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Description of the adult worms of a new Brazilian isolate of *Echinostoma paraensei* (Platyhelminthes:Digenea) from its natural vertebrate host *Nectomys squamipes* by light and scanning electron microscopy and molecular analysis

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Abstract *Echinostoma paraensei* Lie and Basch, 1967 (Echinostomatidae:Platyhelminthes), a 37 collar spine echinostome of the ''revolutum group'', has been used extensively as a model organism to study the interactions of digenetic trematodes with both their snail and vertebrate hosts. This worm was first isolated from the snail *Biomphalaria glabrata* from Belo Horizonte (BH isolate), Minas Gerais State, Brazil, by Lie and Basch [J Parasitol (1967) 53:1192–1199]. The natural definitive host for the BH isolate was never determined, and it has been maintained in the laboratory since 1967 in *B. glabrata* and hamsters. In this study, using light and scanning electron microscopy and molecular analysis, we describe an echinostome recently obtained from its natural vertebrate host, the wild rodent *Nectomys squamipes* (Rodentia: Sigmodontinae) from Sumidouro, Rio de Janeiro State, Brazil (RJ isolate). This echinostome was also compared to the laboratory-maintained BH isolate of *E. paraensei*. We observed that adult worms of both BH and RJ isolates could be differentiated from other echinostome species by the relatively small size of the dorsal collar spines relative to lateral and corner collar spines. SEM confirmed the similarity of this morphological character between the two isolates. As additional diagnostic features, the tegumentary spines are scale-like and the region between the genital pore and the acetabulum lacks scales. There is a folded protuberance with a pore just posterior to the genital pore. The tegument of the acetabulum is unspined and radially wrinkled, and there are numerous randomly distributed small, domed, ciliated papillae. The sequences of the internal transcribed spacers of the nuclear rDNA complex of the RJ and BH isolates are identical. Together these shared features provide strong evidence that both isolates are the same and can be referred to as *E. paraensei*. In conclusion, we have identified, for the first time, one of the natural definitive hosts for *E. paraensei*, the rodent *N. squamipes*, and have extended the known geographical distribution of this species to include Sumidouro in Rio de Janeiro State, Brazil.

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Introduction

Echinostoma paraensei Lie and Basch, 1967 (Echinostomatidae:Platyhelminthes) is a peristomic 37-collar-spined echinostome belonging to the ''revolutum group''. It was first isolated from *Biomphalaria glabrata* Say, 1818 (Mollusca: Pulmonata) from Belo Horizonte, Minas Gerais State, Brazil (Lie and Basch 1967). Its natural definitive host was never determined, but subsequent laboratory work showed that it was only able to infect mammals (see review by Kostadinova and Gibson 2000).

This helminth has been used as a model organism to study the immunobiology of snail–trematode relationships (Adema et al. 1999), and is also extensively used to investigate the interactions between echinostome flukes and experimental vertebrate hosts (Meece and Nollen 1996). It has been maintained in the laboratory since 1967, using hamsters and *B. glabrata* as experimental hosts (Lie and Basch 1967).

Morphological characters have traditionally been used for zechinostome systematics. Many features of adult morphology previously used are now considered unstable and inadequate for identification within species

of the revolutum group (Kanev 1985), indicating that caution is required in interpreting them. One of the key features separating *E. paraensei* from other echinostomes of the revolutum group is the small size of the dorsal collar spines in relation to the lateral and corner spines (Lie and Basch 1967; Kostadinova and Gibson 2000).

Few species of the genus *Echinostoma* have been studied by scanning electron microscopy, and of these, *Echinostoma trivolvis* and *Echinostoma caproni* are the best known (Fried et al. 1990; Krejci and Fried 1994; Ursone and Fried 1995).

Nuclear rDNA ITS sequence variation and random amplification of polymorphic DNA have been used to diagnose species and study phylogenetic relationships among members of the genus *Echinostoma* (Fujino et al. 1995; Morgan and Blair 1995; Petrie et al. 1996). *E. paraensei* DNA sequences differ considerably from most species within the revolutum group but show a close relationship to *E. trivolvis* (Morgan and Blair 1995).

In this study we describe, by light and scanning electron microscopy and by molecular analysis, an echinostome recently isolated from its natural vertebrate host, the wild rodent *Nectomys squamipes*, captured in Sumidouro, Rio de Janeiro State, Brazil. This isolate is compared with the original Belo Horizonte isolate of *E. paraensei* that has been maintained in the laboratory since 1967.

Materials and methods

Parasites

Adult rodents, *Nectomys squamipes,* were captured at Sumidouro in Rio de Janeiro State. They were necropsied and the echinostomes found were either prepared for light microscopy or dissected to collect eggs in order to start a laboratory-maintained life cycle. Over the 2 year period of this study, 111 rodents were captured.

Five hamsters were each exposed per os to 50 metacercariae derived from the isolate obtained from naturally infected *N. squamipes* (RJ isolate). Additional hamsters were exposed per os to 50 metacercariae each of the *E. paraensei* originally isolated by Lie and Basch (1967) from *B. glabrata* from Belo Horizonte, Brazil (BH isolate). The BH isolate has been maintained under laboratory conditions since 1967. For this study it was obtained from the Loker laboratory, University of New Mexico, where it has been maintained since 1985 in hamsters and the M line isolate of *Biomphalaria glabrata*. All hamsters were necropsied 4 weeks after exposure and the worms were collected for morphological and molecular analysis.

