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ORIGINAL ARTICLE / ARTÍCULO ORIGINAL

MITOCHONDRIAL DNA AND MORPHOLOGY DATA OF *HELMINTHOXYS FREITASI* QUENTIN, 1969 REVEALS ITS PHYLOGENETIC RELATIONSHIPS IN THE TRIBE PROTOZOOPHAGINI

LOS DATOS DEL ADN MITOCONDRIAL Y DE LA MORFOLOGÍA DE *HELMINTHOXYS FREITASI* QUENTIN, 1969 REVELAN SUS RELACIONES FILOGENÉTICAS EN LA TRIBU PROTOZOOPHAGINI

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ABSTRACT

Nematodes from the genus *Helminthoxys* Freitas, Lent & Almeida, 1937 are intestinal parasites of caviomorph rodents with a wide Neotropical distribution. This study detailed the morphology of *Helminthoxys freitasi* Quentin, 1969 using light microscopy and scanning electron microscopy (SEM), and inferred a phylogeny for the tribe Protozoophagini using partial mitochondrial cytochrome *c* oxidase subunit I gene sequences (MT-CO1). Rodents *Mesomys hispidus* (Desmarest, 1817) were collect in three distinct areas in the state of Acre, Brazil. The helminths were recovered and morphology of their surfaces, such as lateral alae reaching the level of the anus, the posterior region of the body in the male having three pair of sessile papillae and one pair papillae pedunculated were detailed. Genetic sequence of *H. freitasi* suggested a close relationship with the genus *Wellcomia* Sambon, 1907, corroborating a previous morphological phylogeny. A new host species, *M. hispidus*, and a new locality in the Amazon rainforest is recorded.

Keywords: Cytochrome *c* subunit I – Integrative Taxonomy – *Mesomys hispidus* – Scanning Electron Microscopy – Syphaciinae

RESUMEN

Los nematodos del género *Helminthoxys* Freitas, Lent & Almeida, 1937 son parásitos intestinales de roedores caviomorfos con una amplia distribución neotropical. Este estudio detalló la morfología de *Helminthoxys freitasi* Quentin, 1969 por microscopía óptica y microscopía electrónica de barrido (MEB), e infirió una filogenia para la tribu Protozoophagini con las secuencias del gene parcial Citocromo *c* Oxidasa subunidade 1 (MT-CO1). Los roedores *Mesomys hispidus* (Desmarest, 1817) fueron colectados en tres áreas distintas en el estado de Acre, Brasil. Los helmintos encuentrados observaron alas laterales que alcanzan el nivel del ano, la región posterior del cuerpo en el hombre con tres pares de papilas sésiles y un par de papilas pedunculadas. La secuencia COI de *H. freitasi* revelan una estrecha relación con el género *Wellcomia* Sambon, 1907, corroborando con la filogenia morfológica anterior. Además, reportamos una nueva especie huésped, *M. hispidus*, con una nueva localidad en la selva amazónica.

Palabras clave: Subunidad I del citocromo C – Taxonomía Integrativa – *Mesomys hispidus* – Microscopía Electrónica de Barrido – Syphaciinae

INTRODUCTION

The genus *Helminthoxys* Freitas, Almeida and Lent, 1937 currently comprises eight species. The type species *Helminthoxys caudatus* (syn. *H. pujoli* Quentin, 1973) was first described infecting the rodent *Microcavia australis* in Argentina (Freitas-Texeira *et al.,* 1937). Subsequently other species were described: *H. tiflophila* Vigueras, 1943 in *Mysateles prehensilis* (Vigueras, 1943); *H. effilatus* Schuurmans-Stekhoven, 1951 (syn. *H. velizi* Parra Ormeño, 1953) in *Lagidium viscacia* (Schuurmans-Stekhoven, 1951); *H. urichi* Cameron & Reesal, 1951 in *Dasyprocta leporina* (Cameron & Reesal, 1951); *H. quentini* Barus, 1972 in *Capromys pillorides* (Barus, 1972); *H. gigantea* Quentin, Courtin & Fontecilla 1975 in *Octodon degus* (Quentin, Courtin & Fontecilla 1975); *H. freitasi* Quentin, 1969 in *Thrichomys laurentius* (syn. *Thrichomys apereoides*) (Quentin, 1969); and *H. abrocomae* Hugot & Gardner, 2000 in *Abrocoma cinerea* (Hugot & Gardner, 2000).

These nematodes inhabit the large intestine of caviomorph rodents of seven different families, which include the following: Caviidea, Capromyidae, Chinchilidae, Dasyproctidae, Echimyidae, Octodontidae, and Abrocomidae (Hugot, 1988).

Studies on the morphologic phylogeny of the order Oxyurida based on morphologic characteres of the reproductive structures of the male and cephalic plate, proposed that the tribe Protozoophagini is composed of three genera which include the following: *Helminthoxys* Freitas, Lent & Almeida, 1937; *Wellcomia* Sambon, 1907; and *Protozoophaga* Travassos, 1923 (Hugot, 1988). Later studies based on molecular phylogenetic inference have confirmed the evolutionary relationship between *Wellcomia* Sambon, 1907 and *Protozoophaga* Travassos, 1923 (Nadler *et al*., 2007), but not included *Helminthoxys* Freitas, Almeida & Lent, 1937. Nevertheless, so far, no molecular phylogeny has included molecular sequence *Helminthoxys* and representatives of the sister Syphaciini tribe.

