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MORPHOLOGICAL AND GENETIC CHARACTERIZATION OF NEMATODES OF THE OPOSSUM *Didelphis albiventris* AND THE ARMADILLO *Dasypus novemcinctus* FROM SERRA DA CAPIVARA NATIONAL PARK

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Abstract: Serra da Capivara National Park (SCNP) in Northeast Brazil has the largest concentration of prehistoric sites in the Americas where studies of coprolites of ancestral parasitic fauna reported the presence of morphotypes of nematode eggs identified as Aspidoderidae and Trichostrongyloidea from dasypodid hosts. Among the current mammals inhabiting the park, the opossum *Didelphis albiventris* and the armadillo *Dasypus novemcinctus* were found roadkill in the nearby area. The aim of this work was to perform an integrative taxonomic analysis of adult nematode parasites of *D. albiventris* (N = 3) and *D. novemcinctus* (N = 2) found roadkill in the SCNP using morphological, ultrastructural and genetic approaches. The nematodes studied included *Aspidodera raillieti* and *Aspidodera subulata* (Aspidoderidae) collected from *D. albiventris*, and *Aspidodera binansata, Aspidodera vazi* (Aspidoderidae) and *Hadrostrongylus speciosum* (Trichostrongylidae) from *D. novemcinctus*. A new geographic locality of four species of Aspidoderidae and one Trichostrongylidae species are reported with new ultrastructural data using scanning electron microscopy of *A. binansata* and *A. vazi*. New genetic data of aspidoderids include sequences of partial 18S rRNA (SSU) and partial 28S rRNA (LSU) genes of *A. raillieti*, the first sequence of 16S rRNA of *A. vazi* and additional sequences of mitochondrial DNA cytochrome c-oxidase subunit 1, internal transcribed spacer 1, 2 and 5.8S rRNA gene of *A. raillieti*. These are the first partial sequences of 18S rRNA (SSU) and 28S rRNA (LSU) genes of species of the family Aspidoderidae, linked to a vouchered specimen.

Keywords: Aspidoderidae; genetics; helminths; scanning electron microscopy; Trichostrongylidae.

INTRODUCTION

Serra da Capivara National Park (SCNP) in Northeast Brazil, considered a World Heritage Site by the United Nations Educational, Scientific and Cultural Organization (UNESCO), has the largest concentration of prehistoric sites in the Americas (FUMDHAM 2016). The archeological richness and records putatively dating the presence of humans in the Americas to more than 50,000 years attracted studies of ancestral parasitic fauna (Sianto 2009). Helminthological studies in the region reported the occurrence of larvae of *Strongyloides ferreirai* Rodrigues, Vicente e Gomes, 1985 (Nematoda: Rhabdiasoidea) found in rock cavy *Kerodon rupestris* (Wied-Neuwied, 1820) (Rodentia: Caviidae) coprolites (Araújo *et al.* 1998), and *Trichuris* sp. (Nematoda: Trichuridae) (Ferreira *et al.* 1991, Araújo *et al.* 1993) and *Syphacia* sp. (Oxyurida: Oxyuridae) (Souza *et al.* 2012) eggs also in the rodent coprolites. However, the identification of ancient parasites is rather difficult, especially because only eggs and larvae are currently found. Thus, morphological and molecular studies of helminths of the recent fauna can be additional tools for future comparisons of ancient parasites.

Although the environment of SCNP is semi-arid, its geomorphology with a large plateau cut by several canyons allows the presence of several species of mammals. Among these species, the armadillos are abundant, especially *Dasypus novemcintus* (Linnaeus, 1758) (Cingulata, Dasypodidae) that is distributed broadly by the plateau and canyons (Chame 2007). Among the marsupials, which are rare in the region, *Didelphis albiventris* Lund, 1840 (Didelphimorphia, Didelphidae) occurs closer to the human dwellings and in the mesic enclaves provided by the canyons and edges of the plateau (Chame 2007). Wild animal populations are impacted by growing fragmentation and isolation, as well as hunting and aggravation of drought abnormalities. A federal highway (BR-324) cuts the park and a state highway (PI-140) follows parallel to its limits. Therefore, the wild fauna is occasionally found roadkill on outlying highways.

A previous study performed with fecal samples from dasypodid hosts such as *D. novemcinctus*, *Dasypus septemcinctus* (Linnaeus 1758), *Euphractus sexcinctus* (Linnaeus 1758) and *Tolypeutes tricinctus* (Linnaeus 1758) collected in the park reported the presence of morphotypes of nematode eggs identified as Aspidoderidae Skrjabin and Schikhobalova, 1947 and Trichostrongyloidea Cram, 1927 (Brandão *et al.* 2009). The family Aspidoderidae currently includes 17 species divided into four genera, and species of the genus *Aspidodera* have been reported in a wide range of hosts (Santos *et al.* 1990; Jiménez-Ruiz *et al.* 2012). These parasites are found in the cecum and large intestine of mammals of the orders Cingulata, Didelphimorphia, and Rodentia (Jimenez-Ruiz *et al.* 2012, Chagas-Moutinho *et al.* 2014).

Currently, the species of the genus *Aspidodera* include *A. scoleciformis* (Diesing, 1851), *A. subulata* (Molin, 1860), *A. fasciata* (Schneider, 1866), *A. binansata* Railliet and Henry, 1913, *A. raillieti* Travassos, 1913, *A. ansirupta* Proença, 1937, *A. vazi* Proença, 1937, *A. lacombae* Vicente, 1964, *A. esperanzae* Fujita *et al.* 1995, *A. sogandaresi* Jiménez-Ruiz, Gardner & Varela-Stokes, 2006, *A. kinsellai* Jiménez-Ruiz, Carreno & Gardner, 2013 and *A. lanfredi* Chagas-Moutinho *et al.* 2014. Among these species, the presence of *A. raillieti* has been reported in several localities in a wide variety of hosts (Jiménez-Ruiz *et al.* 2012).

Previous molecular studies of *A. raillieti* were performed by Jiménez-Ruiz *et al.* (2012) and Jiménez-Ruiz *et al.* (2013), who obtained sequences of spanning mitochondrial cytochrome c-oxidase subunit 1 (*cox*-1), 16S rRNA and ITS1, 5.8S and ITS2. On the other hand, *A. binansata* was studied for 16S rRNA and ITS1, 5.8S and ITS2 genes (Jiménez-Ruiz *et al.* 2012). However, there are no sequences for *A. vazi* available in the GenBank database so far. Genetic markers, such as 18S and 28S rRNA, are genes known to infer phylogenetic relationships for phylum and family studies (Nadler 1992, 1998), but there are no reports of 18S rRNA and 28S rRNA for any aspidoderid species.

We had the opportunity to examine roadkilled carcasses of *D. albiventris* and *D. novemcinctus* found in SCNP, which were parasitized by aspidoderid and trichostrongylid nematodes. In this context, the aim of this study was to identify and characterize these nematodes using integrative taxonomic analysis, including morphological and new descriptions of molecular sequences. We expect this study to give support for future parasitological studies in the Caatinga biome on the current and ancient fauna of nematodes.

MATERIAL AND METHODS

Host collection and parasitological examination

SCNP is located from 08º26'50.10" N to 08º54'23.36" S and from 08º36'33.68" E to 08º46'28.38" W in an isolated region of the northeast semiarid in Piauí state, Brazil. Three adult specimens of *D. albiventris* and two of *D. novemcinctus* found roadkill or seized from hunters by agents of Chico Mendes Institute for Conservation of Biodiversity (ICMBio) were donated to the Museum of American Humanity (FUMDHAM). For research purposes, FUMDHAM donated the animals to Fundação Oswaldo Cruz (SISBIO n. 28319-6).

The parasites found in the gastrointestinal content were washed in physiological saline (0.7%) and stored in 70% alcohol. In the laboratory, the parasites were examined after clearing in glycerin for morphological identification. The eggs were obtained from females previously identified and microphotographs were performed using an AxioCam ERc5s coupled to a Zeiss microscope, using the program AV LE (AxioVision Limited Edition). Measurements are given in micrometers (µm) with range in parentheses. Drawings were made with a drawing tube and redrawn using Adobe Illustrator CS6 (Adobe Systems Inc.)

Scanning electron microscopy (SEM)

Nematode specimens were washed in 0.1 M cacodylate buffer pH 7.2, post-fixed in 1% OsO_4 and 0.8% potassium ferrocyanide for 1 h, dehydrated in a graded series of ethanol solutions (30–100%) for 1 h for each step, critical-point dried in CO_2 and sputter coated with gold (Cavalcante *et al.* 2018). The specimens were examined using a Jeol JSM 6390 SEM.

