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# Integrative taxonomy suggests that South American freshwater nematodes *Echinocephalus* and their host stingrays co-originated in late Oligocene to early Miocene

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## Abstract

Nematoda of the genus *Echinocephalus* Molin, 1858 include species from the Gnathostomatidae family, the adult stages of which parasitize the stomach and spiral intestine of elasmobranchs as their final hosts. In the present study, we describe *Echinocephalus spinosus* n. sp. found parasitizing the spiral valve of the freshwater stingray *Potamotrygon motoro* from the Tapajós River, in the Amazon Basin, in the state of Pará, Brazil. In the study we performed morphological (light and scanning electron microscopy) and molecular (small subunit ribosomal ribonucleic acid (SSU rDNA) and mitochondrial cytochrome c oxidase I sequencing) analyses. *E. spinosus* n. sp. is only the second species of the *Echinocephalus* genus described from a strictly freshwater environment. The SSU rDNA based phylogenetic analyses showed *Echinocephalus* clade as a sister lineage of *Gnathostoma*, and that the new species arises as a sister to *Echinocephalus* cf. *pseudouncinatus*. Time-calibrated phylogenetic analysis revealed that the origin of the freshwater *Echinocephalus* coincides with the recently proposed origin of the freshwater host potamotrygonin stingray, namely the late Oligocene to early Miocene, when the western Amazon was dominated by the Pebas wetlands, an epicontinental marine/freshwater system.

## Introduction

Nematodes Gnathostomatidae (Railliet, 1895 emend. Nicoll, 1927) are grouped into seven genera (Bain *et al.*, 2014). Of these, the genus *Echinocephalus* Molin, 1858, harbours 13 species the mature stages of which infect only elasmobranchs, mainly marine rays. These include: *Echinocephalus uncinatus* Molin, 1858 (type species) parasitic in the dasyatid stingray *Bathytoshia centroura* (Syn. *Trygon brucco* Bonaparte, 1834) from Mediterranean sea, *Echinocephalus spinosissimus* von Linstow, 1905, *Echinocephalus multidentatus* Baylis & Lane, 1920, and *Echinocephalus southwelli* Baylis & Lane, 1920, parasitic in the myliobatid and dasyatid rays from India and Sri Lanka; *Echinocephalus pseudouncinatus* Millemann, 1951, parasitic in *Heterodontus francisci* (Heterodontidae) and *Myliobatis california* (Myliobatidae) from Mexico; *Echinocephalus sinensis* Ko, 1975, parasitic in *Aetobatus flagellum* (Aetobatidae) from the South China Sea and the South Pacific; *Echinocephalus pteroplatae* Wang, Zhao & Chen, 1978, from the *Gymnura japonica* (Gymnuridae) from Taiwan; *Echinocephalus janzeni* Hoberg, Books, Molina-Ureña & Erbe, 1998, parasitic in the dasyatid stingray *Styracura pacifica* (Syn. *Himantura pacifica* Beebe & Tee-Van, 1941) from the East Pacific in Costa Rica and Mexico; *Echinocephalus overstreeti* Brooks & Deardorff, 1988 and *Echinocephalus inserratus* Moravec & Justine, 2021 parasitizing dasyatids and heterodontids from the Southwest Pacific; and *Echinocephalus caniculus* Saad *et al.*, 2022, parasitic in the scyliorhinid cat shark *Scyliorhinus canicula* from Tunisia (Baylis & Lane, 1920; Moravec & Justine, 2021; Saad *et al.*, 2022). Reports of *Echinocephalus* species infecting freshwater elasmobranchs are restricted to *Echinocephalus diazi* Troncy, 1970 and *Echinocephalus daileyi* Deardorff *et al.*, 1981, both found in South American stingrays of the Potamotrygonidae Garman, 1877. *Echinocephalus diazi*, however, was described from a ray identified as *Potamotrygon hystrix* caught in Lake Maracaibo (Troncy, 1970), a large brackish lake connected to the Gulf of Venezuela (Laval *et al.*, 2005). Later, this gnathostomatid nematode was twice reported in the marine ray *Styracura schmardae* Werner, 1904 (Syn. *Trygon*

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*schmardae* Werner, 1904; *Himantura schmardae* Werner, 1904) (Deardorff *et al.*, 1981). *Echinocephalus daileyi,* on the other hand, was found infecting *P. hystrix* and *Potamotrygon circularis* (a junior synonym of *Potamotrygon motoro* (Müller & Henle, 1841) from a freshwater environment from the Amazon and Orinoco River basins (Baylis & Lane, 1920; Deardorff *et al.*, 1981; Moravec & Justine, 2021). In addition to these two named species, Lacerda *et al.* (2009) reported *Echinocephalus* sp. parasitizing the spiral valve of the largespot river stingray *Potamotrygon falkneri* Castex & Maciel, 1963, from the upper Paraná River, in southeastern Brazil, although these authors did not identify the parasite on a specific level.