Light microscopy

Echinostoma paraensei adult worms from both isolates were randomly selected, washed briefly in Locke's solution (Fried and Huffman 1996) and fixed under slight cover slip pressure at room temperature, with an alcohol-formalin-acetic acid solution (AFA). They were then stained with chloridic carmine, dehydrated in a graded ethanol series, cleared with methyl salicylate and mounted in Canada balsam. The specimens from the Rio de Janeiro isolate were deposited at the Coleção Helmintológica do Instituto Oswaldo Cruz (CHIOC) with the number CHIOC 34242 a–d. Illustrations were obtained with the aid of a camera lucida, connected to a Zeiss light microscope.

The following museum specimens were also obtained and examined: two paratypes of *E. paraensei* Lie and Basch, 1967 deposited at the United States National Parasite Collections, USNPC number 062968.00, slides 2 and 3 (hamster host); *Pseudechinostomum caballeroi* Kohn and Fernandes 1977, type species, CHIOC 31157 a, and paratypes 31157 b–d; *Echinostoma revolutum* Froelich, 1802, USNPC 083343.00, 076146.00, 074696.00, 039263.00, and CHIOC 30321 a and b, 30322 a and b, and 30323 a and b; *Echinostoma barbosai* Lie and Basch, 1966, USNPC 060796.00 and 060795.00; *Echinostona rodriguesi* Hsu, Lie and Basch, 1968, USNPC 070938.00; *Echinostoma neglectum* Lutz, 1924, CHIOC 30752 b and d; *Echinostoma erraticum* Lutz, 1924, CHIOC 17121 a and d; and *Echinostoma nephocystis* Lutz, 1924, CHIOC 17322 a–c, 17326 b–c, 17321 a and 17325b.

Scanning electron microscopy

Adults worms previously fixed in AFA solution (Mafra and Lanfredi 1998) were washed twice in sodium cacodylate buffer, pH 7.2 for 24 h at room temperature, post-fixed in 1% $Os0_4$ for 3 h, and washed with sodium cacodylate buffer. The helminths were then dehydrated in an ethanol series, critical point dried on CPD Blazers equipment, and sputter-coated with gold. Specimens were examined in a JEOL scanning electron microscope at 15 kV.

DNA extraction

DNA was extracted from individual adult worms from three sources: specimens obtained directly from field-caught *N. squamipes*, specimens of the RJ isolate maintained under laboratory conditions in hamsters, and specimens of the BH isolate maintained in laboratory conditions in hamsters. Alcohol was removed from the ethanol stored specimens prior to extraction by leaving them overnight in 1×TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA was extracted using the non-ionic detergent CTAB (cetyltrimethylammonium bromide). The following protocol was modified from that described by Winnepenninckx et al. (1993) for genomic DNA isolation from gastropods. The sample was homogenized in 500 µl of CTAB buffer pH 8.0 (2% CTAB, 1.4 M NaCl, 0.2% β-mercapto-ethanol, 20 mM EDTA, 100 mM Tris-HCl) containing 200 µg of proteinase K. This mix was incubated at 60°C until the sample had completely dissolved (1–2 h). CTAB was removed from the lysate by adding an equal volume of chloroform, then centrifuging the mixture at 14,000 rpm for 5 min, and removing the aqueous layer. Any remaining protein was removed using a standard phenol/chloroform extraction. The remaining DNA was precipitated for 5 min at room temperature with 0.6 volumes of 100% isopropanol. The solution was centrifuged at 14,000 rpm for 10 min and the pellet washed with 70% ethanol then air dried before resuspending in 50 µl of milli-Q water.

DNA amplification

The polymerase chain reaction (PCR) was used to amplify the ribosomal DNA ITS1, 5.8S and ITS2 region between the forward primer BD1 (5′ GTC GTA ACA AGG TTT CCG TA 3′) and reverse primer ITS2 (5′ CCT GGT TAG TTT CTT TTC CTC CGC 3′). Initial PCR reactions were carried out in a total volume of 20 µl. Each reaction contained 0.5 µM of each primer pair, combined with 10–100 ng of template DNA, 10× *Taq* buffer, 0.8 mM dNTP, 2.5 mM magnesium and 0.2 units of *Taq* polymerase (Promega). This mix was processed in a Whatman Biometrea T Gradient thermocycler for 31 cycles, programmed to ramp between temperatures at 10°C per second. Cycle 1 was 95°C for 60 s, 56°C for 45 s and 72°C for 90 s. This was followed by 29 shorter cycles, 95°C for 30 s, 56°C for 30 s and 72°C for 90 s. This mixture was held at 72°C for 7 min to complete extension,

then dropped to 4°C. Products were viewed on an ethidium bromide stained 1% agarose TAE gel. Successful PCR reactions were repeated in 100 µl volumes to obtain enough DNA for sequencing.