DNA extraction, polymerase chain reaction, and sequencing

The Genomic DNA of the nematodes was extracted from one entire specimen of each species using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's recommendations. PCR assays were carried out in a total volume of 15 µl containing 7.5 µl of 2× GoTaq® Colorless Master Mix (Promega), 1.5 µl of primers with final concentration of 0.4-1.0 µM, 1.0-3.0 µl of cDNA sample and ultrapure water to complete the volume. The 16S rRNA was amplified by PCR using the forward primer 16SCE (5'- ATTCTATCTCACAATGAATTAAAC-3') and reverse primer C2F3 (5'-CGTCAATGTTCAGAAATTTGTGG-3') (Jiménez-Ruiz et al. 2012). The partial region spanning 18S rRNA was amplified by PCR using a new primer designed for this study: forward Asp_18SF (5'-CGTTCCGTCGGCGGTAAATATG-3'), and the known reverse 136 (5'-TGATCCTTCTGCAGGTTCACCTAC-3'), following the conditions described by Nadler *et al.* (2007). The rRNA region spanning ITS1, 5.8S and ITS2 was amplified by PCR using a newly designed set of primers: Asp_ITS_F (5'-GTTGCTGCATGCTTGAAAGT-3') and Asp_ITS_R (5'GCACTAGCGGAATACTCCTAAC-3'), with the following conditions: 94°C for 2 min, followed by 40 cycles at 95°C for 30 s, 56°C for 30 s, 72°C for 60 s, and 72°C for 7 min. The mitochondrial cytochrome c-oxidase subunit 1 (cox-1) spanning region was amplified by PCR using a cocktail of primers (Prosser et al. 2013). The new region spanning partial 28S rRNA was amplified by PCR using the newly designed set of primers Asp_28SF (5'- AAGCCCAGCGCTGAATCCATTA -3') and Asp_28SR (5'- TCAACTTTCACACCGAGAGGCTA -3'), with the following conditions: 94 °C for 2 min, followed by 40 cycles at 95°C for 30 s, 56°C for 30 s, 72°C for 60 s, and 72°C for 7 min.

Amplicons were analyzed by electrophoresis in 1.5% agarose gels, stained with SyberGreen (Invitrogen) and photographed under UV transillumination. Amplified PCR products were purified with ExoSAP-IT (Affymetrix) and sequenced using the same primer set. DNA cycle sequencing reactions were performed using BigDye v.3.1 chemistry (Applied Biosystems, Foster City, CA, USA). Sequences of both strands were generated, edited and aligned using the CLUSTAL W algorithm implemented in MEGA 6.0. The sequence was compared to other sequences available in the GenBank database using BLAST (Altschul *et al.* 1990). The sequences obtained in this study were deposited in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) as: *A. raillieti* KX962175 (mtDNA *cox*-1), KX954131 (16S rRNA), KX954128 (18S rRNA), KX954129 (ITS1, 5.8S and ITS2 rRNA) and KX954130 (28S rRNA); for *A. vazi*, KX951458 (16S rRNA).

Phylogenetic analysis

Genetic distance (pairwise) was inferred to establish the divergences among species of *A. raillieti* from this study and specimens deposited in GenBank database using Tamura-Nei model for mtDNA *cox1*, ITS1, 5.8S and ITS2 rRNA and 16S rRNA. The results in percentage are followed by standard errors in parentheses. The best substitution model for partial 28S rRNA for Bayesian inference (BI) and maximum likelihood (ML) dataset was the GTR+I+G model of nucleotide substitution selected under the Akaike information criterion (AIC) by MrModelTest 2 with the aid PAUP4.0a147 (Nylander 2004). Phylogenetic reconstructions were carried out using BI using MrBayes 3.2.6, where the Markov chain Monte Carlo (MCMC) was set to 4×10^6 generations, every 4×10^3 tree was sampled and the first 1×10^6 generations were omitted from phylogeny reconstruction. The remaining trees were used to generate a consensus tree and to calculate the Bayesian posterior probabilities of all branches using a majority-rule consensus approach. Maximum likelihood analysis was performed with PhyML 3.1 and nodal support was estimated by performing 1,000 bootstrap replicates (Swofford 2002). An additional phylogenetic reconstruction using concatenated mtDNA *cox1* and 16S rRNA sequences of *A. raillieti* isolated from different hosts and countries was constructed including the sister group *Nematomystes* spp. and as outgroup, *Heterakis gallinarum*. Tree topologies were visualized in FigTree 1.4.2. Taxa for which sequences have been reported in the GenBank are listed in Table 1.

RESULTS

Morphology and ultrastructure

The specimens of *D. albiventris* examined harbored *A. subulata* and *A. raillieti*. The specimens of *D. novemcinctus* were parasitized by *A. binansata*, *A. vazi* and *Hadrostrongylus speciosum*.

***Aspidodera raillieti* Travassos, 1913** (Figures 1A-B, 2A, and 3A-D)

Site of infection: large intestine and cecum.
Prevalence: two of three specimens infected (66.6%).
Host: *Didelphis albiventris*
Intensity: 26 and 72 parasites.
Specimen deposited: CHIOC no. 38.353.
Locality: Serra da Capivara National Park, new geographical distribution.

Male (based on ten specimens): Body length 6445 (5600–7550), width at midbody 577 (500–665). Cephalic cap 130 (110–140) long and cephalic cordons 74 (60–90). Body length/cephalic cap ratio1: 40.7 to 57.6. Esophagus 779 (700–970) long; bulb 207 (200–225) long and 177 (165–195) wide. Nerve ring at 261 (250–275) and excretory pore at 616 (585–650) from anterior end (Figure 1A). Spicules equal in size and shape, 1025 (950–1200) long with a slightly rounded tip. Gubernaculum 195 (185–205) long. Genital sucker 117 (105–135) long, situated 80 (70–90) from the cloaca. Caudal spine 41 (35–50) long. We observed 17 pairs of papillae: 5 pairs precloacal, 2 adcloacal and 11 postcloacal (Figure 1B).

Female (based on ten specimens): Body length 8748 (7800–9790), width 858 (740–970) at vulva region. Cephalic cap 174 (155–200) and cephalic cordons 96 (85–110) long. Body length/cephalic cap ratio 1:40.4 to 55.9. Esophagus 832 (775–925) long; bulb 258 (200–300) long and 195 (175–205) wide. Nerve ring at 225 (190–235) and excretory pore at 447 (425–475) from anterior end. Vulva at 2990 (2300–3430) from cephalic end. Tail long, 1273 (1190–1390). Eggs thin-shelled, elliptic, 63.9 (50–72.5) long and 45 (42.5–47.5) wide (Figure 2A).

The micrographs of *A. raillieti* show a cephalic end formed by three lips, one dorsal and two ventrolateral, lateral groove and excretory pore (Figure 3A). Each lip has two slender projections in posterior region (Figure 3B). The ventrolateral lips present with a pair of papillae and one amphid. Between the lips, we observed an interlabial projection with an apical pore-like structure. The interlabial projection between the ventrolateral and dorsal lips was thinner than that observed between the ventrolateral lips (Figure 3B). Some papillae were found right below the cephalic cap. Tail presented a striated cuticle and a spine, genital sucker precloacal delimited with two pairs of papillae (one pair at the anterior edge and one posterior) and cloaca (Figures 3C and D).

Table 1. List of species of Nematodes used in the phylogenetic analysis of the aspidoderids using 16S, 28S rRNA and mtDNA *cox1*.