Potamotrygonidae harbours two subfamilies: the recently created Styracurinae, which is composed of *S. schmardae* (type species) and *Styracura pacificus* Beebe & Tee-Van, 1941 (Syn. *Dasyatis pacificus* Beebe & Tee-Van, 1941; *Himantura pacificus* Beebe & Tee-Van, 1941) (Carvalho *et al.*, 2016); and Potamotrygoninae, a marine-derived lineage composed of 38 exclusively freshwater stingray species distributed in four genera and occurring in the main South American watersheds, with the greatest diversity observed in the Amazon (Lovejoy *et al.*, 1998, 2006; Carvalho *et al.*, 2003; Silva & Loboda, 2019; Fontenelle *et al.*, 2021; Loboda *et al.*, 2021).

The present study reports, using light and scanning electron microscopy, and DNA based phylogenetic approach based on multiple genes (small subunit ribosomal ribonucleic acid (SSU rDNA) and mitochondrial cytochrome c oxidase I (COI)) and dating inference analyses, a new *Echinocephalus* species infecting the spiral intestines of specimens of the potamotrygonin species *P. motoro* from the Tapajós River, in the Amazon basin, Brazil.

## Material and methods

### *Sampling and morphological studies*

A total of 35 specimens of *P. motoro* were caught using hook and line or longline in the Tapajós River, in the municipal region of Santarém, in October 2018 (2°26′23.30″S; 54°53′39,67″W and 2°20′34.68″S; 54°53′27.69″W) and October 2021 (2°23′34.41″S; 54°43′57.53″W) (fig. 1). The fish were transported alive to a field laboratory on the shores of the Tapajós River and the methodology was approved by the ethics research committee of the Federal University of São Paulo (CEUA N 4457040219). Capture and genetic heritage access were authorized by the Brazilian Ministry of the Environment (SISBIO no 66053–1 and SisGen no AD28DC2, respectively).

Nematode specimens ($n = 39$) found in the spiral intestine of the stingrays were fixed in 96% ethanols for light and scanning electron microscopy analyses and DNA sequencing.

The morphological analyses with light microscopy were based on three males and three females. Specimens were cleared with lactophenol on temporary slides and analysed in a V3 Leica