DNA sequencing and alignment

PCR products were concentrated and desalted prior to sequencing using a Centricon centrifugal filter device (Millipore). The concentration of DNA was quantified by running the product on an ethidium bromide stained 1% agarose gel alongside a marker (*Hin*dIII). PCR products were sequenced using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE applied Biosystems). Half volume (10 µl) reactions containing 1 µl of Big Dye Ready Reaction Mix, 3 µl of Big Dye buffer (200 mM Tris pH 8.5, 5 mM $MgCl_2$), 15–45 ng of PCR products and 3.2 pmol of one PCR primer were cycled in a Whatman Biometra T Gradient thermocycler following the kit's recommended protocol for a TC1 thermal cycler. This was followed by an ethanol/sodium acetate precipitation (using the protocol for microcentrifuge tubes). Products were run on an ABI 377 automated sequencer. Two internal primers, 3S (5′ GGT ACC GGT GGA TCA CGT GGC TAG TG 3′) and 4SR (5′ GCA ATT TCC GTT CAA GAT GTC GAT G 3′), were used to obtain the complete sequence from both DNA strands. DNA sequences were aligned by eye using Sequencher (tm) version 4.0.5 (Gene Code Corp, Ann Arbor, Mich., USA, 1999). Sequence comparisons were made with published echinostome sequences available from GenBank.

Results

Taxonomic position

Echinostoma paraensei Lie and Basch, 1967

Natural vertebrate host: *Nectomys squamipes* (Rodentia:Sigmodontinae).

Natural invertebrate host: unknown.

Experimental invertebrate hosts: *Biomphalaria glabrata*, *Physa marmorata* and *Lymnaea columella* (Maldonado et al. 2001).

Locality: Sumidouro, Rio de Janeiro State, Brazil. 22' 02'' S and 42' 41'' W.

Light microscopy

The dimensions of the adult worms based on 30 specimens (RJ isolate) are presented in Table 1. The morphological characteristics for the RJ isolate fall within the range recorded for species within the 37-spine revolutum group (Fig. 1a–c).

The body was slender and elongate. The peristomic collar was ventrally interrupted with 35, 36 or 37 spines distributed along its margins (Fig. 1a) with the frequency of 10%, 20% and 70%, respectively. Among the 30 specimens studied, the lack of one or two spines was always in the dorsal-most region of adult worms at 4 weeks of infection. The ratio of the dimensions of the dorsolateral and dorsal-most spines was 1.01:1.0 (Table 1). The lateral and corner spines were slightly longer (Fig. 1b, Table 1). The oral sucker was subterminal and surrounded by the peristomic collar. The prepharynx was short and the pharynx bulbar and muscular. The esophagus was long and bifurcated anterior to the acetabulum, forming two ceca that ran laterally along the body to the posterior end (Fig. 1a). The acetabulum was big and muscular; its relation with the oral sucker was 1.4:1. The testes were smooth, tandem and oblong, with the posterior testis frequently bigger and bilobed, and located posterior to the middle of the hindbody. The cirrus sac was well developed and ovoid, and located dorso-laterally to the genital atrium and the acetabulum, with the seminal vesicle, prostatic gland and unspined cirrus conspicuous (Fig. 1a, c). The genital pore was median and anterior to the acetabulum. Vitelline follicles were lateral, dorsal and ventral to the ceca, and ran from the acetabulum to near the posterior end of the body. The ovary was spherical or ovoid and anterior to the testes. The Mehlis' gland complex was located between the ovary and the testes. The uterus was long, coiled, full of eggs, and intercecal, post-acetabular and pre-ovarian in its location (Fig. 1a). The excretory system was straight, from the posterior testis to the subterminal excretory pore.

The morphological features of the BH isolate are similar to those described for the RJ isolate and the morphometric data, based in 6 adults, can be seen in Table 1. It was remarkable that only 3 of the dorsolateral spines on each side of the collar of 4-week-old adult worms were visible by light microscopy. They were slightly smaller than the lateral spines. The dorsal-most spines were not visible. For the *E. paraensei* paratype 062968.00 (slide 2), which was collected on the 27th day of infection, the 4 dorso-lateral spines were visible, and their sizes were similar to the laterals. Also, 4 of the 7 dorsal-most spines were visible and were smaller than the dorso-lateral spines (Table 1); the ratio of their lengths was 2.6:1.0. The paratype 062968.00 (slide 3), collected after 12 days of infection, showed the same pattern, but differences in the dimensions of the dorsal spines were less evident, with the ratio being 1.08:1.0 (Table 1). There was no evidence that the small dorsal-most spines were broken.

We also examined the holotype and paratypes of *Pseudechinostomum caballeroi* because this species was described from worms collected from *Nectomys squamipes*, also captured at Sumidouro, Rio de Janeiro State (Kohn and Fernandes 1977). This species is morphologically similar to *E. paraensei*, but lacks spines around the peristomic collar.

Scanning electron microscopy

Electron micrographs of the RJ and BH isolates of *E. paraensei* are presented in Fig. 2a–l and Fig. 3a–h, respectively. The RJ and BH isolates were very similar, showing the anterior part of the elongate body with the lateral borders curved ventrally on the longitudinal axes, enlarging from the acetabulum onwards, especially when the cirrus was protruded (Figs. 2c, 3a).