Species	Host	228S rRNA	16S rRNA	mtDNA cox1	References
Heterakoidea					
Aspidodera raillieti	*Didelphis albiventris* (Brazil)	**KX954130**	**KX954131**	**KX962175**	Present study
Aspidodera vazi	*Dasypus novemcinctus* (Brazil)		**KX951458**		Present study
Aspidodera raillieti	*Didelphis virginiana* (U.S.A)			KC470127	Jiménez-Ruiz *et al.* 2012, 2013
Aspidodera raillieti	*Didelphis virginiana* (Panama)*			KC470128	Jiménez-Ruiz *et al.* 2012, 2013
Aspidodera raillieti	*Didelphis pernigra* (Bolivia)		JN852770	KC470125	Jiménez-Ruiz *et al.* 2012, 2013
Aspidodera raillieti	*Didelphis pernigra* (Bolivia)		JN852768	KC470126	Jiménez-Ruiz *et al.* 2012, 2013
Aspidodera raillieti	*Didelphis pernigra* (Bolivia)		JN852769		Jiménez-Ruiz *et al.* 2012
Aspidodera raillieti	*Didelphis marsupialis* (Guatemala)		JN852767		Jiménez-Ruiz *et al.* 2012
Aspidodera binansata	*Dasypus novemcinctus* (Peru)		JN852758		Jiménez-Ruiz *et al.* 2012
Aspidodera sogandaresi	*Dasypus novemcinctus* (Mexico)		JN852771	KC470131	Jiménez-Ruiz *et al.* 2012
Aspidodera sogandaresi	*Dasypus novemcinctus* (Mexico)		JN852772		Jiménez-Ruiz *et al.* 2012
Aspidodera sp.	*Dasypus novemcinctus* (Costa Rica)			KC470132	Jiménez-Ruiz *et al.* 2012
Aspidodera sp.	*Dasypus novemcinctus* (Mexico)			KC470133	Jiménez-Ruiz *et al.* 2012
Aspidodera sp.	*Dasypus novemcinctus* (Mexico)			KC470134	Jiménez-Ruiz *et al.* 2012
Aspidoder scoleciformis	*Euphractus sexcinctus* (Paraguay)		JN852757		Jiménez-Ruiz *et al.* 2012
Strongyluris calotis	*Pseudocalotes brevipes*	LC133188			Tran *et* al. 2016
Ramsonematoidea					
Carnoya filipjevi	diplopod *Saipidobolus* sp.	JX946703			Malysheva, 2014
Cattiena fansipanis	diplopod Pseudospirobolellidae	JX436470			Malysheva *et al.* 2012
Heth taybaci	diplopod Harpagophoridae	JX946704			Malysheva *et al.* 2012
Oxyuroidea					
Thelandros tinerfensis	*Tarentola gomerensis*	KJ778089			Jorge *et al.* 2014
Parapharyngodon echinatus	*Gallotia atlantica mahoratae*	JF829240			Jorge *et al.* 2011
Aspiculuris tetraptera	*Mus musculus*	AB500179			Okamoto *et al.* 2009
Ascaridoidea					
Heterakis gallinarum	*Gallus gallus*		JN852754	KP308363	Jiménez-Ruiz *et al.* 2013, Gu *et al.* 2015
Heterocheilus tunicatus	No information	U94759			Nadler & Hudspeth, 1998

*Currently *Didelphis marsupialis*.

***Aspidodera subulata* Railliet and Henry, 1912**
(Figures 1C and 2B)

Site of infection: cecum and large intestine.
Prevalence: one of three specimens infected (33.3%).
Host: *Didelphis albiventris*
Intensity: one parasite.
Specimen deposited: CHIOC no. 38.353.
Locality: Serra da Capivara National Park, new geographical distribution.

Female (based on one specimen): Body length 7100, width 830 at vulva region. Cephalic cap 140 and cephalic cordons 85 long. Body length/cephalic cap ratio 1:50.7. Esophagus 775 long; bulb 225 long and 200 wide. Nerve ring at 250 from anterior end. Excretory pore not observed (Figure 1C). Vulva at 2490 from cephalic end. Tail at 650 from tip. Eggs thin-shelled, elliptic and irregular, 67 (62.5–72.5) long and 45.8 (42.5–50) wide (Figure 2B).

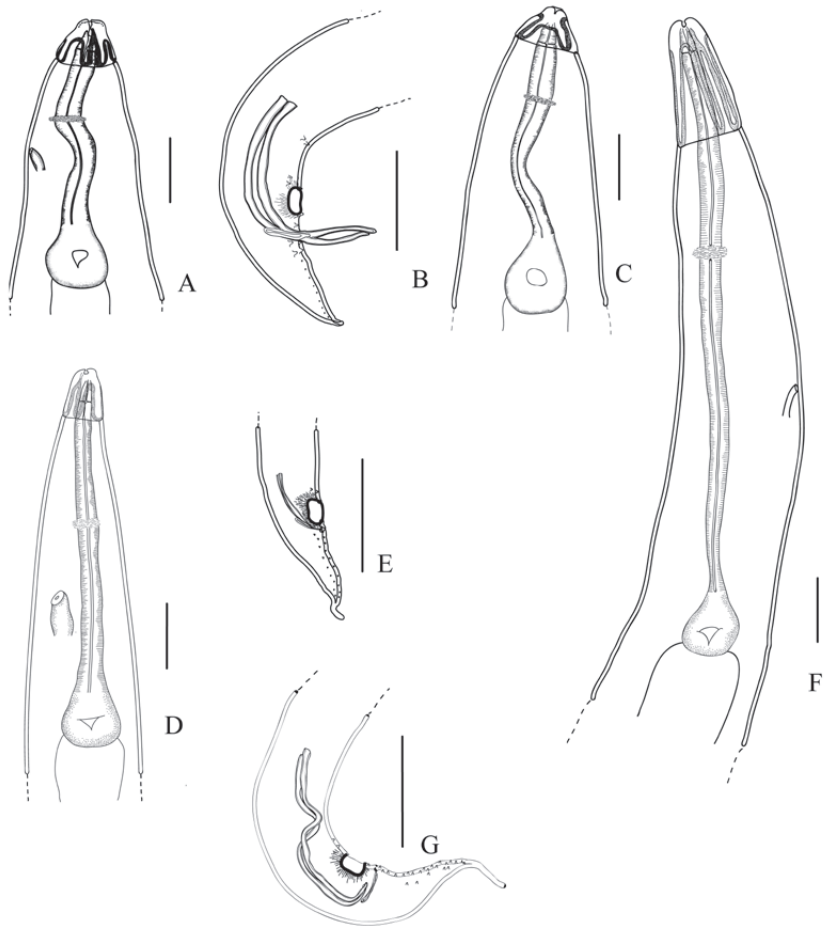

Figure 1. Drawing from light microscopy: **A –** *Aspidodera raillieti* (Ascaridida, Aspidoderidae): general view of anterior region, cephalic cordons, cephalic cap, nerve ring, excretory pore, esophagus with a terminal bulb; **B –** tail of male showing genital sucker, cloaca, spicules, gubernaculum, caudal projection, and caudal papillae; **C –** *Aspidodera subulata*: cephalic cordons, cephalic cap, nerve ring and esophagus; **D –** *Aspidodera binansata*: cephalic cordons, cephalic cap, nerve ring and esophagus with a terminal bulb; **E –** tail of male showing genital sucker, cloaca, spicules, gubernaculum, caudal projection, and caudal papillae; **F –** *Aspidodera vazi*: view of anterior region showing the cephalic cordons, cephalic cap, nerve ring, excretory pore, esophagus with a terminal bulb and **G –** tail of male showing genital sucker, cloaca, spicules, gubernaculum, caudal projection, and caudal papillae. Scale bar of 0.2 mm in A, C, D and F and bar of 0.5 mm in B, E and G.

***Aspidodera binansata* Railliet and Henry, 1913**
(Figures 1D-E, 2C, and 4A-B)

Site of infection: cecum and large intestine.
Prevalence: two of two specimens infected (100%).
Host: *Dasypus novemcinctus*
Intensity: 10 and 26 parasites.
Specimen deposited: CHIOC nº 38.351.
Locality: Serra da Capivara National Park, new geographical distribution.

Male (based on ten specimens): Body length 6483 (6000–7550), width at midbody 368 (320–460). Cephalic cap 150 (130–160) long and cephalic cordons 104 (88–115). Body length/cephalic cap ratio 1:37.5 to 50. Esophagus 1148 (1020–1280) long; bulb 198 (173–250) long and 178 (145–220) wide. Nerve ring at 444 (350–500) and excretory pore at 607 (550–660) from anterior end (Figure 1D). Spicules equal in size and shape, 322 (300–335) long with a slightly rounded tip. Gubernaculum 156 (140–165) long. Genital sucker 116 (105–135) long, situated 23 (10–40) from the cloacal aperture. Caudal spine 112 (100–125) long. Caudal papillae, 33 pairs: 8 pairs precloacal, 1 pair adcloacal and 24 postcloacal (Figure 1 E).

Female (based on ten specimens): Body length 7093 (5625–7750), width 457 (370–510) at vulva region. Cephalic cap 163 (125–175) and cephalic cordons 113 (100–125) long. Body length/cephalic cap ratio 1:40.4 to 46.4. Esophagus 1206 (1100–1270) long; bulb 196 (163–225) long and 195 (175–208) wide. Nerve ring at 536 (500–600) and excretory pore at 720 (670–800) from anterior end. Vulva at 4050 (3500–4750) from cephalic end. Tail long, thin, 1008 (825–1200) long. Thin-shelled, elliptic and irregular eggs 64.3 (57.5–70) long and 45.6 (42.5–50) wide (Figure 2C).

The ultrastructure from the anterior region showed a cephalic cap with three lips and digitiform projection lateral to lips. Long interlabial projections reached the base of the cephalic cap; two anastomosing cordons can be seen on the dorsal side of the cap. Small papillae were observed below to cephalic cap (Figure 4A). The posterior region showed the ventral sucker near the cloaca and precloacal, adcloacal and postcloacal papillae (Figure 4B).