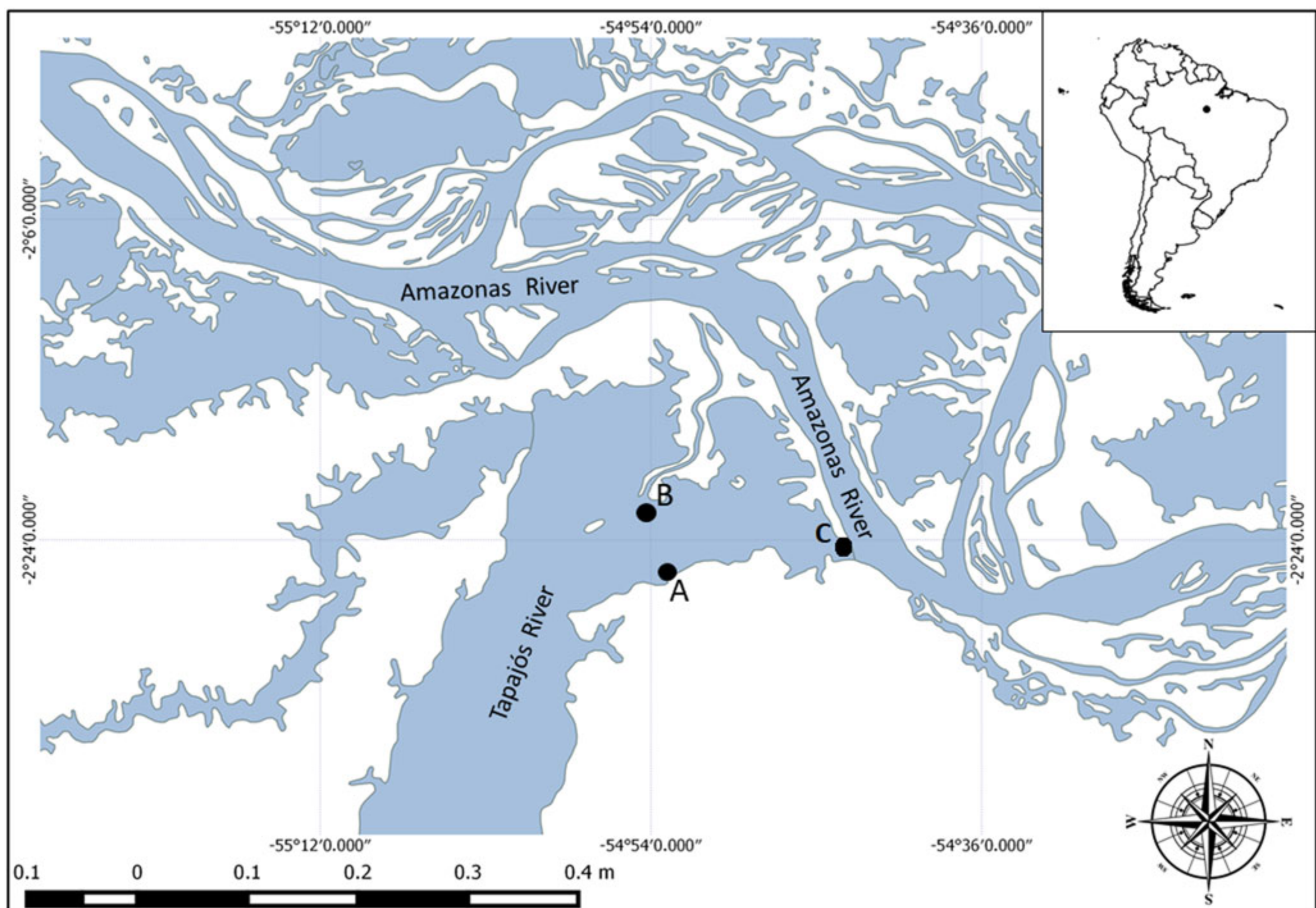


**Fig. 1.** Map of collection localities in the Amazon basin: Tapajós River, Pará State. (A) 2°26′23.30″S; 54°53′39,67″W; (B) 2°20′34.68″S; 54°53′27.69″W; and (C) 2°23′34.41″S; 54°43′57.53″W.

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Application Suite computerized system for image analysis with differential interference contrast. The range measurements ($n = 6$) are provided in micrometres unless otherwise specified. Holotypes and paratypes were deposited in the collection of the 'Museu de Diversidade Biologica- MDBio' (the Museum of Biological Diversity) of the Institute of Biology (IB) of the *University of Campinas* (UNICAMP), Campinas, São Paulo State, Brazil.

For scanning electron microscopy, one specimen of each sex was examined. Samples previously fixed in ethanol were transferred to a glutaraldehyde solution (2.5%) in 0.15 M phosphate buffer (pH 7.3) for 24 h, and then to an osmium tetroxide solution (1%) in the same buffer, for 2 h. The samples were washed in phosphate buffer, dehydrated in graduated ethanol (70, 80, 90, 95 and 100% at intervals of 15 min each), and finally in hexamethyldisilazane (HMDS) for around 5 min, in a methodology adapted from Bray *et al.* (1993). The dried samples were glued on a stub using double-sided tape, covered with gold (200 s/40 mA), and examined under a scanning electron microscope Leo Stereoscan S-440 of the São Paulo Federal University (UNIFESP), Campus Diadema.

Identification was based on the key to species of the *Echinocephalus* genus as proposed by Moravec & Justine (2021) and Saad *et al.* (2022).

### DNA extraction and amplification

The DNA analysis used nematode specimens and *P. motoro* muscle tissue (due to difficult morphological identification) previously fixed in ethanol. The worm specimens were cut on the medial section, the anterior portions were retained in absolute ethanol as hologenophores (see Pleijel *et al.*, 2008) and deposited in the 'Museu de Diversidade Biologica- MDBio'-IB/UNICAMP, while the posterior portions were used for DNA extraction. Nematode and stingray DNA extraction was performed with the Qiagen D Neasy® Blood & Tissue kit, in accordance with the manufacturer's instructions.