Table 1 Morphometric comparison of *Echinostoma paraensei* adult worms, original description (Lie and Basch, 1967), *E. paraensei* Belo Horizonte isolate and *E. paraensei* Rio de Janeiro isolate. *np* Not present in original paper; *NV* not visible. Measurements in micrometers

Morphological characters	*E. paraensei* Lie and Basch (1967)	*E. paraensei* BH isolate ($n=6$) present work	*E. paraensei* RJ isolate ($n=30$) present work
Body length	7,490–16,033	16,323 ± 159	15,492 ± 1.416
Body width	792–1,950	2,399 ± 80	2,407 ± 157
Collar (width)	396–746	545 ± 79	657 ± 96
Oral sucker	216–408×216–408	255 ± 28×274 ± 33	338 ± 50×384 ± 60
Spines (number)	37	26	35–37
Dorsal spines	22–33		
Dorsal-most	*22.9 ± 3.7/** 16.6 ± 4.2	NV	37.5 ± 7.6
Dorso-lateral	*21.1 ± 4.8/**43.5 ± 8.2	56.9 ± 6.9	38.0 ± 8.1
Lateral spines	36–99 *36.6 ± 5.5/**53.3 ± 3.7	74.1 ± 13.6	54.2 ± 5.1
Corner spines oral	36–99 *37.0 ± 2.0/**52.5 ± 4.4	77.7 ± 5.5	44.2 ± 5.8
Corner spines aboral	36–99 *33.8 ± 6.1/**53.2 ± 4.5	79.4 ± 9.4	45.5 ± 5.6
Prepharynx (length)	72–120	139 ± 26	129 ± 42
Pharynx	180–348×180–288	198 ± 26×161 ± 16	287 ± 57×231 ± 42
Esophagus	360–664	388 ± 54	405 ± 73
Acetabulum	465–1008 (diameter)	870 ± 38×917 ± 75	824 ± 65×930 ± 108
Testes anterior	384–732×312–612	752 ± 179×717 ± 108	844 ± 132×763 ± 95
Posterior	408–852×312–516	917 ± 99×741 ± 113	940 ± 110×750 ± 83
Ovary	250–408 (diameter)	490 ± 39×682 ± 51	476 ± 86×712 ± 54
Uterus coils	Up to 22	20 ± 1.6	18 ± 3
Uterine eggs/worm	Np		2,416 ± 417
Measure	104–122×74–86		120 ± 5×85 ± 4

*, ** Measurement after paratypes 062.968.00, 12 and 28 days old respectively

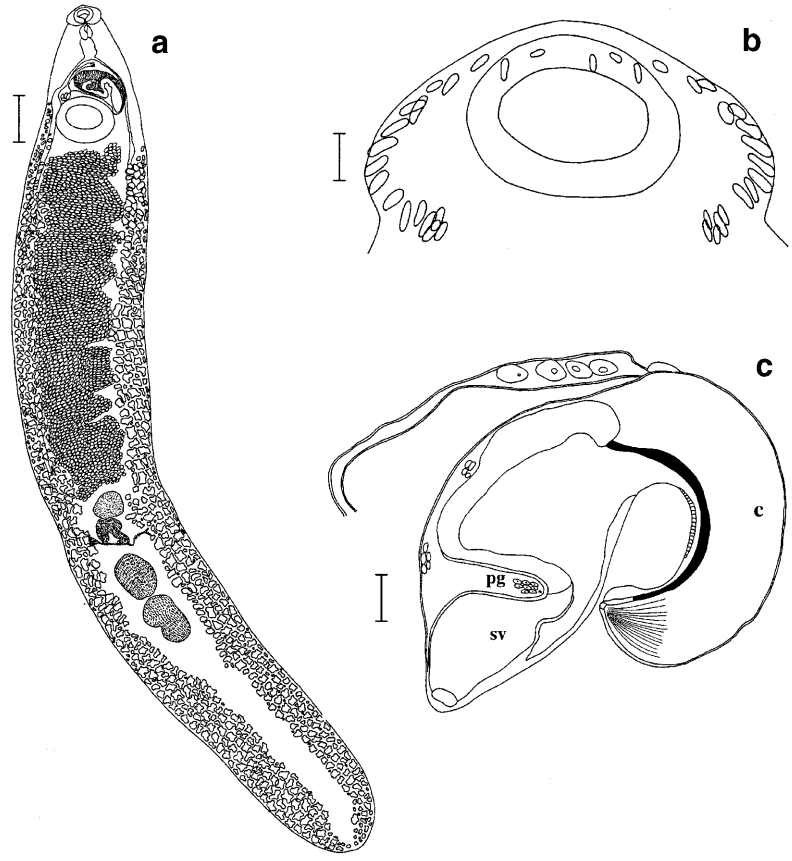

Fig. 1a–c *Echinostoma paraensei* isolate from Rio de Janeiro passed through a hamster (scales bars are 100 μm except for adult worm that is 1 mm).**a** Adult worm, 28 days old, **b** collar spines, **c** cirrus (*c*) sac with unspined surface, seminal vesicle (*sv*), prostate gland (*pg*) and terminal uterus with eggs