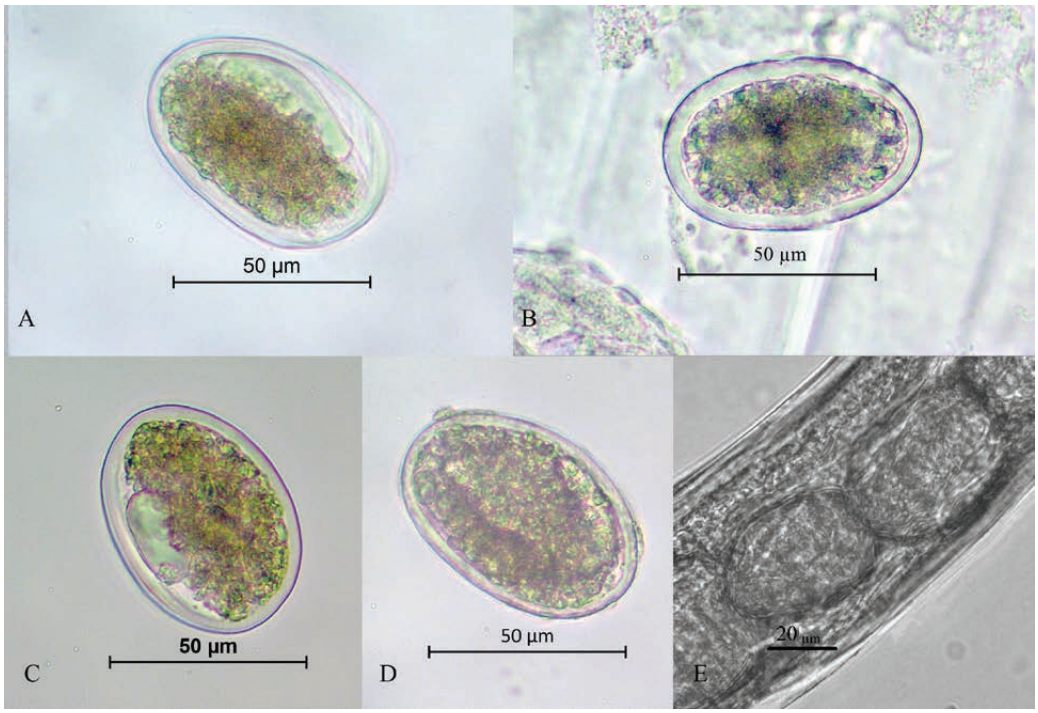

Figure 2. Micrographs of eggs of the nematodes from Serra da Capivara National Park, Piauí state, Brazil: **A –** *Aspidodera raillieti* (Ascaridida, Aspidoderidae); **B –** *Aspidodera subulata*; **C –** *Aspidodera binansata*; **D –** *Aspidodera vazi*; **E –** *Hadrostrongylus speciosum* (Strongylida, Trichostrongylidae).

***Aspidodera vazi* Proença, 1937** (Figures 1F-G, 2D, and 5A-F)

Site of infection: cecum and large intestine.
Prevalence: two of two specimens infected (100%).
Host: *Dasypus novemcinctus*
Intensity: 5 and 25 parasites.
Specimen deposited: CHIOC no. 38.354.
Locality: Serra da Capivara National Park, new geographical distribution.

Male (based on fifteen specimens): Body length 7485 (6250–8625), width at midbody 347 (250–510). Cephalic cap 363 (337–395) long and cephalic cordons 311 (277–333) long. Body length/ cephalic cap ratio 1:17.7 to 23.2. Esophagus 1159 (1000–1400) long; bulb 182 (150–250) long and 166 (125–250) wide. Nerve ring at 717 (650–750) and excretory pore at 993 (780–1200) from anterior end (Figure 1F). Spicules equal in size and shape, 822 (650–1200) long with a slightly rounded tip. Gubernaculum 156 (145–180) long. Genital sucker 122 (100–150) long, situated 45 (25–55) from the cloaca. Caudal spine 118 (50–200) long. Caudal papillae 26 pairs: 3 pairs precloacal, 1 pair adcloacal and 23 postcloacal (Figure 1G).

Female (based on ten specimens): Body length 7345 (6950–8000), width 3 55 (280–490) at vulva region. Cephalic cap 438 (378–478) and cephalic cordons 338 (313–375) long. Body length/ cephalic cap ratio 1:15.1 to 19.6. Esophagus 1379 (1210–1530) long; bulb 211 (175–238) long and 192 (173–213) wide. Nerve ring at 723 (680–800) and excretory pore at 892 (800–980) from anterior end. Vulva at 2763 (2500–3500) from cephalic end. Tail 677 (575–750) long. Eggs thin-shelled, elliptic 60 (58–63) long and 52 (48.5–55) wide (Figure 2D).

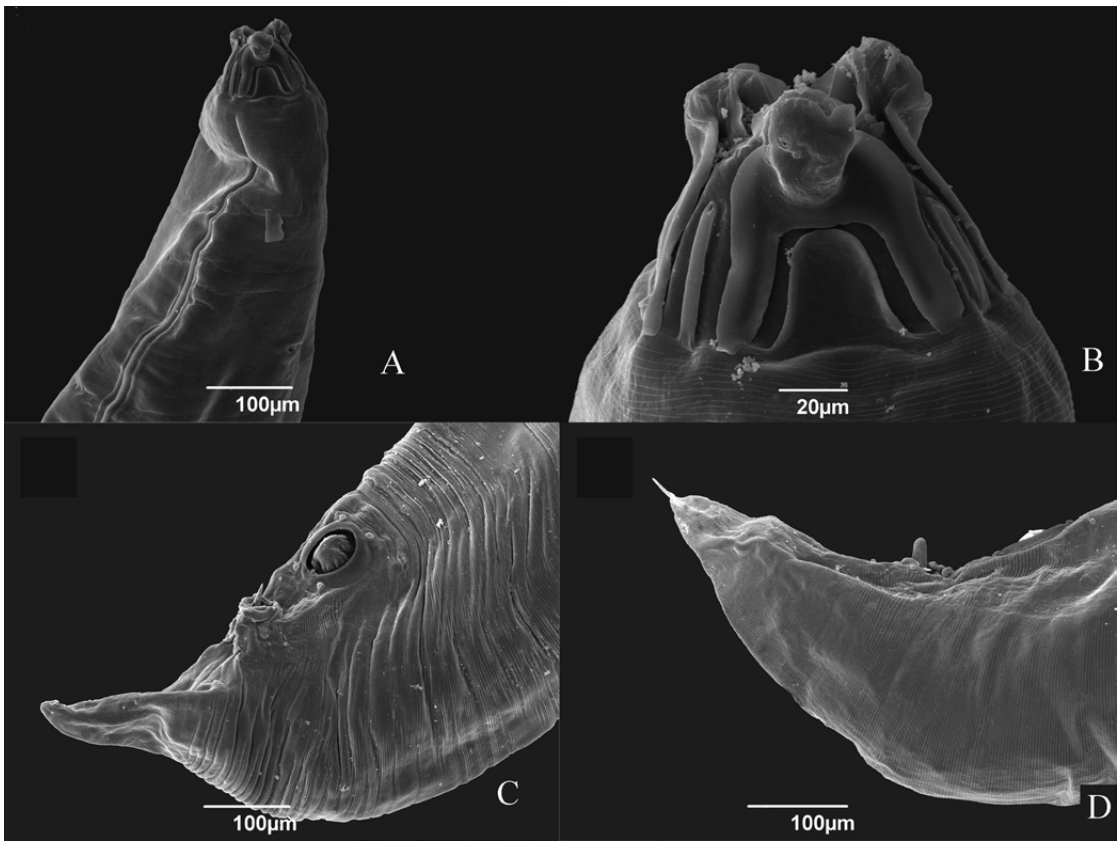

Figure 3. Scanning electron microscopy of *Aspidodera raillieti* (Ascaridida, Aspidoderidae) from *Didelphis albiventris* (Didelphimorphia, Didelphidae), Serra da Capivara National Park, Piauí state, Brazil: **A –** anterior region showing cephalic cap with three lips, lateral groove and excretory pore; **B –** detail of cephalic cap with three lips, interlabial projection, slender when appears between ventrolateral and dorsal lips and with a pore-like structure at its apex; left ventrolateral lip with amphid and papilla. Ventrolateral lips emitting two slender projections to posterior region; **C –** posterior region of male with a pre-cloacal sucker and papillae and **D –** lateral view of male posterior region with a caudal spine.

