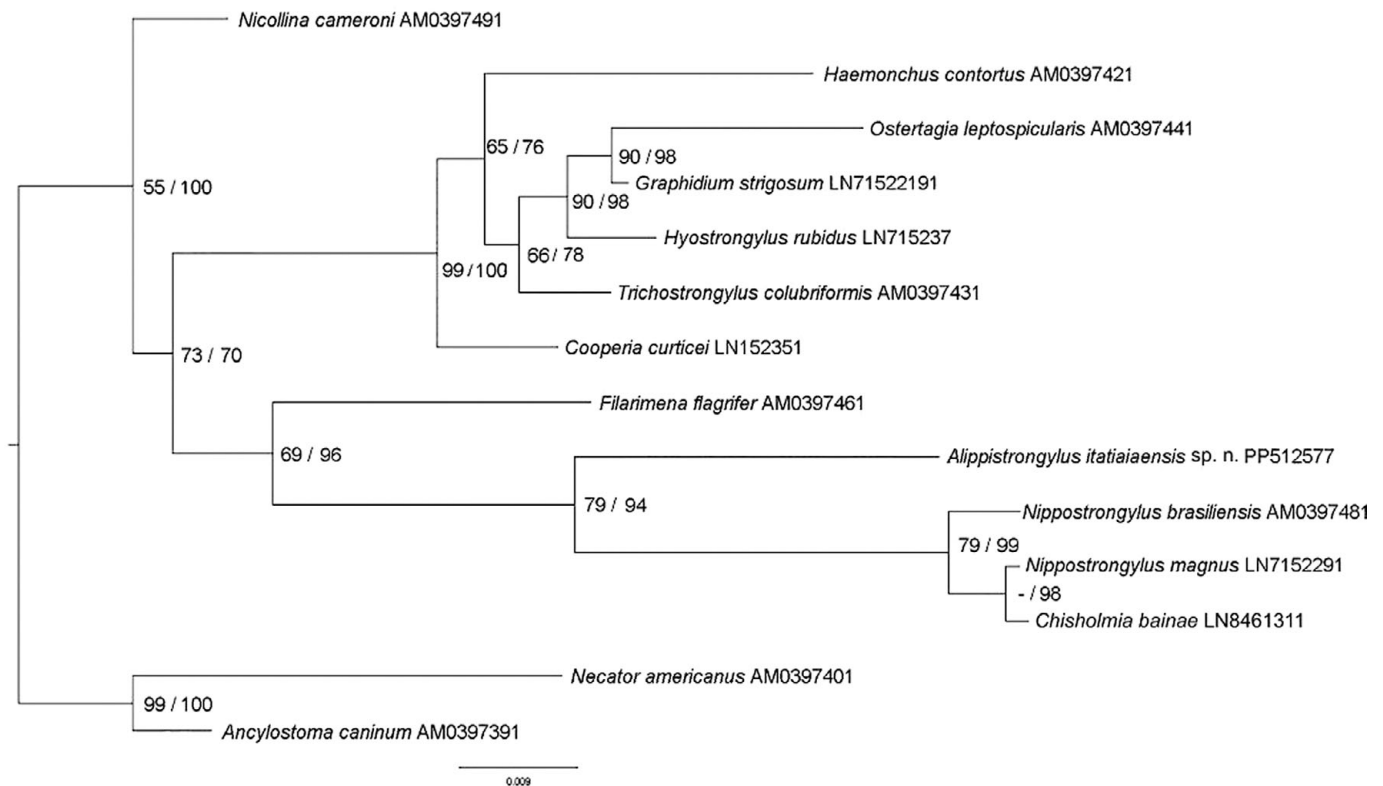


Figure 4. Bayesian phylogenetic tree for the 28S rRNA gene. The numbers at the nodes are aLRT (left) and BPPs (right). The scale bar represents the number of substitutions per site.

Synlophe: (Studied in 1 male and 1 female). In both sexes, cuticles with uninterrupted longitudinal ridges gradually appearing posterior to the cephalic vesicle, disappearing immediately anterior to the bursa in the male and reaching the caudal appendage in the female. Synlophe with 19 ridges in female (8 dorsal/11 ventral) (Figure 2a) and 21 in male (9 dorsal/12 ventral) (Figure 2b) between deirid and esophagus; 22 ridges in female (9 dorsal/13 ventral) (Figure 2c) and 24 ridges in male (11 dorsal/13 ventral) (Figure 2d) at mid-body; 22 ridges in female (9 dorsal/13 ventral) (Figure 2e) anterior to vulva and 24 in male (10 dorsal/14 ventral) (Figure 2f) anterior to caudal bursa. In the middle of the body, the size and shape of the ridges present limited variation with ridges in the right ventral and dorsal quadrant slightly larger. Single axis of orientation of ridges inclined at about 80° to sagittal axis in both sexes.