Nematode DNA samples were quantified and visualized on 2% agarose gel. SSU rDNA (800 base pairs (bp) ) and COI (780 bp) fragments were amplified using the Qiagen polymerase chain reaction (PCR) Master Mix kit. For SSU rDNA amplification the primers Nem18SF (5′ CGCGAATRGCTCATTACAACAGC-3′) and Nem18SR (5′-GGGCGGTATCTGATCGCC-3′) were used (Floyd *et al.*, 2005). The reactions in final volumes of 20 µl were constituted of: 7.7 µl of ultrapure water; 10 µl of Master Mix Qiagen 2X; 0.4 µl of Nem18SF (5 µM) primer; 0.4 µl of Nem18SR (5 µM) primer; and 1.5 µl of DNA template. The PCR conditions were 94°C/5 min., (94°C/30seg., 54°C/30seg., 72°C/60seg., repeated 35X) and 72°C/10 min. For COI amplification the primers amplification of the *Cox1* gene the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) were used in accordance with Folmer *et al.* (1994), for reactions with a final volume of 20 µl, consisting of 7.5 µl ultrapure water, 10 µl of Master Mix Qiagen 2X, 0.4 µl of HCO2198 (5 µM), 0.4 µl of LCO1490 (5 µM), 1.5 µl of DNA and 0.2 µl of *Taq* DNA polymerase (5 U/µl) (Kappa Biosystems), and processed by the following cycling profile: (94°C/3 min., (94°C/50seg., 54° C/60seg., 72°C/60seg., repeated 35X), 72°C/5 min). The PCR products were visualized on 2% agarose gel for the confirmation of yield. Positive reactions were further purified using the 20% polyethylene glycol (PEG8000) protocol adapted from Dunn & Blattner (1987). Sequencing reactions were performed by the Sanger di-deoxiterminal method and were processed with ABI PRISM® Big Dye™ Terminator V.3 kit (Applied Biosystems™) following the manufacturer's instructions. Reactions were precipitated by the ethanol/ethylene diamine tetra acetic acid method and resuspended in Formamida Hi-Di for application of the ABI3500 genetic analyser (Applied Biosystems™). Sequences were assembled using Sequencher v.5.2.4 (Gene Codes, Ann Arbor, MI, USA) and submitted to GenBank. Fragments of the stingray COI mtDNA, used to confirm taxonomic status, were amplified and sequenced according to Ward *et al.* (2005).

### Phylogenetic analysis and divergence time estimation

Two phylogenetic analyses were performed for the gnathostomatid studied herein, one using sequences of partial SSU rDNA and another the mitochondrial COI gene. The alignments were carried out using the sequences obtained in this study, plus that of Nematoda downloaded from GenBank (supplementary table S1). For COI data analyses the outgroup chosen were *Necator americanus* (AF303153) and *Heligmosomoides polygyrus* KJ994544. For SSU rDNA data analyses, the outgroup chosen was *Tylocephalus auriculatus* Bütschli, 1873 and *Plectus aquatilis* Andrássy, 1985 according to Nadler *et al.* (2007). Sequences representing different genes were separately aligned using the default parameters of the Muscle algorithm (Edgar, 2004) implemented in Geneious 7.1.3 (Kearse *et al.*, 2012). To evaluate the occurrence of substitution saturation, each aligned matrix was tested and the index of substitution saturation (ISS) was estimated using DAMBE 5 software (Xia, 2013). The number of base substitutions per site between the sequences was calculated. Standard error estimates were obtained using a bootstrap procedure with 2000 replicates. Analyses were conducted using the Kimura 2-parameter model using MEGA5 software (Kimura, 1980; Tamura *et al.*, 2013).

The best-fit model for nucleotide evolution in the resulting matrices was determined using the Akaike information criterion in the jModelTest software package (Posada, 2008). Phylogenetic analyses were performed using Bayesian inference (BI) and maximum likelihood (ML) inference methods. The BI analyses were implemented in MrBayes 3.2 (Ronquist & Huelsenbeck, 2003). The model indicated by JModelTest for the COI gene dataset was GTR + I + G. For the SSU gene dataset it was TIM2 + I + G. As this is not implemented in MrBayes 3.2, it was replaced by the closest over-parameterized model available, which was GTR + I + G. BI were run using the following nucleotide substitution model settings for both datasets: lset nst = 6; rates = invariable; ncat = 4; shape = estimate; inferrates = yes; and basefreq = empirical. For the Markov chain Monte Carlo search, chains were run with 10,000,000 generations, saving one tree every 1500 generations. On the burn-in, the first 25% of generations were discarded and the consensus tree (majority rule) was estimated using the remaining topologies; only nodes with posterior probabilities greater than 90% were considered well supported. ML were carried out using RAxML (Guindon & Gascuel, 2003) with bootstrap support values of 1000 repetitions, and only nodes with bootstrap values greater than 70% were considered well supported. The trees were visualized using FigTree v.1.3.1 (Rambaut, 2009).