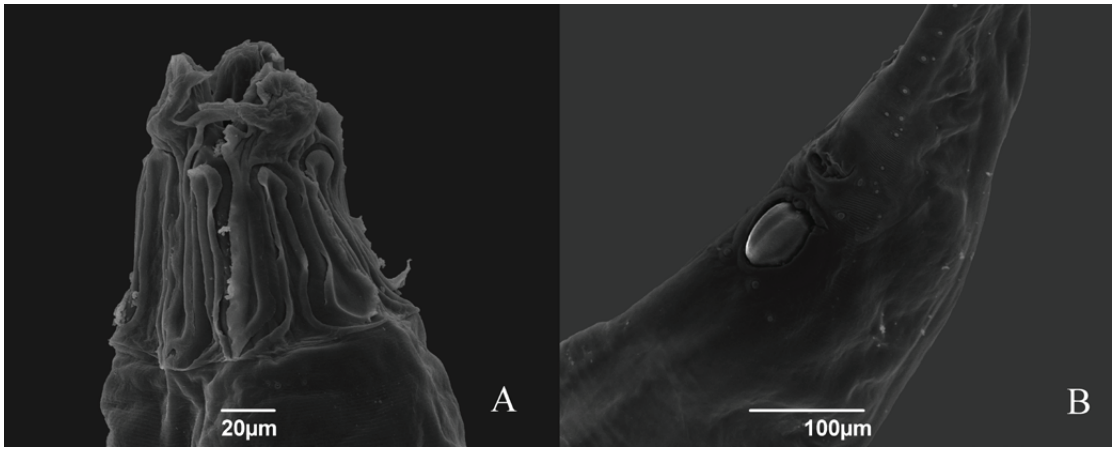

Figure 4. Scanning electron microscopy of *Aspidodera binansata* (Ascaridida, Aspidoderidae) from *Dasypus novemcinctus* (Cingulata, Dasypodidae), Serra da Capivara National Park, Piauí state, Brazil: **A –** cephalic cap with three lips; ventrolateral lip emitting two slender projections to posterior region and one projection arising from the base of the cephalic cap; long interlabial projection. Some papillae observed below the cephalic cap and **B –** posterior region of male with pre-cloacal sucker and numerous papillae.

Scanning electron microscopy shows an anterior extremity with an elongated cephalic cap. Arising from the basis of cephalic cap, a large projection directed to the anterior extremity was observed on each lip (Figure 5A). Three lips, one dorsal and two ventrolateral, each one with two slender and long projections directed posteriorly (Figure 5B). Ventrolateral lips with one amphid and one papilla each (Figure 5C). The right side of the apex of the lateral lips presented digitiform projections. Lateral ala in the posterior region (Figure 5D). Oral vestibule lined by a cuticular membrane (Figure 5E). Small papillae were observed below the cephalic cap and near the excretory pore (Figure 5F).

***Hadrostrongylus speciosum* Hoppe and Nascimento, 2007** (Figures 2E, and 6A-D)
Site of infection: cecum and large intestine.
Prevalence: one of two specimens infected (50%).
Host: *Dasypus novemcinctus*
Intensity: 37 parasites.
Specimen deposited: CHIOC no. 38.355.
Locality: Serra da Capivara National Park, new geographical distribution.

Male (based on ten specimens): Body length 4287 (3825–4750) and 126 (105–150) wide. Cephalic region 39. Nerve ring 153 (145–160) from anterior end and excretory pore close to junction esophagus-intestine. Esophagus 287 (275–310) long (Figure 6A). Male with trilobate copulatory bursa, formula 2-1-2. The lateral lobes joined. Rays 2 and 3 are joined in all extension and with origin in a common trunk. Rays 4 and 6 emerge in common trunk. Ray 4 short and thicker than the other and are not joined with 5 and 6 close to their base. Dorsal ray is thick, bifurcated in its extremity is distal. Spicules unequal, complex, well ornamented: larger 196 (187–212) and smaller 155 (137–170) long. Gubernaculum simple, 123 (112–137) long. Copulatory bursa 134 (117–150) long and 167 (125–192) wide (Figure 6B).

Female (based on ten specimens): Body length 5740 (5125–6625) and 142 (100–165) wide in vulva region. Esophagus 330 (280–360) long. Nerve ring 130 (100–165) and excretory pore 170 from anterior end. Female are didelphic and amphidelphic. Vulva at 1110 (875–1250) from the anterior region. Branches of vagina vera are unequal: anterior 201 (180–225) long and posterior 135 (125–145) long (Figure 6C). Anal opening subterminal 119 (110–150) from tail tip (Figure 6D). Tail with terminal spine. Eggs elliptic in uterus, 54 (50–57.5) long and 31.1 (27.5–32.5) wide, feature a hyaline space between the embryo and the thin shell (Figure 2E).

Molecular analysis
The sequences of *A. raillieti* obtained in this study were deposited in the GenBank as mtDNA *cox*-1 region accession number KX962175 with 600 bp; partial 16S rRNA number KX954131 with 930 bp;

new sequence of partial 18S rRNA number KX954128 with 722 bp; ITS1, 5.8S and ITS2 rRNA number KX954129 with 765 bp and new sequence of partial 28S rRNA KX954130 with 726 bp. Additionally, a new sequence of *A. vazi* was deposited in the GenBank as 16S rRNA region accession number KX951458 with 743 bp.

Sequences of *A. raillieti* of the mtDNA *cox*-1 and 16S rRNA and ITS1, 5.8S and ITS2 rRNA were similar to *A. raillieti* accession numbers KC470125, EF180070 and JQ995300, respectively (Table 1). The new sequence of the 18S rRNA obtained in our study were similar to *Aspidodera* sp. (EF180070), and the partial 28S rRNA obtained was more similar to *Strongyluris calotis* (Heterakoidea: Heterakidae) (LC133188).

The mtDNA *cox*-1 BLAST analysis indicated 94% identity with 81% query cover and a maximum score of 734 of with *A. raillieti* (KC470125). The partial 16S rRNA BLAST analysis indicated 96% identity with

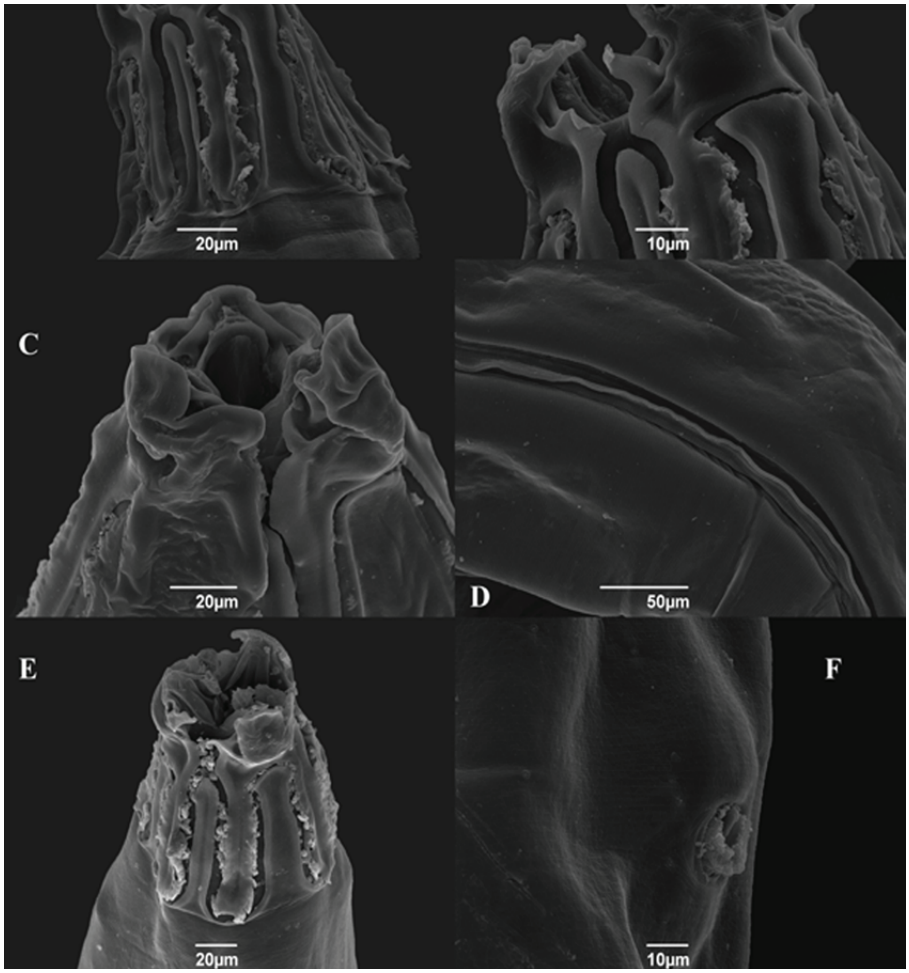

Figure 5. Scanning electron microscopy of *Aspidodera vazi* (Ascaridida, Aspidoderidae) from *Dasypus novemcinctus* (Cingulata, Dasypodidae) Serra da Capivara National Park, Piauí state, Brazil: **A –** cephalic cap showing lips with two long and slender projections directed to posterior region and a larger projection arising from the base of cephalic cap; long and slender interlabial projection; ventrolateral lip with one amphid and one papilla; **B –** detail of the lips showing amphid and papilla on the ventrolateral lip and the digitiform projection on the lateral apex of the lips; **C –** apical view of oral aperture showing the oral vestibule lined by a cuticular membrane; **D –** detail of lateral groove; **E –** anterior region with cephalic cap with three lips long and slender interlabial projection. Each lip emits two long and slender projections to posterior region and, at the base of cephalic cap, one long projection arises directed to anterior extremity and **F –** detail of excretory pore with some papillae nearby.