Male (holotype and 10 paratypes): 3.36 (2.33–3.51) mm long and 51 (44–78) wide at mid-body. Cephalic vesicle 23 (21–44) long and 18 (15–27) wide. Nerve ring, deirids, and excretory pore situated at 120 (128–301), 178 (133–308), and 228 (145–370) from anterior region, respectively. Oesophagus 293 (196–368) long. Dissymmetrical caudal bursa, with the right lobe larger than the left (Figure 1c). Left and right lobe with 4-1 pattern (Figure 1d, 1e). Right ray 2 is longer than left ray 2, reaching the edge of the caudal bursa. Right and left rays 3, 4, and 5 are similar sizes. Rays 6, in both lobes, are smaller than the others. Dorsal lobe short. In the medial region of the right lobe, the end of ray 8 reaches ray 6, but in the right lobe, the rays do not reach each other. Dorsal ray long and thinner, divided at about distal third into 2 branches. Each branch divided at tip into 2 different branches: ray 10 (internal) slightly larger than ray 9 (external). Genital cone well-developed with apex bifurcated 68 (55–85) long and 47 (32–53) wide, with visible papillae 7 (Figure 1f). Spicules 361 (228–361) long ending in a bevel

shape (Figure 3a). Spicule length in relation to body length (SpL/BL) 9.3% (9.7%–10.2%). Gubernaculum 10 (8–16) long and 34 (24–44) wide (Figure 15).

Female (allotype and 10 paratypes): 3.20 (2.56–3.39) mm long and 47 (47–82) wide at mid-body. Cephalic vesicle 34 (30–46) long and 19 (19–31) wide. Nerve ring, deirids, and excretory pore situated at 167 (105–279), 202 (116–347), and 216 (135–292) from anterior region, respectively. Oesophagus 315 (160–434) long. Monodelphic (Figure 1g). Vulva situated at 117 (75–184) from caudal extremity. Vagina vera 21 (18–27) long, vestibule 49 (32–77) long and 42 (24–50) wide, sphincter 50 (42–76) long and 27 (22–68) wide, infundibulum 118 (113–218) long. Uterus 940 (711–1.07) long, containing approximately 4 (1–6) eggs with 46 (34–62) long and 25 (24–51) wide. The posterior region bifurcates into a ventral conical portion and a dorsal postvulvar rounded appendage (Figure 3b). Ventral portion (mucron included) 62 (61–76) long. Tail (mucron included) 46 (39–59) long. Distal mucron 10 (9–15) long. Distance anus to end of tail 30 (24–36). Rounded appendage 96 (82–133) long and 89 (74–116) wide.

Taxonomic summary

Type host: *Delomys dorsalis* (Hensel, 1873)

Site of infection: Small intestine

Type locality: vicinity of Abrigo Lamego, Itatiaia National Park, municipality of Itatiaia, state of Rio de Janeiro, Brazil

Prevalence: 20% (11 of 56 rodents collected)

Mean intensity: 31 (339 helminths in the 11 infected rodents).

Mean abundance: 6 (339 helminths in the 56 rodents collected)

Specimens deposited: Holotype: CHIOC 39673a (male); allotype: CHIOC 39673b (female); paratypes: CHIOC 39674 (10 males and 10 females)

Etymology: The specific epithet name refers to the locality where the nematode was found.

Differential diagnosis

Alippistrongylus itatiaiaensis sp. n. can be distinguished from *A. bicaudatus* and *A. marki* based on the morphology of the female appendage. Unlike the other species, the present species exhibits a dorsal appendage that is not elongated or digitiform, but rather rounded shortly after bifurcation, assuming a spherical shape. Additionally, it has the appendage located dorsally similarly to *A. bicaudatus* and in contrast to *A. marki*, in which it ventrally positioned. The patterns of the caudal bursa of *Alippistrongylus itatiaiaensis* sp. n. consist of 4-1 in both lobes, unlike *A. bicaudatus* which presents a configuration of 1-4 in the left lobe and 4-1 in the right lobe. The new species can be further differentiated from the other two species through the dorsal ray, which is smaller and asymmetrical. In *Alippistrongylus itatiaiaensis* sp. n., the ends of rays 8 reach rays 6, resembling *A. bicaudatus*, whereas in *A. marki*, they emerge at the distal half of the dorsal trunk. Moreover, *Alippistrongylus itatiaiaensis* sp. n. differs from *A. bicaudatus* due to the presence of a gubernaculum and a well-developed genital cone, features absent in *A. marki*. In addition, the nerve ring of *Alippistrongylus itatiaiaensis* sp. n. is located anterior to the excretory pore, whereas in *A. bicaudatus*, it is closer to the excretory pore. The orientation of the ridges on the synlophes differs between the two species. *Alippistrongylus itatiaiaensis* sp. n. exhibits a single ridge orientation in both sexes, at about 80° in relation to the sagittal axis. In contrast, *A. bicaudatus* possesses ridges oriented at a 60° in both sexes. Table 1 provides a morphometric comparison among the species.