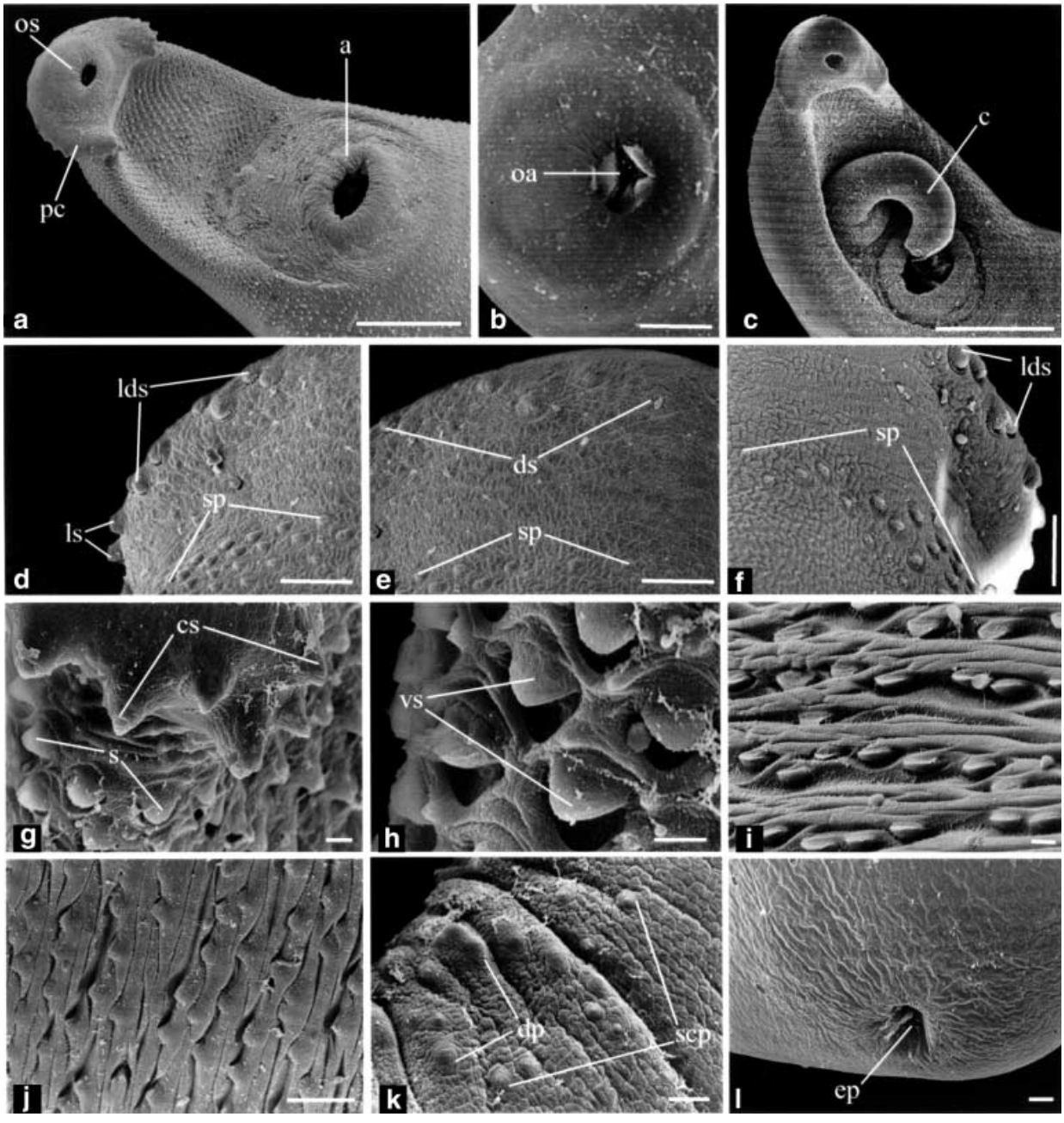

Fig. 2a–l Electron micrographs of *Echinostoma paraensei* Rio de Janeiro isolate. **a** Ventral view of *E. paraensei* showing oral sucker (*os*), peristomic collar (*pc*), acetabulum (*a*). **b** Detail of the oral sucker showing the oral aperture (*oa*). **c** Ventral view showing peristomic collar and protruded unspined cirrus (*c*). **d–f** Lateral spines (ls), laterodorsal spines (lds) and dorsal spines (ds) showing the concavity on the bottom and three rows of sensory papillae (sp). **g** Corner spines partially covered by tegument (cs) and scales (s). **h** Ventral scales (vs) between the collar and the acetabulum. **i** Scales and folded tegument of ventral region on the middle of the body. **j** Ventral spines flat with rounded edges. **k** Unspined acetabular tegument wrinkled with domed (*small arrow*) and small ciliated papillae (*arrowhead*) randomly distributed. **l** Excretory pore (*ep*)

The oral sucker was better defined in the RJ isolate (Fig. 2b), and it merged with the collar. The oral aperture, inside the oral sucker, is bordered by three lips in the RJ isolate (Fig. 2b). The collar of spines of the RJ isolate was complete (Fig. 2a–c), however, the dorsal spines were smaller and were retracted inside a concavity or pocket formed by the tegument (Fig. 2e). The dorsal-most spines of the BH isolate were not visible, although sometimes it was possible to observe the orifices of their pockets (Fig. 3b). The corner spines were smaller (Fig. 3c) than for the RJ isolate (Fig. 2g). The spines of both isolates were partially covered by tegument with the distal spine end emerging from the tegument (Figs. 2g, 3c). The collar of both isolates had a papillate sensory region between the spines of the collar and the sucker lips. RJ and BH isolates presented three rows of papillae in the dorsal region, a short distance from the dorsal collar spines (Figs. 2d–f, 3b).