95% query cover and a maximum score of 1408 with *A. raillieti* (JN852767). The BLAST alignment for partial 18S rRNA with *Aspidodera* sp. (EF180070) showed 99% identity, 100% query cover and maximum score of 1328. The BLAST alignment for ITS1, 5.8S and ITS2 rRNA with *A. raillieti* (JQ995300) showed 99% identity, 100% query cover and maximum score of 1402. The partial 28S rRNA region aligned with *Strongyluris calotis* (LC133188) with 90% identity, 100% query cover and maximum score of 950. This is the first report of a sequence of 18S rRNA for *A. raillieti* and 28S rRNA sequence for Aspidoderidae. In addition, the new sequence of *A. vazi* of 16S rRNA was similar to *Koerneria sudhausi* accession number KT355738, indicating 76% identity, 82% query cover and maximum score of 291.

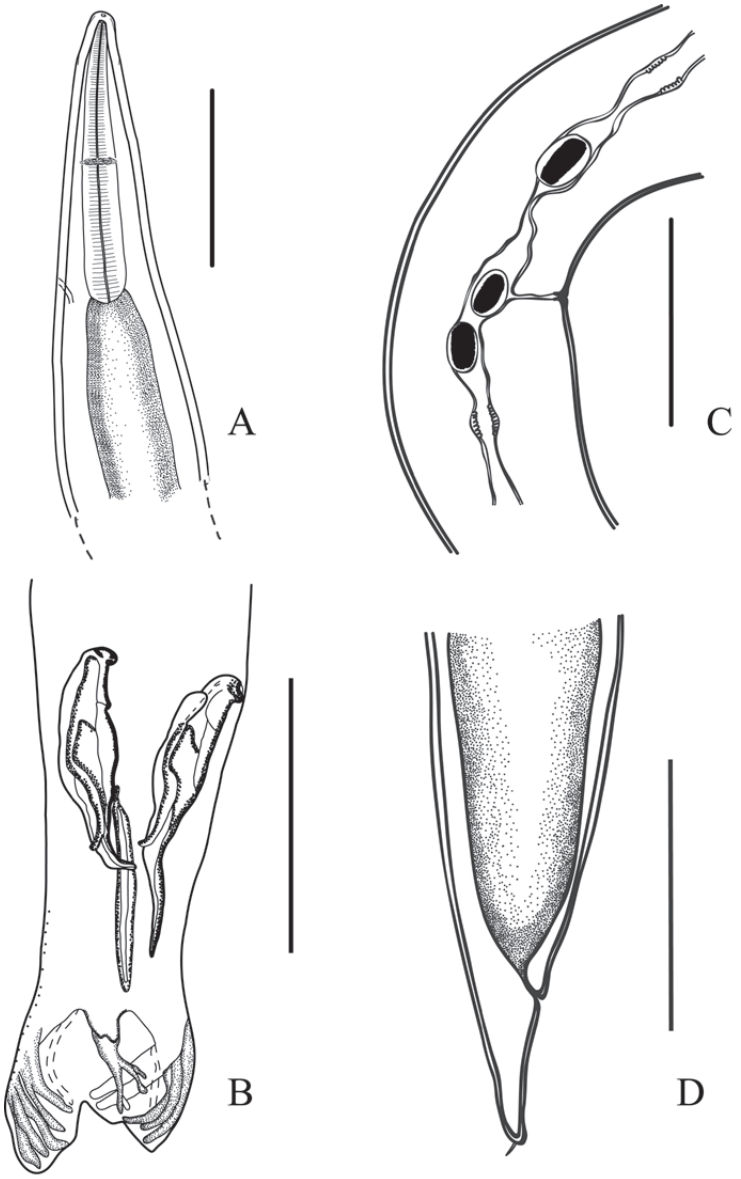

Figure 6. Drawing from light microscopy of *Hadrostrongylus speciosum* (Strongylida, Trichostrongylidae), Serra da Capivara National Park, Piauí state, Brazil. **A** –anterior region showing nerve ring and excretory pore close to junction esophagus-intestinal (Scale bar: 0.3 mm). **B** – copulatory bursa, unequal spicule and gubernaculum (Scale bar: 0.2 mm). **C** – Uterus with eggs and anterior branch *vagina vera* larger than posterior (Scale bar: 0.2 mm). **D** – anal opening subterminal with the tail tip (Scale bar: 0.2 mm).

The genetic distance based on mtDNA *cox1* of *A. raillieti* from different hosts and geographical areas varied from 0.3–9.5%. The divergences from our specimens were 7.9 % (± 1.4) from specimens isolated from *D. virginiana* from USA, 7.1% (± 1.3), 7.2% (± 1.2) from specimens of *D. virginiana* (currently *D. marsupialis*) from Panama and 7.3% (± 1.3) from specimens from *D. pernigra* (Bolivia). The overall genetic distance of the mtDNA *cox1* dataset from specimens of *A. raillieti* was 7.2%. For ITS1, 5.8S and ITS2 rRNA region the genetic distance ranged from 0.1 to 1.0% among all specimens of *A. raillieti*. Genetic distance between *A. raillieti* from this study and those from GenBank was 0.3–0.7% (± 0.3) from specimens isolated of *D. pernigra* (Bolivia) and 0.4% (± 0.2) from specimens isolated of *D. marsupialis* (Guatemala). The overall genetic distance of the ITS1, 5.8S and ITS2 rRNA was 0.5%. For 16S rRNA, the genetic distance among all available sequences of *A. raillieti* ranged from 0–5.7%. The comparison of *A. raillieti* from this study and those previously obtained from GenBank was 5.4–5.7% (±0.9) from specimens isolated of *D. pernigra* (Bolivia) and 4.8% (± 0.8) from specimens isolated of *D. marsupialis* (Guatemala). The overall genetic distance of the rRNA 16S dataset was 4.1%.

The topologies of the phylogenetic reconstruction using ML and BI were similar in both analyses. Phylogenetic reconstruction showed that *Aspidodera raillieti* (Aspidoderidae) and *Strongyluris calotis* (Heterakidae) belonging to the Heterakoidea were closely related with support (BI = 1 and ML = 96%). Heterakoidea (represented by Aspidoderidae and Heterakidae) was more closely related to Oxyuroidea (Pharyngodonidae and Heteroxynematidae) than Ransomnematoidea (represented by Carnoyidae and Hethidae) (BI = 1 and ML = <70%). Within Oxyuroidea, *Thelandros tinerfensis* and *Parapharyngodon echinatus* belonging to the Pharyngodonidae appear in a separate clade. Similarly, within Ransomnematoidea, *Carnoya filipjevi* and *Cattiena fansipanis* belonging to the Carnoyidae appear in separate clades (Figure 7).