This study detailed the morphology of *Helminthoxys freitasi* Quentin, 1969 by light microscopy and scanning electron microscopy (SEM), adding further taxonomic characteristics for species and inferred a phylogeny for the tribe Protozoophagini using partial mitochondrial cytochrome *c* oxidase sub unit I gene (MT-CO1). In addition, both a new host species and new geographic locality were recorded.

MATERIALS AND METHODS

Collection sites

This study was developed in three distinct areas within the Amazon rainforest in the state of Acre, in the municipalities of Porto Acre (9°54'17.70"S;67°17'8.01"W), Senador Guiomard (10°09'39.0"S;67°44'17.6"W), and Xapuri (10°49'40.79"S; 68°21'38.89"W). Rodents were

trapped using Tomahawk (model 201, Hazelhurst, Wisconsin) and Sherman (model XLK, H.B. Sherman Traps, Tallahassee, Florida) live traps. To capture arboreal mammals, traps were tied to tree branches placed in the forest understory. Captures occurred during five consecutive nights in 2014, 2015, and 2016. Euthanasia followed the guidelines of the American Society of Mammologists for the use of wild mammals in research and the Brazilian Guide to Good Practices for Euthanasia in Animals (Sikes, 2016). Permits for rodent capture and handling were issued by the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio), and experimental procedures on animals were approved by the Ethics Committee on Animal Use (CEUA) of the *Instituto Oswaldo Cruz*.

Helminths collection

After collection, worms were washed in saline, sodium chloride solution (NaCl 0.9%) and maintained in 70% ethanol solution. For examination under light microscopy, nematodes were clarified in lactophenol 90% and drawings were produced with aid of a Camera Lucida, attached to a Zeiss Scope Z1 light microscope (Zeiss, Göttingen, Germany). All measurements were in micrometers. The structures were measured through digital images captured by a Zeiss Axio Cam HRC (Zeiss, Germany) using the accessory software Axio Vision Rel. 4.7 (2009).

For SEM analyses, six specimens (three males and three females) of post-fixed helminths were dehydrated in increasing ethanolic series (70%, 80%, 90%, and absolute ethanol), for 20 min at each stage, and dried by the critical point method with CO_2 (Souza *et al*., 2017). The samples were then submitted to gold metallization with layer thickness of approximately 20nm. The specimens were then analyzed in a SEM JEOL JSM6390LV at the *Plataforma de Microscopia Eletrônica Rudolf Barth*, *Instituto Oswaldo Cruz*, Fiocruz (Electron Microscopy Platform of the Oswaldo Cruz Institute).

A paratype of *H. freitasi* from the *Coleção Helmintológica do Instituto Oswaldo Cruz* – CHIOC (Nº 30936) was used to compare morphological characteristics. Voucher specimens were deposited in CHIOC under the following number: CHIOC 38502.

Molecular and phylogenetic analyses

Genomic DNA was isolated from three individual pinworms from the Senador Guiomard and Porto Acre localities using the QIAamp DNA Mini Kit, applying the manufacturer's protocol (QIAGEN, Hilden, Germany).

DNA amplification by polymerase chain reaction (PCR) was conducted using the following primers: SyphaCO1_F (5'-ACCCGCTGAATTTAA-GCAT-3') and SyphaCO1_R (5'-AACC-ACCCAACGTAAACATAAA-3') to produce amplicons of the mitochondrial cytochrome *c* oxidase subunit I gene (MT-CO1) fragments (Okamoto *et al*., 2007).

Each PCR contained 1x PCR buffer, 4 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM of each deoxynucleotide triphosphate solution (dNTPs), 1U of Platinum *Taq*DNA polymerase (Invitrogen, São Paulo, Brazil), 2.0 µL of genomic DNA, and ultrapure water, in a total reaction volume of 25 µL. PCR-cycling parameters followed Okamoto *et al.*, (2007). Resulting amplicons were visualized on 1.5% agarose gels after electrophoresis, using Gel Red™ nucleic acid gel stains (Biotium, Hayward, California, USA), and UV transilluminator. Successfully amplified amplicons were purified using the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit following 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 better data accuracy. Sequencing was performed using the ABI3730 DNA Analyzer. Both procedures and cycle-sequenced products precipitation were conducted at the *Plataforma de Sequenciamento de DNA do Instituto Oswaldo Cruz*, PDTIS/Fiocruz (DNA Sequencing Platform of the Oswaldo Cruz Institute). Fragments were assembled into contigs and edited for ambiguities using the software Geneious 9.1.8 (Kearse *et al.,* 2012), resulting in consensus sequences.

Our dataset included sequences from the closest relatives of *Helmintoxy* genus, oxyurids of the subfamily Syphaciinae. These oxyurids belong to four genera each representing a different tribe as follows: *Passalurus* Dujardin, 1845;

Rauschtineria Hugot, 1980; *Syphacia* Seurat, 1916, and *Wellcomia* Sambon, 1907. We also included sequences of genera *Enterobius* Leach, 1853 and *Lemuricola* Chabaud et Petter, 1959 as a representative of the oxyurid subfamily Enterobiinae. The oxyuroid *Aspiculuris tetraptera* Schulz, 1924, from the family Heteroxynematidae, was included as outgroup. The subfamilies, tribes of Syphaciinae, species, GenBank accession numbers, and references of specimens used in this study are listed in Table 1.

We aligned the MT-CO1 sequences using the Translator X online software (Abascal *et al.*, 2011). Resulting alignments were trimmed of poorly aligned regions using the Mesquite package software (Maddison & Maddison, 2011). Substitution saturation in the dataset was assessed via the Test by Xia (Xia *et al*., 2003; Xia & Lemey, 2009) using the DAMBE program, Version 6.4.79 (Xia & Xie, 2001).