Molecular Characterisation

One partial 28S gene consensus sequence of *Alippistrongylus itatiaiaensis* sp. n., obtained in this study, comprised 546 base pairs in length. The sequence has been deposited in the GenBank database (<http://www.ncbi.nlm.nih.gov>) under accession number PP512577.

The sequence alignment in our dataset resulted, after trimming, in a matrix with 14 taxa. The best-fitting model, calculated by SMS in PhyML based on AIC, was HKY85 + G + I (free taxa model), presenting four free taxa categories, a proportion of invariant sites (P_{inv}) of 0.644 and a gamma (α) shape parameter of 0.117. This generated a maximum likelihood tree with lnL = -6257.889292. In the Bayesian analysis, after removing 25% of the burn-in data, the MCMC samples resulted in a mean marginal likelihood estimate of lnL = -6264.9745 (standard error = 0.0298; median = -6264.627). ESS values were robust for all parameters.

The tree topologies produced with different optimality criteria (ML and BI) were largely congruent, showing Trichostrongylina as monophyletic group, confirming *Alippistrongylus itatiaiaensis* sp. n. in the subfamily Nippostrongylinae clade with moderate to high support values (aLRT= 79%, BPP= 94%) (Figure 4).

Discussion

The genus *Alippistrongylus* has been sparsely documented in the literature, with only two species described to date: *Alippistrongylus*

bicaudatus Digiani and Kinsella, 2014 and *Alippistrongylus marki* Drabik, Vivar and Jiménez, 2022. Digiani and Kinsella (2014) described the genus *Alippistrongylus* and the species *A. bicaudatus* in the rodent *Delomys dorsalis*, the first helminth reported in this host, captured in the province of Misiones, Argentina. Drabik *et al.* (2022) described *A. marki* in the rodent *Euryoryzomys nitidus* (Thomas, 1884) found in the province of La Convención, Peru. The generic characterisation by Digiani and Kinsella (2014) outlined distinctive features, such as 21 uneven synlophes ridges in both sexes; males exhibiting an asymmetrical caudal bursa, with the right lobe hypertrophied, with a 1-4 or 4-1 pattern; and females with a long conical dorsal appendage, posterior to the vulva, forming a two-tailed appearance. The species *A. marki* shares diagnostic characteristics with the genus but differs from *A. bicaudatus*, notably in the female's ventrally positioned conical appendage and males with a symmetrical caudal bursa having a 2-2-1 pattern (Drabik *et al.* 2022).

Delomys dorsalis, the host species, is a terrestrial rodent found in tropical and subtropical humid forests of high and low altitudes in the South and Southeast regions of Brazil, as well as in the Northeast region of Argentina (Cademartori *et al.* 2005; Bonvicino *et al.* 2008). Our study describes a novel parasitic species sharing the same host as *A. bicaudatus*. However, *A. itatiaiaensis* sp. n. was discovered at high altitudes within the Itatiaia National Park, a pristine area of Atlantic Forest nestled in the municipality of Itatiaia, state of Rio de Janeiro. Noteworthy diagnostic distinctions of the new species include the appendix's rounded, non-conical shape and the caudal bursa's asymmetry, exhibiting a 4-1 pattern in both lobes, thus confirming its novelty.

Although our morphological analysis was comprehensive, the inclusion of molecular genetic data for the species could enrich our findings. Despite the limitations of the sequences obtained in this study, which could not be aligned with some belonging to the family Heligmonellidae, such as *Hassalstrongylus* and *Heligmosomoides*, the genetic sequence of *A. itatiaiaensis* sp. n. remains valuable to facilitate the identification and molecular differentiation of *Alippistrongylus* species. In addition, it helps to elucidate the systematic status of the subfamily Nippostrongylinae in future studies.

The understanding of helminths in small mammals within preserved areas remains scant, as evidenced by recent species disclosures (Cardoso *et al.* 2018). Indeed, the examination of parasites in wild animals is vital, serving as bioindicators to assess parasite-host dynamics and environmental health (Simões *et al.* 2010; Castro *et al.* 2017). Moreover, given the threats posed by climate change and forest fires, which contribute to biodiversity loss and parasite extinction, comprehensive biodiversity data is imperative and urgent.

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Competing interest. The authors declare that they have no conflict of interest.

Ethical standard. Permits for the collection and handling of the animals were issued by the Chico Mendes Institute for Biodiversity Conservation (ICMBio),

under authorisation N° 74498-11, and the Ethics Committee on the Use of Animals (CEUA/IOC), with license N° L-036/2018-A1.

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