The phylogenetic reconstruction based on 16S rRNA and mtDNA *cox1* sequences of aspidoderids from different hosts and countries showed a clade with *Aspidodera raillieti* from didelphid hosts linked to clades of *Aspidodera* spp. from dasypodid hosts (Figure 8 A-B). In the 16S rRNA tree, *A. raillieti* sequences from Bolivia, Brazil and Guatemala are clustered with high support separated from *A. binansata, A. sogandaersi, A. scoleciformis* and *A.*

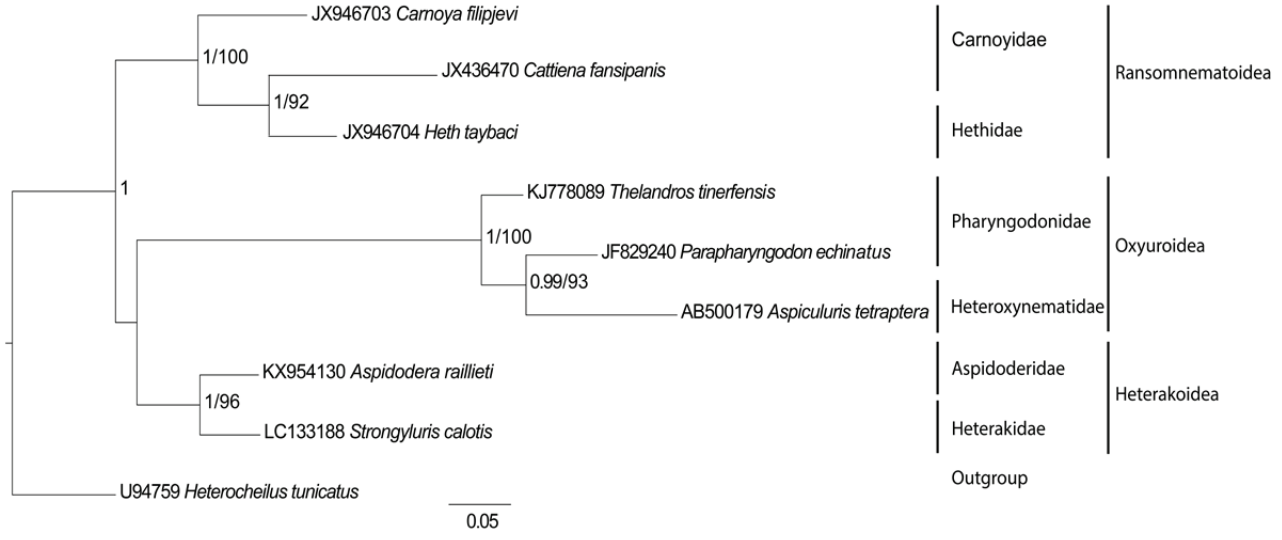

Figure 7. Phylogenetic relationship among aspidoderid nematodes based on the maximum likelihood (ML) and Bayesian inference (BI) of the partial 28S rRNA gene. Nodal support associated with branches is listed as ML bootstrap support / Bayesian posterior probability (Bpp). Statistical support values lower than 0.80 in Bpp and 70% in bootstrap are not shown. Scale bars indicate the nucleotide mutations per site.

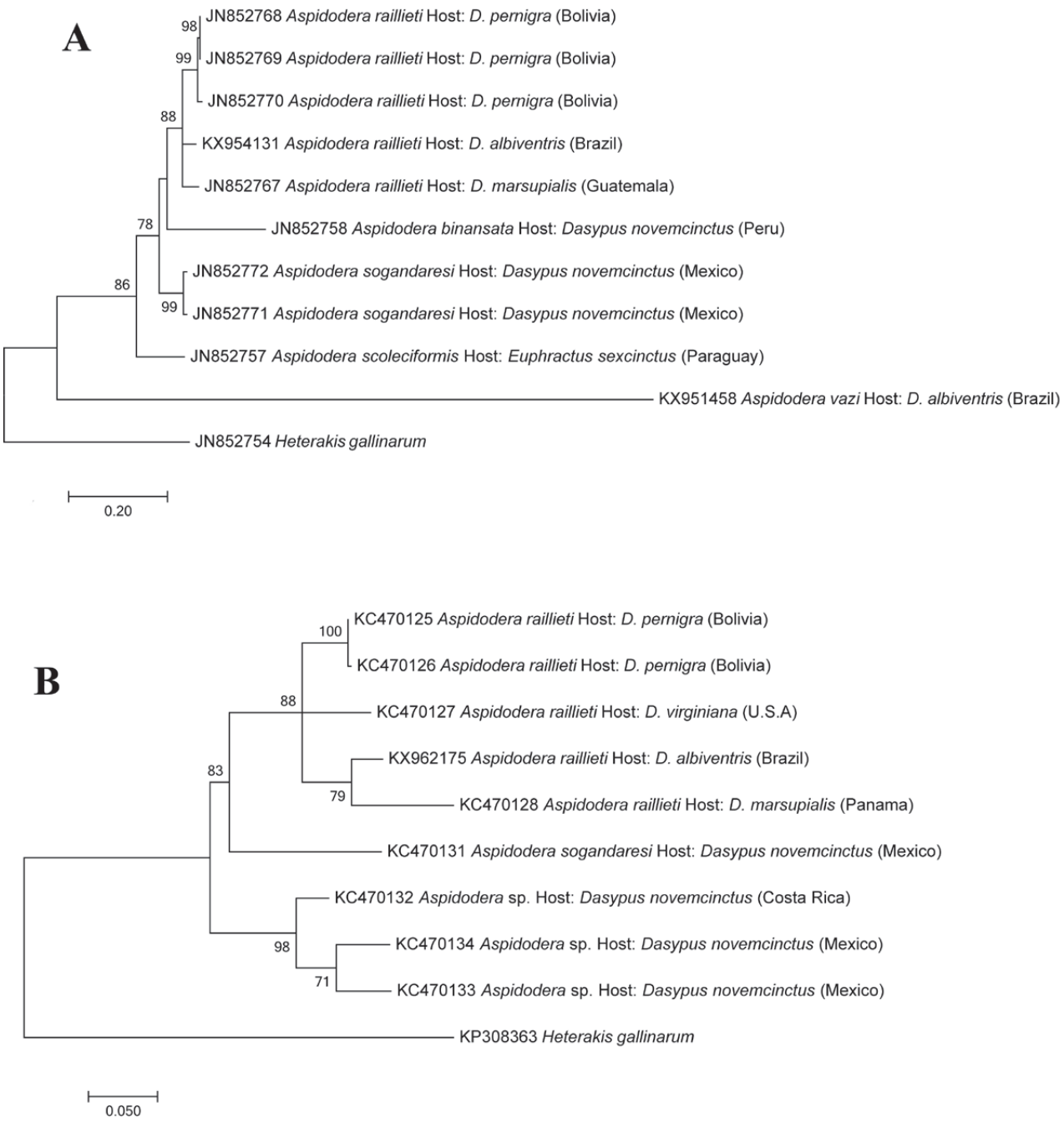

Figure 8. Phylogenetic relationship (ML) among aspidoderid nematodes based on the (A) 16S rRNA and (B) mtDNA *cox1*. Nodal support is labelled around the nodes. Scale bars indicate the nucleotide mutations per site. *Didelphis virginiana* (Didelphimorphia, Didelphidae) from Panamá is currently *D. marsupialis*.

vazi (Figure 8A). In the mtDNA *cox1* tree, *A. raillieti* from *D. albiventris* from Brazil and *D. virginiana* (currently *D. marsupialis*) from Panama clustered together separated from the isolates from *D. albiventris* from U.S.A and *D. pernigra* from Bolivia.

DISCUSSION

Taxonomic studies of mammalian parasites in the Caatinga biome are rare and sparse. Although it is expected that helminth species, well distributed among several hosts, are found in the region, the environmental and climatic transformation in the last 40,000 years points to the population isolation (Sianto 2009). This isolation can, in addition to the few studies, evidence novelties in the helminthological fauna, as shown by ours results. The present study constitutes a new geographic locality of four species of Aspidoderidae, a Trichostrongylidae species, and presents new