Phylogenetic reconstructions using maximum likelihood (ML) were carried out using PhyML 3.0 software (Guindon *et al.*, 2010). Nucleotide evolutionary model selection was executed with SMS (Smart Model Selection) (Lefort *et al*., 2017) in PhyML, using the Bayesian information criterion (BIC). Node support in ML trees was assessed by the Approximate Likelihood-Ratio Test for Branches (aLRT) (Anisimova & Gascuel, 2006) and by nonparametric bootstrap percentages (ML-BP) after 1000 pseudoreplications. Bayesian phylogenetic inference (BI) was carried out using the MrBayes program, version 3.2.6 (Ronquist *et al*., 2012) on XSEDE using the CIPRES Science Gateway (Miller *et al*., 2010). To account for different evolutionary processes at each of the three codon positions, BI analyses were performed using the GTR+G model for each codon position, with unlinked base frequencies and parameters. Markov chain Monte Carlo samplings were performed for 10,000,000 generations with four simultaneous chains in two runs. The robustness of nodes was assessed by Bayesian posterior probabilities (Bpp) calculated from tree samples every 100 generations, after removal of a "burn-in" fraction of 25%. To assess the adequacy of our sampling, we used the Tracer v1.6 program (Rambaut *et al*., 2014) to calculate the Effective Sample Sizes (ESS) of parameters. Values above 1000 effectively independent samples were considered sufficient. To assess the level of variation in the (COI) among the selected samples of different taxa, uncorrected (*p*) pairwise genetic distances were calculated using PAUP* 4.0b10 software (Swofford, 2002).

Ethical standards

License for animal capture was provided by the *Instituto Chico Mendes de Conservação da Biodiversidade*- ICMBio (permanent license 13373-1). All protocols followed the guidelines for capture, hand ling and care of the Ethics Committee on Animal Use of the Oswaldo Cruz Institute (according to license L-049/08) (protocol P-70/13-2; license LW-39/14).

Conflict of interest

The authors declare no conflict of interest.

RESULTS

Morphology by scanning electron and light microscopy

Adult helminths exhibited sexual dimorphism. In both sexes (Figures 1A and 3A) the anterior extremity had three prominent pseudolabia, one ventral and two dorsolateral, interspersed with three strong conical esophageal teeth (Figure 3B), which were intercalated with cuticularized thickenings of the inner part of the pseudolabia and the vestibule (Figure 1B). In the external part, surrounded by a rough cuticular area located on each dorsolateral pseudolabia, two labial papillae were closely grouped laterally with corresponding amphids (Figure 1C). Morphological analysis by scanning electron microscopy showed the cuticular expansions which formed the cervical alae extend in lateral alae reaching the level of the anus, in the light microscope only the cervical wing was clearly seen (Figure 3A).

Males (Figures 1A and 2A) had two cuticular mamelons protruding as cuticular expansions with longitudinal ridges located in the posterior part of the body (Figures 2C and 3F). There was 18 ventral trimmings after the second mamelon in the form of small longitudinal cuticular ridges (Figures 2B and 3E), one long spicule, gubernaculum, accessory