Low magnification SEM micrographs of adults of the RJ and BH isolates revealed shell-like scales that lay over a tegumentary cavity that covered the ventral surface of the anterior part of the body (Fig.2h–j). Scales were arranged in rows, with an organized distribution (Fig. 2j). The scales were more widely scattered at the middle and the lateral edges of the body (Figs. 2c, 3b, g). At this level, the scales presented a flat aspect with rounded edges, intercalated by transversally folded tegument (Fig. 2h, j). No scales were observed on the dorsal-most side of the body (Figs. 2e, 3b, g), or on the ventral surface, from the anterior to the genital pore to the posterior region of the acetabulum (Figs. 2a, c, 3a, d–f). Scales were also absent at the posterior end of the body (Figs. 2l, 3h).

The genital pore was located ventrally, distant from the anterior margin of the acetabulum, thus permitting the distal end of the cirrus to curl inside it (Figs. 2a, c, 3a, d). Representative specimens of both isolates had the cirrus protruded and it was possible to see that its tegument was not spined. One specimen presented only a protuberance with the genital pore on its top (Fig. 3g). Between the acetabulum and the genital aperture, a folded protuberance was present with a smooth surface and an aperture on its top (Fig. 3f).

The tegument of the acetabulum was unspined and radially wrinkled (Figs. 2k, 3f). The rim had a plaited border and there were numerous randomly distributed domed and small ciliated papillae (Fig. 2k).

The excretory pore was subterminal in both isolates and covered by a smooth tegument without any characteristic structure (Figs. 2l, 3h).

Molecular analysis

The ribosomal DNA ITS1, 5.8S and ITS2 sequences of all three isolates originating from Brazil (RJ isolate, BH isolate and the wild isolate from *N. squamipes*) were identical. The few bases that differ between these samples and the published *E. paraensei* ITS sequence (BH isolate sequenced by Morgan and Blair 1995) are most likely artifacts from the older, less efficient, method of sequencing. The GenBank accession numbers for the three isolates are: wild type – AF336232, RJ isolate – AF 336233 and BH isolate – AF 336234.

Discussion

Most echinostome species described from Brazil have, until now, been isolated from naturally infected snails. *Echinostoma paraensei* was first described from material obtained from naturally infected *Biomphalaria glabrata* snails shipped to the United States, where Lie and Basch (1967) described the species and its life history using experimental vertebrate hosts. Since then, this trematode has been maintained under laboratory conditions and has been used in several biological investigations. It would be helpful to know the natural vertebrate host for this model organism.

Recently, we isolated a member of the family Echinostomatidae from the rodent *Nectomys squamipes*, near a vegetable plantation crossed by a stream, at the Sumidouro, Rio de Janeiro State. Initially we identified this worm as *Pseudechinostomum caballeroi*, because this species had been collected from the same vertebrate host at the same locality in the past. We observed a larger number of the worms by light microscopy and compared them to the type and paratypes of *P. caballeroi*. We noticed morphological and morphometric similarities between our new specimens and *P. caballeroi*, but noted that our specimens had spines around the peristomal collar whereas *P. caballeroi*, as typical of the genus *Pseudechinostomum*, does not. During the past 2 years we have captured and examined additional *N. squamipes* at Sumidouro. Almost 25% of them were infected with this species of *Echinostoma* which we are now describing, and identifying as *E. paraensei*. None of the rodents we examined had worms with an unspined peristomic collar.

Kanev and Busta (1992) referred to abnormalities of the collar spination of echinostomes. Among them was the loss of spines at the peristomic collar and the possibility that spines can be captured in their tegumental pockets as a result of improper manipulation or fixation. They also found worms missing spines due to malformation. Lie and Basch (1967) commented on the minute size of the dorsal-most collar spines of adult *E. paraensei* and noted they were totally absent in worms older than 3 weeks. We feel that the lack of collar spines on *P. caballeroi* should be reconsidered in light of these observations.

Using light microscopy we compared our isolate to paratypes of several other species including *E. paraensei*, *E. revolutum*, *E. barbosai* and *E. rodriguesi*. We obtained morphological and morphometric evidence that our new isolate was *E. paraensei*, despite some differences that will be discussed later. We also compared our sample with the BH isolate of *E. paraensei* maintained under laboratory conditions at the Department of Biology, University of New Mexico. As can be seen in Table 1, there are differences in the dimensions of the three *E. paraensei* samples compared: the original description (Lie and Basch 1967), laboratory-reared BH isolate and RJ isolate). This is probably explained by the fact that Lie and Basch (1967) described the species based upon unflattened adult worms, with a large range of ages (12–109 days). In contrast, BH and RJ worms were 28 days old. Many of the remaining characters were in the same range. The ability to contract and distend the body when alive or during fixation reduces the value of body dimensions as characters for distinguishing among species and isolates. The differences found among the samples compared are probably mostly due to the way in which the material was prepared.