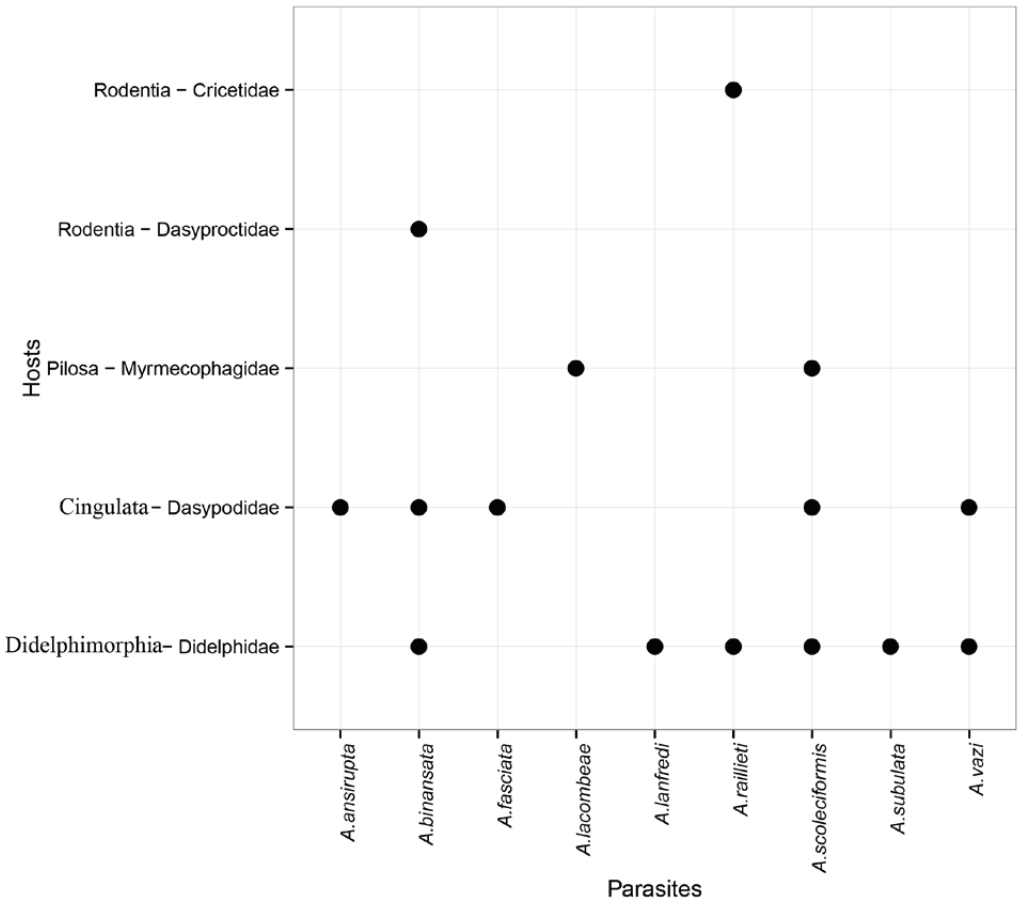

Figure 9. Scatter plot reporting different mammal hosts of *Aspidodera* spp. (Ascaridida) in Brazil.

molecular data of *A. raillieti* from *D. albiventris* and *A. vazi* from *D. novemcinctus* from Brazil.

Species of *Aspidodera* parasitize a wide range of mammal hosts in Brazil (see Figure 9), which shows the known distribution of these parasites, especially in members of Didelphidae and Dasypodidae. The species of *Aspidodera* can be differentiated by the morphology of cephalic cap and cordons, which contain six longitudinal loops, shape and size of esophagus with a terminal bulb, ventral sucker on males, spicules and gubernaculum, and digitiform projection on the posterior end (Santos *et al.* 1990, Chagas-Moutinho *et al.* 2007, Chagas-Moutinho *et al.* 2014). The morphological identification of *A. raillieti* is well established and the species can be characterized by the anterior region bearing a cuticular expansion or cap that surpasses the length of the vestibule, adorned with cephalic cordons bearing six longitudinal loops that touch the base of the cephalic cap (Santos *et al.* 1990). The morphological profile of *A. raillieti* is closely related to *A. lanfredi*, but can be readily differentiated by having interlabial projections larger than in *A. raillieti* but both of them with a pore-like structure at the apex. It differs from *A. soganderensi* as this species has larger cephalic cap, smaller spicules and higher number of caudal papillae (23–29 pairs). *Aspidodera raillieti* and *A. binansata* are characterized by having a cap that never surpasses the length of the vestibule but in *A. binansata* the longitudinal loops do not touch the base of the cephalic cap (see Santos *et al.* 1990). *Aspidodera vazi* has smaller cephalic cap/body length ratio, 1:13 to 1:27, than other aspidoderids. The measurements and morphological characteristics of the species of the present study are in accordance with the data from Vicente *et al.* (1966, 1997), Santos *et al.* (1990), Chagas-Moutinho *et al.* (2007) and Hoppe & Nascimento (2007). The eggs of the aspidoderids studied here are very similar in size and shape (elliptic, thin-shelled),

but the new genetic sequences now available will certify their future diagnosis in coprolites.

The little nematode *H. speciosum* is characterized by a cuticle longitudinally striated; cephalic dilatation finely striated, simple oral aperture; didelphic, amphidelphic females; male with copulatory bursa type 2-1-2, spicules different in size and shape, and dorsal ray asymmetric (Hoppe & Nascimento, 2007). *Hadrostrongylus ransomi* (Travassos, 1937), the only other species of the genus is differentiated mainly by having equal spicules and symmetrical dorsal ray. Due to the quality of the material, differences in the synlophe could not be observed.

The first genetic sequences of *A. raillieti* from *D. albiventris* and the first sequences of the 18S rRNA and 28S rRNA gene for aspidoderids are presented herein. Previous sequences of *A. raillieti* were performed for mitochondrial *cox*-1 and 16S genes and ribosomal ITS1, 5.8S and ITS2 region from different hosts and localities, such as *Didelphis pernigra* from Bolivia, *D. marsupialis* from Guatemala and *D. virginia* from United States (Jiménez-Ruiz *et al*. 2012).

The mitochondrial *cox*-1 gene has been initially proposed as a standard reliable genetic marker to elucidate cryptic, diversity and barcode eukaryote species (Subbotin *et al*. 2015). Studies using *cox*1 as genetic marker for nematodes have displayed a low level of intraspecific variation and higher level interspecificity, but there are exceptions (Blouin 2002). Previous studies revealed a high percent sequence difference, up to 6.0% from *Ostertagia ostertagia* (Nematoda: Trichostrongylidae) for mtDNA sequence (Blouin 1998). Similarly, the region *cox*-1 from *A. raillieti* obtained in this study had low identity score (94%) for a conspecific sequence deposited in the GenBank (*A. raillieti* accession number KC470125) found in the opossum *D. pernigra* from Bolivia. The high dissimilarity level could have occurred due to isolation of the parasite population in SCNP, in addition to evolutionary processes undergone by parasites in different hosts. The data on *cox*-1 could also cast doubt on the reliability of Nematoda DNA barcoding, which can adversely affect the accuracy of Nematoda identification as discussed by Gutiérrez-Gutiérrez *et al*. (2013) and Subbotin *et al*. (2015). The same process could have occurred with the 16S rRNA gene for *A. raillieti* from *D. albiventris* and *A. vazi* from *D. novemcinctus* from Brazil.

The sequence variations observed for internal transcribed spacer rRNA in individuals are typically low, approximately ≤ 1% (Nadler *et al.* 2000). However, D'Amelio *et al*. (2002) found high difference of percentage of nucleotide in the entire ITS ribosomal DNA region in populations of *Pseudoterranova decipiens* (Anisakidae) from different host species (up to 5.2%). In our studies, ITS1, 5.8S and ITS2 genes presented 99% identity with the conspecific sequence deposited in the GenBank (JQ995300). The intraspecific genetic distance among *A. raillieti* species for ITS1, 5.8S and ITS2 rRNA region was low (0.1 – 1.0 ± 0.3). Similar results were reported by Klimpel *et al*. (2007) when assessed the genetic variability for ITS rRNA of nematodes from different geographical areas. The lowest intraspecific genetic distance among *A. raillieti* species was 0.1 (± 0.2) for both specimens of *D. pernigra* from Bolivia. Moreover, *A. raillieti* isolated in *D. albiventris* from Brazil obtained from this study have a low genetic distance with same species isolated from different geographical areas or hosts (0.3–0.7 ± 0.3).

Concerning the genetic distance based on mtDNA *cox-1*, *A. raillieti* seems to present distinct lineages with high genetic distance values. The lowest genetic distance found for *A. raillieti* isolates was 0.3% (± 0.2%) found between two parasite specimens of *D. pernigra* from Bolivia. On the other hand, the highest intraspecific genetic distance occurred between the specimens collected from *D. virginiana* in Panama (currently *D. marsupialis*) and U.S.A. (9.5±1.4). Similar results were found in the analysis of 16S rRNA where relatively high values were found between specimens, except between isolates from Bolivia. Specimens from United States were originally described as *Aspidodera harwoodi* Chandler, 1932 and based on morphological characters was synonymized to *A. raillieti* by Santos *et al*. (1990). The new genetic results suggest that specimens of *D. virginiana* from United States should be reanalyzed integrating morphological and molecular data to certify their taxonomic status.

The topology of the phylogenetic reconstruction for partial 28S rRNA showed high statistical support for clades, with few exceptions. In general, ML appears with lower statistical support values than BI. The tree based on the new

sequence from Aspidoderidae partial 28S rRNA showed that Heterakoidea, Ransomnematoidea and Oxyuroidea appear in separate clades. In this study, Heterakoidea appears more closely related to Oxyuroidea than Ransomnematoidea. This corroborates the findings of Nadler *et al.* (2007), when described these relations using 18S rRNA. In addition, partial 28S rRNA gene was a good genetic marker to distinguish phylogenetically these superfamilies, but not the families included in this study.

The phylogenetic reconstruction using mtDNA *cox1* and 16s rRNA showed separate clades for the aspidoderid specimens thus suggesting the existence of distinct lineages of *A. raillieti* in the Americas. This integrative study contributes to better understand the helminth fauna of *D. albiventris* and *D. novemcinctus* with new genetic profiles of the actual fauna of aspidoderids from SCNP, giving support for future paleoparasitological studies comparing with the ancient fauna found in coprolites in the region.

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