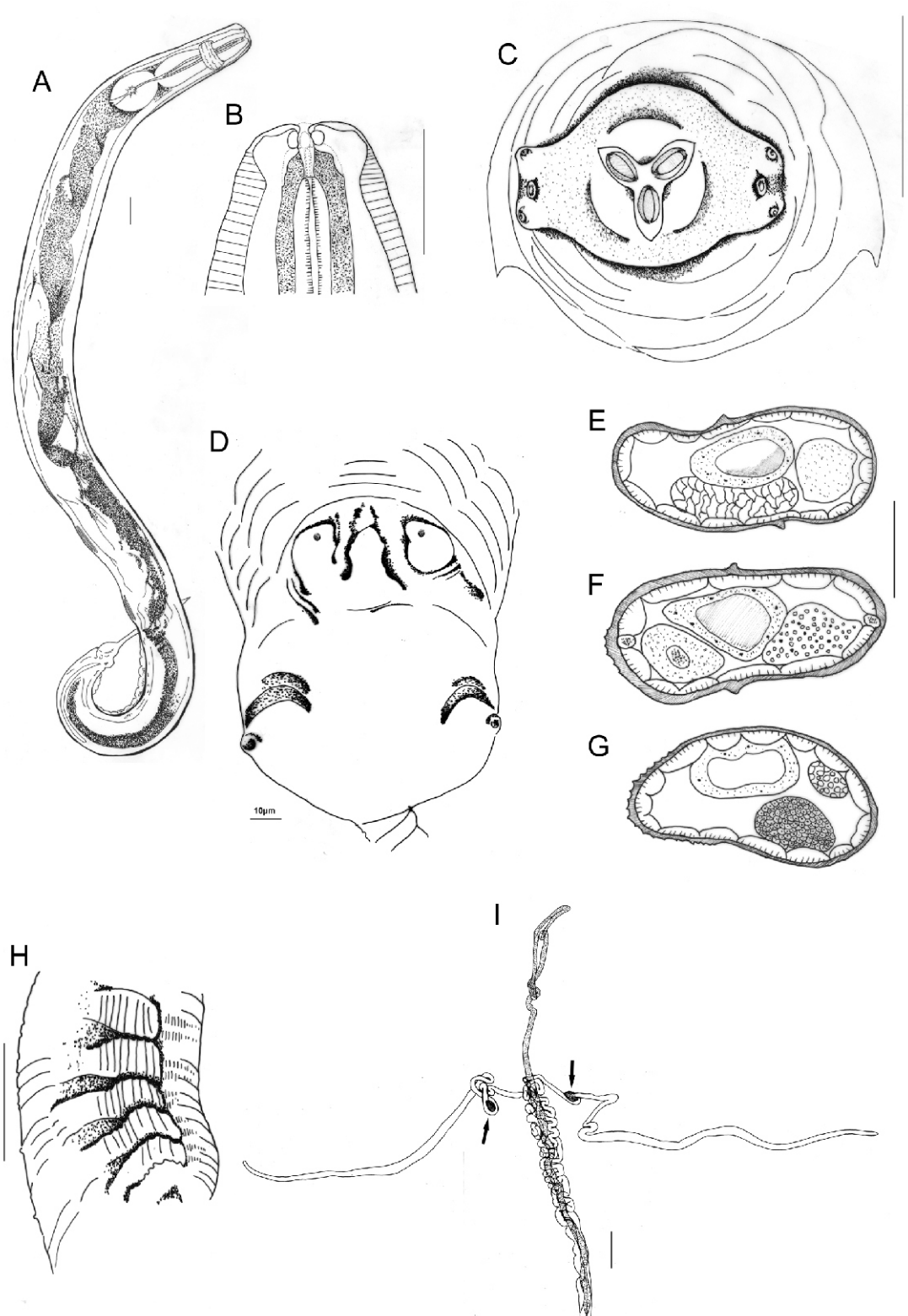

Figure 1. *Helminthoxys freitasi* A) Complete male view; B) Head, left lateral view; C) En face view of head; D) Ventral view of caudal bursa showing one pair of sessile papillae and one pair of pedunculate papillae; E) Cross-section of the body at the level of the cervical alae; F) Cross-section of the body at the level of the first mamelon; G) Cross-section of the body at the level of the area rugosa posterior to the second mamelon; H) Area rugosa posterior to the second mamelon, ventral view; I) Uterus didelphic and a pair of spermatheca (arrow). *Scale*: A, E, F, G, H, I, 50µm; B, C, D, 10µm.