Lie and Basch (1967) recognized 37 spines in the peristomic collar of the larval stages and young adults

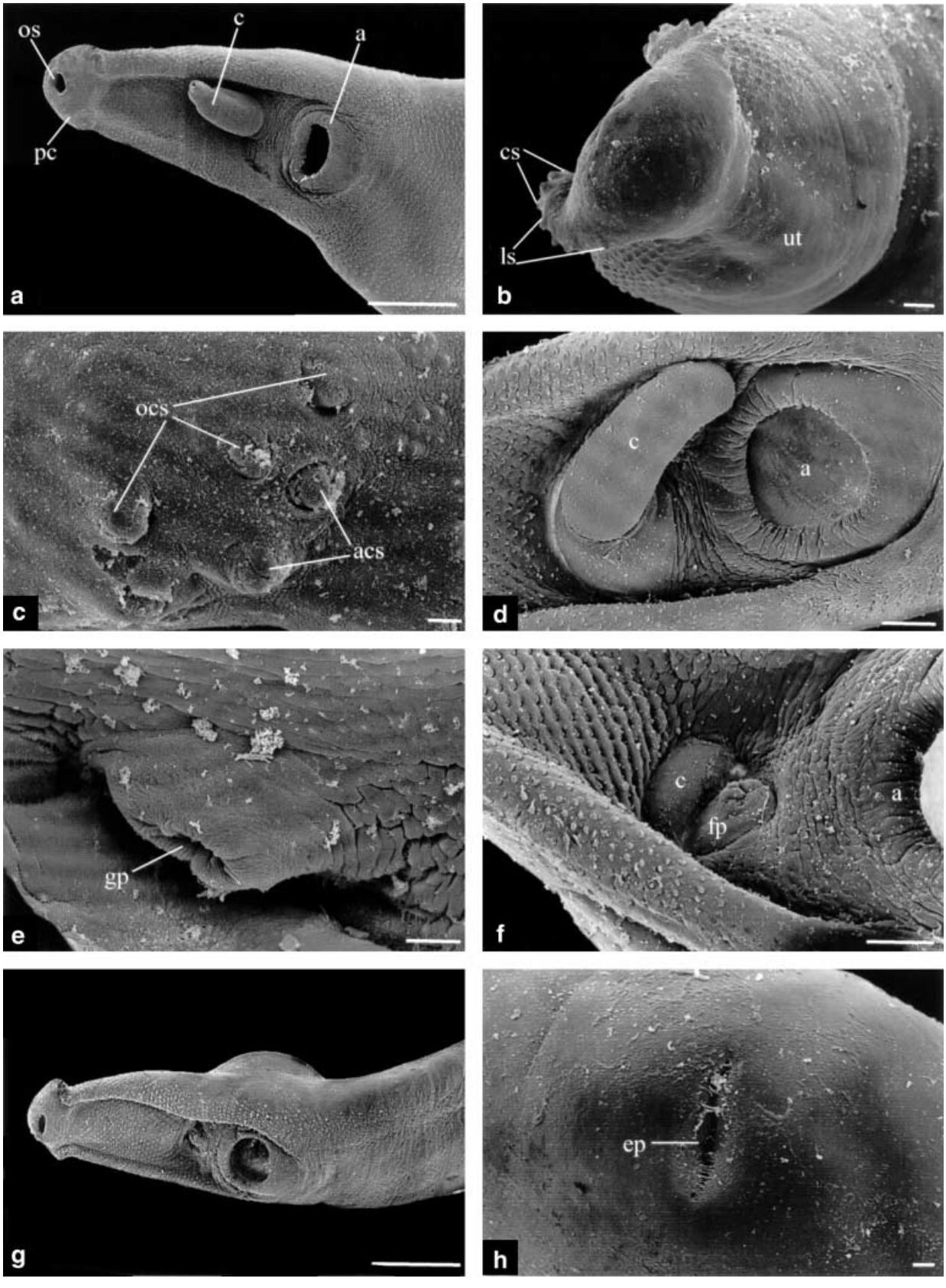
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Fig. 3a–h Electron micrographs of *Echinostoma paraensei* Belo Horizonte isolate. **a** *E. paraensei* ventral view showing oral sucker (*os*), peristomic collar (*pc*), cirrus (*c*) and acetabulum (*a*). **b** Dorsal view showing the unspined tegument (*ut*), the orifices of the dorsal spines, corner spines (*cs*) and lateral spines (*ls*) of the peristomic collar. **c** Oral corner spines (*ocs*), aboral corner spines (*acs*). **d** Ventral view showing the unspined protruded cirrus (*c*) and the acetabulum (*a*); notice the unspined tegument at the raised genital and acetabulum areas. **e** Genital pore (*gp*) and the unspined tegument around it. **f** Cirrus (*c*) and folded protuberance (*fp*) with an aperture on its top. **g** Latero-ventral view of the body showing the distribution of the scales on the surface and raised dorsal regions. **h** Excretory pore (*ep*)

but also noted that adult worms, older than 3 weeks, might possess fewer spines in the dorsal region. We noted the same with the BH isolate as most adult worms (4 weeks old) lacked spines in the dorsal-most region. Most of the individuals of the RJ isolate presented 37 spines, although some lacked one or two spines in the same region.

Another interesting observation concerned the dimension of the spines of the peristomic collar. Lie and Basch (1967) noted that the dorsal spines of *E. paraensei* were very small compared to the spines of other regions of the peristomic collar. Unfortunately, they presented only the range of the lengths of spines from worms aged 12–109 days. Measurements of the spines of the two paratypes studied in the present work (Table 1) showed that the 27-day-old worm had dorsal-most spines that were smaller than the dorso-lateral spines while for the 12-day-old worm, all of the dorsal spines were the same length. The dorsal-most spines of the BH isolate were not visible and the dorso-lateral spines were almost the same length as the laterals. The dorsal-most and dorso-lateral spines of the RJ isolate were similar in length and slightly shorter than the oral and aboral spines (Table 1). The main differences which we observed between the RJ and BH isolates were in the dorsal spines. These differences may be explained by the fact that the BH isolate has been maintained in a laboratory for over 35 years and the RJ worms were only recently isolated from the field.