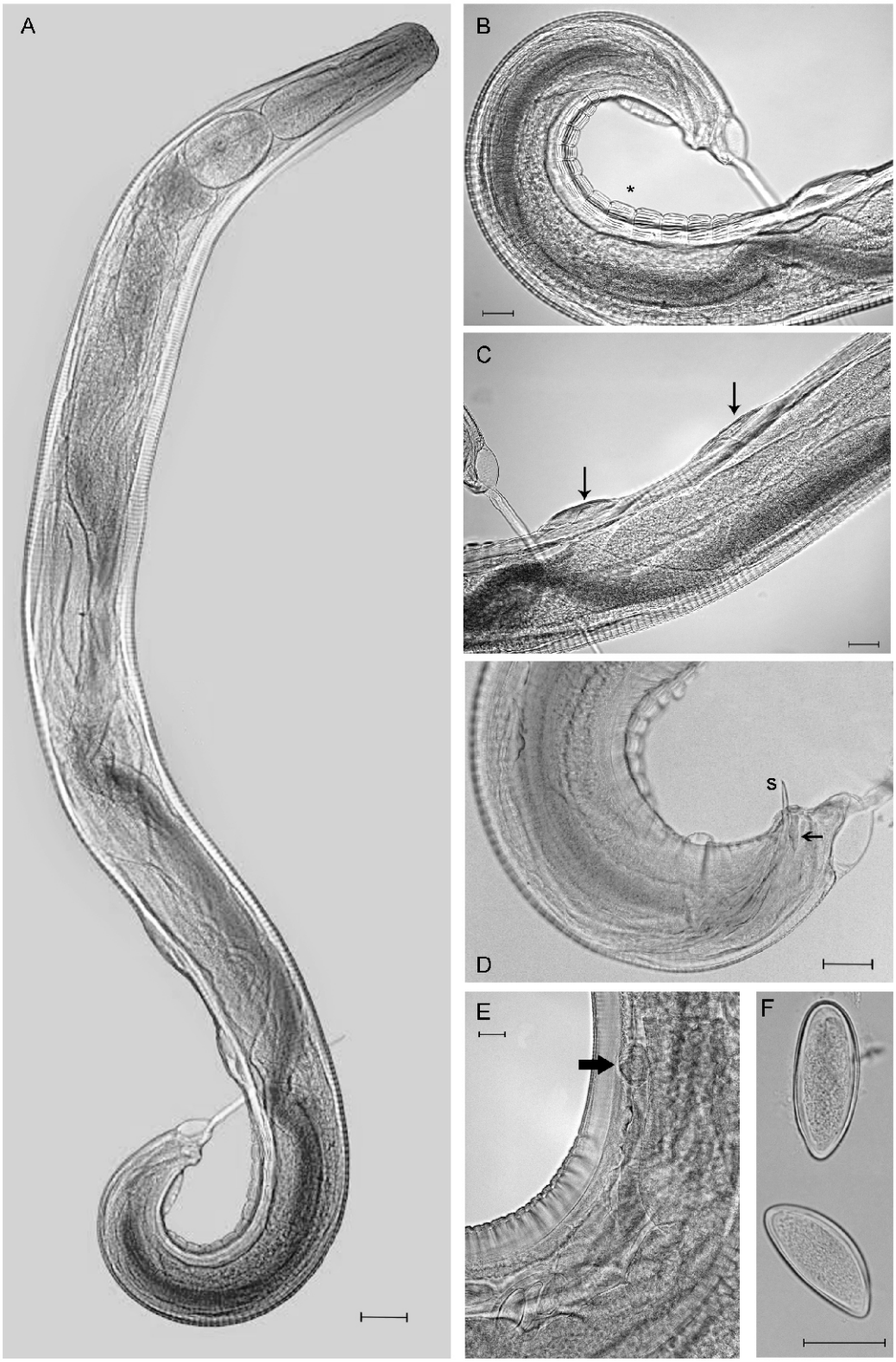

Figure 2. *Helminthoxys freitasi* A) Adult male, general view; B) Bursal caudal, detail of the area rugosa posterior to second mamelon (asterisk); C) First and second mamelon (arrow), lateral view; D) Detail, spicule (S) and gubernaculum (arrow), lateral view; E) Detail, vulva (large arrow), lateral view; F-G) Eggs. *Scale*: A, 100µm; B, C, D, E, 50µm; F, 10µm.

hooks at the base of the cloacal opening, three pair of sessile ad-cloacal papillae, and one pair of pedunculate posterior papillae (Figures 1D, 1H, 2D, 3C, and 3D). Phasmids were located anteriorly to the pedunculate pair of papillae.

In the females, the vulva was in the posterior part of the body (Figure 2E). The uterus folded on itself, opening in two oviducts with a pair of spermatheca (Figure 1I). Eggs were asymmetrical and not operculated (Figure 2F).

Morphometric data including all the species of the genus *Helminthoxys*, from their original descriptions were compared, emphasizing distinctions of our specimen and added new data with the measurements of eggs not previously described (listed in Table 2).

Taxonomic Summary

Host: *Mesomys hispidus* (Desmarest, 1817) (Rodentia: Echimyidae).
Site infection: large intestine
Locality: Municipalities of Porto Acre (9°54'17.70"S; 67°17'8.01"W), Senador Guimard (10º09'39.0''S;67º44'17.6''W), and Xapuri (10°49'40.79"S; 68°21'38.89"W), State of Acre, Brazil.
Mean intensity: 6.2 (31 specimens out of 5 host infected)
Prevalence: 45.4 (5 host infected out of 11 host examined)
Abundance: 2.81 (31 specimens out of 11 host examined).
Specimens deposited: CHIOC Nº 38502

Molecular and phylogenetic analyses

We obtained consensus MT-CO1 sequences from three adult *Helminthoxys freitasi* recovered from two hosts from different localities. Two consensus sequences were obtained from Porto Acre (A) and one was obtained from Senador Guiomard (B). The sequence from A were identical 975 bp whereas the sequence from B had 954 bp and differed from A by a single transition, representing two distinct MT-CO1 haplotypes. Both sequences were deposited in the GenBank database under accession numbers: MH212135 and MH212136.

The resulting aligned matrix with GenBank sequences comprised of 18 taxa (shown in Table 1) and 819 characters, of which 482 characters were constant, 118 variable characters were parsimony-uninformative, and 201 were parsimony informative. The test by Xia (Xia *et al.,* 2003; Xia & Lemey, 2009) provided evidence for substantial saturation only at the third codon positions, whereas at the first and second positions, and overall there was little saturation in the matrix.

As the best-fit model, PhyML-SMS selected the GTR+G model nucleotide substitution, with ML optimized frequencies, estimated Gamma-shape parameter (α=0.280), and four rate categories. The best log-likelihood ML tree score was -4469.546140.

For the BI, the mean estimated marginal likelihood was -4077.7366 and the median was -4077.421. ESSs for all parameters were above 1000 effectively independent samples and for most parameters, indicating the robustness of our sampling.

The pairwise uncorrected *p*-distances for representatives of tribes of the subfamily Syphaciinae and subfamily Enterobiinae are summarized in Supplementary Table S1. Overall, our matrix had pairwise genetic intraspecific *p*-distances from 0.1% to *Helminthoxys* and to *Passarulus* genera and 17.9% interspecific distances between *W. siamensis* Nadler, 2007 and *E. vermicularis* (Linneu, 1758) Leach, 1853 (mean = 13.1%). The genetic distance between Syphaciinae and Enterobiinae ranged from 11.6% between *R. eutamii* (Tiner, 1948) and *E. macaci* Yen, 1973, to 17.9% between *P. ambiguus* Rudolphi, 1819 and *E. vermicularis* (mean = 14.5%).

The genetic distance between *H. freitasi* and *W. siamensis* (i. e. within the tribe Protozoophagini) ranged from 11.5–11.6% (mean=11.5%). The distance between Protozoophagini and Hilgertini ranged from 12.7% between *H. freitasi* and *R. eutamii,* to 16.5% between *W. siamensis* and *R. eutamii* (mean = 14.3%). The distances between Protozoophagini and Syphacini ranged from 12.7% between *H. freitasi* and *S. stroma,* to 17.5% between *W. siamensis* and *S. agrarian* (mean = 14.7%). The distances between Protozoophagini and Passalurini ranged from 12.7% between *H. freitasi* and *P. ambiguus,* to 15.3% between *W. siamensis* and *P. ambiguous* (mean = 13.7%).

ML and BI phylogenies resulted in similar topologies with little variation in nodes and support values, as shown in Figure 4. All analyses agreed with. *H. freitasi* haplotypes forming a monophyletic group, sister to *W. siamensis* with strong support (aLRT=99%, BP-ML=100%, BPP=100%). The tribes Hilgertiini, Protozoophagini, and Syphaciini formed a monophyletic group with strong support only in the aLRT (99%) and the BPP (99%). Passalurini was a sister group to the other tribes. The subfamily Syphaciinae thus formed a monophyletic group, including all four tribes represented in our sample, although with weak to moderate support (aLRT = 89%, BP-ML = 51%, BPP = 63%). The subfamily Enterobiinae also formed a monophyletic group, although with weak to moderate support (aLRT = 77%, BP-ML = 39%, BPP = 62%).

Table – 1 Subfamilies, tribes of Syphaciinae, species, GenBank accession numbers, and references of specimens used in this study.

Subfamily	Syphaciinae Tribes	Species	Genbank accession number	Reference
Heteroxynematinae		*Aspiculuris tetraptera*	KT764937	Wang *et al.* (2016)
Enterobiinae		*Enterobius macaci*	AB626858	Hasegawa *et al.* (2012)
		Enterobius vermicularis	EU281143	Kang *et al.* (2016)
Syphaciinae	Passalurini	*Passarulus ambiguus*	KT879302	Liu *et al.* (2016)
		Passarulus ambiguus	KF472059	Sheng *et al.* (2014)
	Hilgertiini	*Rauschtineria eutamii*	KT875323	Bell *et al.* (2016)
		Rauschtineria eutamii	KT875241	Bell *et al.* (2016)
	Shypaciini	*Syphacia frederici*	MF142425	Stewart *et al.* (2016)
		Syphacia montana	AB282581	Okamoto *et al.* (2007)
		Syphacia obvelata	KT900946	Wang *et al.* (2016)
		Syphacia agraria	AB282589	Okamoto *et al.* (2007)
		Syphacia emileromani	AB282590	Okamoto *et al.* (2007)
		Syphacia ohtaorum	AB282592	Okamoto *et al.* (2007)
		Syphacia stroma	MF142420	Stewart *et al.* (2016)
	Protozoophagini	*Helminthoxys freitasi A*	MF212135	This study
		Helminthoxys freitasi B	MF212136	This study
		Wellcomia siamensis	GQ332427	Park *et al.* (2011)

Table 2 - Measurements, in micrometers, of male and female of all species of genus *Helminthoxys*, plus the specimens in study.

Male	*H. caudatus (H.pujoli)*	*H. tiflophila*	*H. effilatus (H.velizy)*	*H. urichi*	*H. quentini*	*H. gigantea*	*H. abrocomae*	*H. freitasi*	*H. freitasi*
Body length (L)	5.500	6.550	6.800	3.000	5.240	6.320	11.672	5.030	3.725
Body width (W)	330	340	360	200	-	-	367	230	230
Nervous ring	190	240	200	120	200	180	306	170	183
Excretory pore	-	1.400	1.250	900	1.200	1.200	1.151	930	840
Oesophage (L)	660	860	800	350	730	700	957	370	546
Bulb (L x W)	170x160	200x150	290x200	130x110	220x160	200x115	285x153	150x120	172x124
Tail (L)	930	650	550	410	490	900	1.365	475	390
Tip of tail (L)	830	-	420	350	-	850	1.243	430	319
1st mamelon to tip tail	3.100	-	3.400	-	-	3.400	5.938	2.450	2.325
2nd mamelon to tip tail	3.500	-	3.880	-	-	3.900	7.099	2.730	2.565
Spicule	320	420	295	533*Hugot,1986	175	234	866	630	580
Gubernaculum	45	120	50	45	38	80	152	60	42.5
Body size/spicules (%)	5.8	6.4	4.3	17.6	3.3	3.7	7.4	12.5	15.5
Female									
Body length (L)	11.620	19.200	20.660	8.640	12.480	13.500	21.211	13.000	10.325
Body width(W)	530	800	800	600	790	420	654	525	407
Nervous ring	270	200	400	110	270	270	512	215	249
Excretory pore	-	2.250	2.470	1.400	1.170	1.830	3.023	1.700	1.200
Oesophage (L)	710	1.250	1.300	580	990	1.150	1.383	650	697
Bulb (L x W)	250x190	350x-	380x250	180x180	290x270	270x180	359x205	225x200	219x179
Tail (L)	1.150	1.600	3.130	1.220	1.310	1.900	3.381	1.720	562
Vulva to tip tail	4.160	8.000	7.680	5.570	5.300	5.000	8.146	8.000	4.657
Anus to tip tail	212	-	-	-	-	-	-	-	-
Eggs (L x W)	104x41	90x40	115x65	-	-	-	77x33		88x38
Host	*Microcavia australis*	*Mysateles prehensilis*	*Lagidium viscacia*	*Dasyprocta leporina*	*Capromys pilorides*	*Octodon degus*	*Abrocoma cinerea*	*Thrichomys laurentius*	*Mesomys hispidus*
Locality	Argentina	Cuba	Argentina	Trindade	Cuba	Argentina	Andes da Bolívia	Brazil	Brazil
Author/Year Anus to tip tail	Freitas -Texeira *et al.* (1937)	Vigueras (1943)	Schuurmans -Stekhoven (1951)	Cameron& Reesal (1951) *Hugot (1986)	Barus (1972)	Quentin *et al.* (1975)	Hugot & Gardner (2000)	Quentin (1969)	Present study

* Measurements in micrometers.