Variation also occurred in the relative size of the lateral and corner spines. In the RJ isolate aboral and oral corner spines were the same length and the lateral ones were slightly bigger; however the BH isolate showed an inverse relation of the size of the lateral and corner spines, and they were always bigger than the spines of the RJ isolate (Table 1). Lie and Basch (1967) also commented that the spines were bigger in older worms (up to 99 μm). The drawings in their paper do not have scale values, so it is impossible to confirm their measurements.

A surprising feature was observed when 12- and 27-day-old paratypes of *E. paraensei* were compared. During worm development, the dimensions of the body, the length of the dorso-lateral, lateral and corner spines increased, while the dorsal-most spines decreased in size or simply disappeared. The present work was performed studying two allopatric isolates of *E. paraensei* of the same age, using hamsters as vertebrate experimental hosts. We tried to reproduce the biological conditions that Lie and Basch (1967) used when they isolated and started the cycle of this species. These authors described the species using a pool of worms aged from 12 to 27 days, which made some of the comparisons with the present study difficult. This observation agrees with Kostadinova and Gibson (2000) who stated that pooling data from worms of different ages further increases variation and makes comparisons difficult.

The minute size of the dorsal spines of *E. paraensei* has been identified as a criterion for adult diagnosis (Kostadinova and Gibson 2000). The new specimens of *E. paraensei* described in this study had dorsal spines that were shorter than lateral and corner spines and, although they were not "minute", they do suggest that dorsal spines that are short relative to other collar spines are a consistent hallmark of this species.

Scanning electron microscopy

The observations on the BH isolate by SEM confirmed the light microscopy observation that the dorsal-most spines of the peristomic collar may be absent or invisible at times. Using SEM it was sometimes possible to visualize the opening of the spine pockets.

The oral sucker of the RJ isolate was more conspicuous than for BH worms, but both possessed papillae on the area around the rim and near the lateral and corner spines at the lap of the collar. The RJ isolate also had 3 rows of papillae posterior to the dorsal spines of the collar, a feature not seen in *E. caproni* or *E. trivolvis* (Fried et al. 1990).

The longitudinal ventral depression between the oral sucker and the acetabulum that was observed in *E. caproni* and *E. trivolvis* by Fried et al. (1990) was also present in the BH isolate. The RJ isolate did not have this depression. Both isolates had numerous domed and short ciliated sensory papillae on the rim of the acetabulum, but there were no acetabular spines, a character that distinguished them from *E. caproni*.

The cirrus of both RJ and BH worms could be protruded, as was observed by Ursone and Fried (1995) for *E. caproni*. The genital area was raised as with *E. caproni* and *E. trivolvis* (Fried et al. 1990). Some specimens of the BH isolate also had a raised dorsal area at the same level. A highly folded protuberance posterior to the cirrus was also observed in both isolates. This structure resembles the genital aperture when the cirrus is not protruded, and it seems that this is the opening of a duct. Fried et al. (1990) noted a similar structure in *E. caproni* and commented that its function is not known.

The distribution and shape of the tegument scales of both isolates were similar to those of *E. trivolvis* (Fried et al. 1995). The main difference was that the scales were

rare or invisible at the middle of the dorsal region of the body of both isolates. The RJ isolate also showed shell-like scales on the anterior, ventral, and lateral regions of the body. This variation was probably the result of the contraction or relaxation of the body. Adults of *E. paraensei* that were 4 weeks old did not have bifurcated or multi-pointed spines along the body as observed in *E. caproni* adults that were 5–10 weeks old (Ursone and Fried 1995). In addition, the shape of the scales, although similar to *E. trivolvis* (Fried et al. 1990), was slightly pointed. Both isolates lacked tegumental spines from the region immediately anterior of the cirrus to the posterior of the acetabulum. In contrast, *E. caproni* has conspicuous spines around the acetabulum.

The similarity of the morphological characters observed by SEM in the two isolates of *E. paraensei* and *E. trivolvis* (Fried et al. 1990) is in concordance with phylogenetic studies, using mitochondrial ND1 and ribosomal ITS1 and two markers (Morgan and Blair 1998), that showed the relatedness of these two species.

Molecular analysis

The molecular data confirm that the three Brazilian isolates (RJ, BH and worms taken straight from *N. squamipes*) are all *E. paraensei*. The nuclear rDNA internal transcribed spacers are not good markers for studying population differences, so other sequences, such as mitochondrial DNA markers, would be better suited for further studies at the population level.

Based on the present data, we conclude that *E. paraensei*, a species of the ''revolutum group'', has a unique morphological feature, namely the small size of the dorsal-most spines of the peristomic collar. Some variation was detected in the present study, which analyzed the paratypes of different ages of two isolates (the BH isolate and RJ isolate). This morphological feature can be used as a diagnostic character for species identification of the mature adult worms. Our findings were corroborated by the presence of identical internal transcribed spacer sequences. Further studies of worms from different wild populations and of different ages from laboratory infections will help us to understand the morphological variations of adult worms, including variations in collar spines.

We also report for the first time a natural definitive host for *E. paraensei*, namely *N. squamipes*. This interesting aquatic rodent also serves as a definitive host for the medically important digenean *Schistosoma mansoni* in Brazil.

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