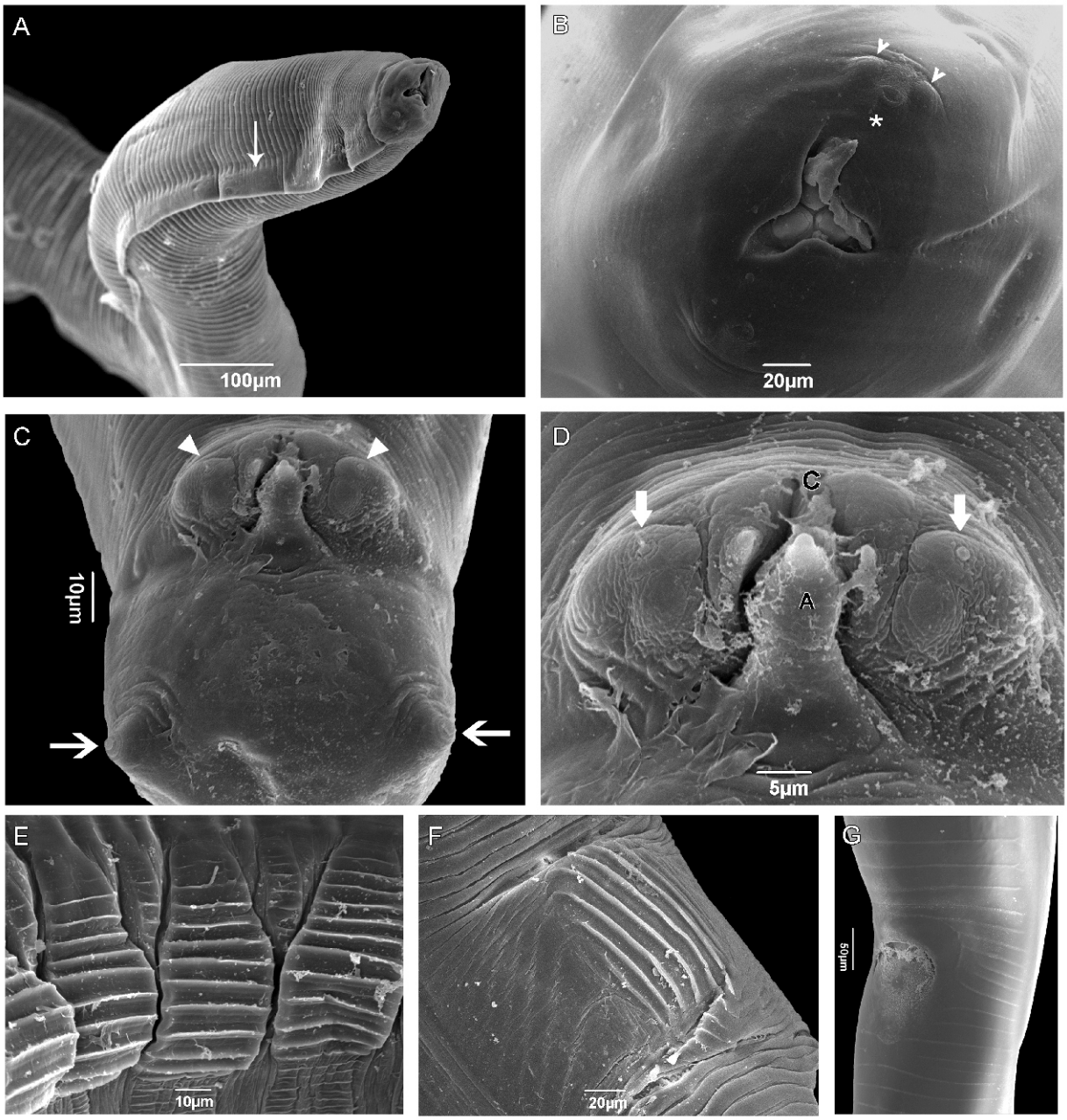

Figure 3. *Helminthoxys freitasi* A) Adult female, general view of the anterior part of the body and alae lateral (thin arrow); B) Cephalic plate, apical view, amphid (asterisk) and two papillae (head arrow); C) Bursal cauda, one pair of papillae (head arrow) and one papillae pedunculate (arrow); D) Detail of the cloaca (C) showing one pair of sessile papillae (large arrow), accessory hook of gubernaculum A); E) Area of the rugosa posterior to the second mamelon; F) Detail of the mamelon surface showing longitudinal striations; G) Anus of the female, ventral view.

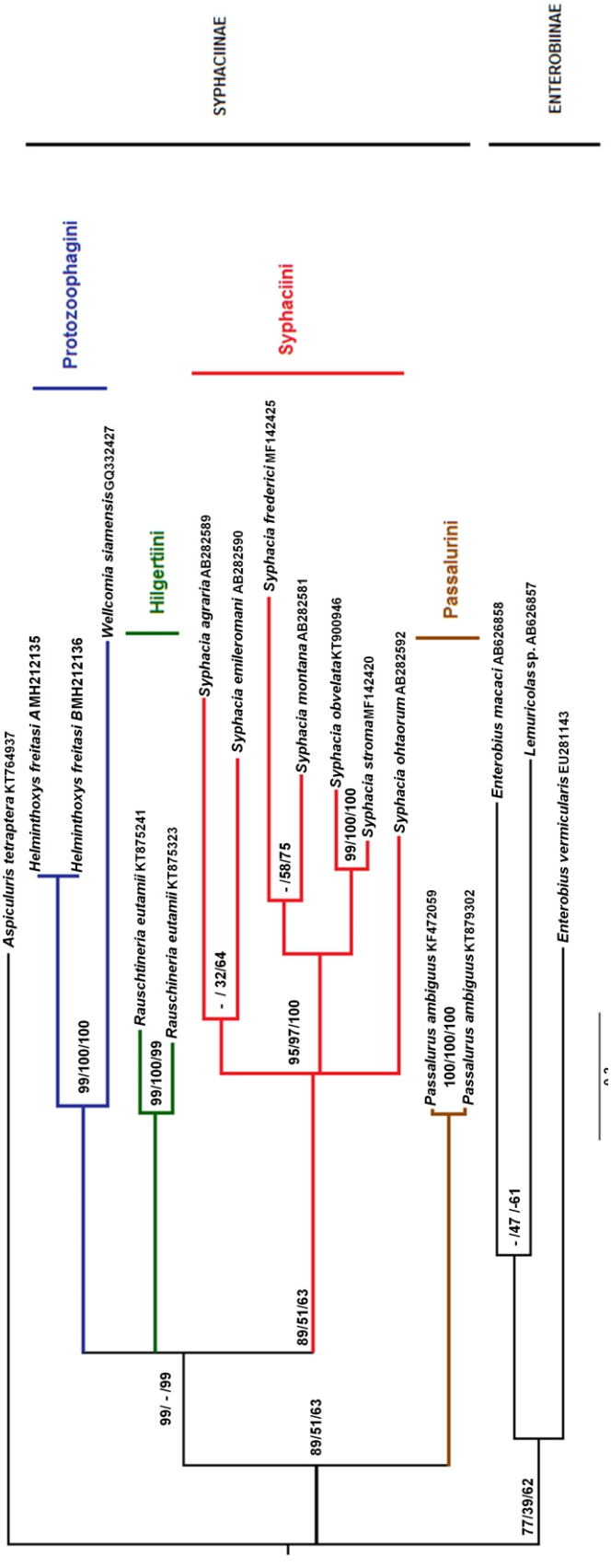

Figure 4. Phylogenetic relationships of *Helminthoxys freitasi* isolates from this study and other oxyuroids using the MT-CO1. Bayesian 50% majority rule consensus tree after burn-in. Support values are shown at the following nodes, respectively: aLRT, ML-BP, and BPP. Branch lengths are proportional to the mean posterior probabilities of the branch lengths of the sampled trees (scale bar, substitutions per site).

DISCUSSION

The taxonomic characteristics of the genus *Helminthoxys* were the presence of two mamelons in the sub-ventral region of the body, ventral ornamentation, size of the spicule, presence of gubernaculum, and cervical and lateral alae according Quentin, 1973. Morphological characteristics that identify *H. freitasi* are the size of the spicule in male and the position of the vulva in relation to the body length in the female, situated at the posterior part of the body.

In comparison, *H. urichi* is the closest species it resembles based on the proportion of the spicule length to the body length. This characteristic corroborates the evolutionary hypothesis proposed by Hugot (1986), which states that the opening of the posterior vulva in the female body is associated with the elongation of the spicules in males of such species (Hugot, 1986). These two species may be separated by the position and size of the mamelons, in which the males of the species *H. urichi* are further developed.

The genus *Helminthoxys* have a wide distribution within the Neotropical region, extending throughout Central and South America, with each species found associated to a family of host. The species *H. freitasi* was first described infecting the echimyid rodent *Thrichomys laurentius* (Trouessart, 1880). The genus *Thrichomys* is found in open and forested areas in the Caatinga, Cerrado, Chaco, and Pantanal biomes in Brazil, Bolivia, and Paraguay (Patton *et al.,* 2015). The present study reports a new host, the rodent, *Mesomys hispidus* (Desmarest, 1817) 18 also belonging to family Echimyidae, and a new geographical distribution in the Amazon Forest in Brazil. Our findings suggest an association of *H. freitasi* and hosts of the family Echimyidae, enable that this specie could also parasitize others echimyids.

However, the differences in the measurements of morphological structures of the specimens studied compared tothe original descriptions may be associated with intraspecific variations relative to adaptive modification to its hosts, and may also be associated with the difference between the body mass of host *Mesomys hispidus* (140–202mm, 160g) and *Thrichomys laurentius* (197–209mm, 267 g) (Patton *et al.,* 2015).

The subfamily Syphaciinae Railliet, 1916 comprises five tribes, including the tribe Protozoophagini composed of three genera, which include the following: *Helminthoxys* Freitas, Lent, and Almeida, 1937; *Wellcomia* Sambon, 1907; and *Protozoophaga* Travassos, 1923, based on morphological characteristics of the genital structures of males and cephalic plaques, according to some synapomorphies that both genera share (Hugot, 1988). Studies based on molecular phylogenies have shown the evolutionary affinity between *Wellcomia* and *Protozoophaga* genera (Nadler *et al.,* 2007). There is, however, no phylogenetic study based on DNA sequences including the genus *Helminthoxys*. In this work, in spite of a limited number of GenBank sequences available, we inferred the phylogenetic relationships of representatives of the tribes Hilgertiini, Passalurini, Syphacinii and Protozoophagiini based on the MT-CO1 gene. The phylogenetic reconstructions obtained by ML and BI revealed *Wellcomia siamensis* as a sister to *H. freitasi*, with high support in all analyses, thus providing support for the tribe Protozoophagiini as a natural group. Our phylogenetic data thus confirms previous works based on morphology (Hugot, 1988; Hugot *et al.,* 2013).

In conclusion, the present study details some morphological characteristics of *H. freitasi* using SEM and light microscopy and contributed with additional taxonomic characters of male and female. This study also contributed the first genetic information for *Helminthoxys*. Our new DNA data suggests a close relationship of *H. freitasi* with the genus *Wellcomia*, corroborating the morphological phylogeny proposed by Hugot (1988). Additionally, *H. freitasi* was recorded for the first time in the Amazon region and parasitizing a new host, *M. hispidus*. This work expands the geographic distribution of the species *H. freitasi* both occurring in a new biome and reported in a new host.

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