For divergence time estimation, a new alignment considering only the SSU rDNA sequences of nematode species were used in BI analysis. For calibrating the molecular clock, the sequence of *Oxyxiuris equi* (Schrank, 17188) (EF180062) and *Dentostomella* sp. (AF036590) were used as the representative species of the Order Oxyurida, which has a fossil record for the species *Paleoxyuris cockburni* (Hugot *et al.*, 2014). *Plectus aquatilis* (GQ892827) and *Tylocephalus auriculatus* (AF202155) were used as an external group.

The analysis was performed using the BEAST v2.4.3 software package (Bouckaert *et al.*, 2014), with the species tree inference method and a relaxed lognormal clock, set to the Yule process option (Drummond *et al.*, 2006). Two runs of 100 million chains were carried out and the quality of the convergences was verified using the Tracer 1.7 software package (Rambaut *et al.*, 2018). Tracing qualities were considered when effective sample size values above 200,000 were observed. The extracts resulting from the trees were created in TreeAnnotator 2.3.1 (Bouckaert *et al.*, 2014) with a burn-in of 10% of the total number of trees generated.

Phylogenetics and divergence time estimation trees generated in this study were visualized in FigTree version 1.3.1 (Rambaut, 2020).

## Results

Fourteen of the 35 examined stingrays, 20 females and 15 males, measuring approximately 31.5 cm (ranging from 17–54 cm) in length and 29.5 cm (ranging from 19–49 cm) in width and (40%) had from 1 to 23 gnathostomatid nematodes in their spiral intestine. Morphological and molecular analyses revealed these to be adult specimens of an undescribed *Echinocephalus* species. Morphological analysis and DNA based phylogenetic and dating inferences are presented below (figs 2–7).

### Taxonomic summary

Phylum: Nematoda Diesing, 1861.
Family: Gnathostomatidae Railliet, 1895.
Genus: *Echinocephalus* Molin, 1858.
*Echinocephalus spinosus*. n. sp. (figs 2–5)
*Host type*: *Potamotrygon motoro* Müller & Henle 1841 (the identification of the host was based on morphology and

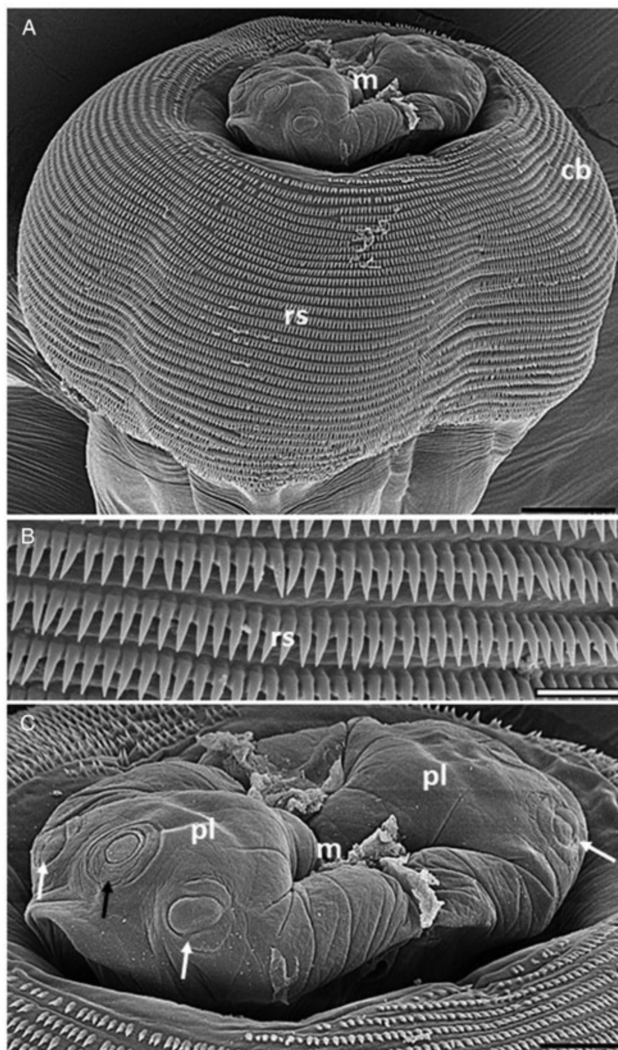


**Fig. 2.** Electron micrographs of *Echinocephalus spinosus* n. sp. parasite of spiral intestine of *Potamotrygon motoro*: (A) male anterior region showing cephalic bulb (cb) with its rows of cephalic spines (rs) and mouth region (m). Scale bar = 50 μm; (B) details of disposition of the cephalic spines. Scale bar = 10 μm; And (C) details of the mouth region (m) showing pseudolabium (pl) with sub-median papillae (white arrows) and amphid (black arrow). Scale bar = 20 μm.

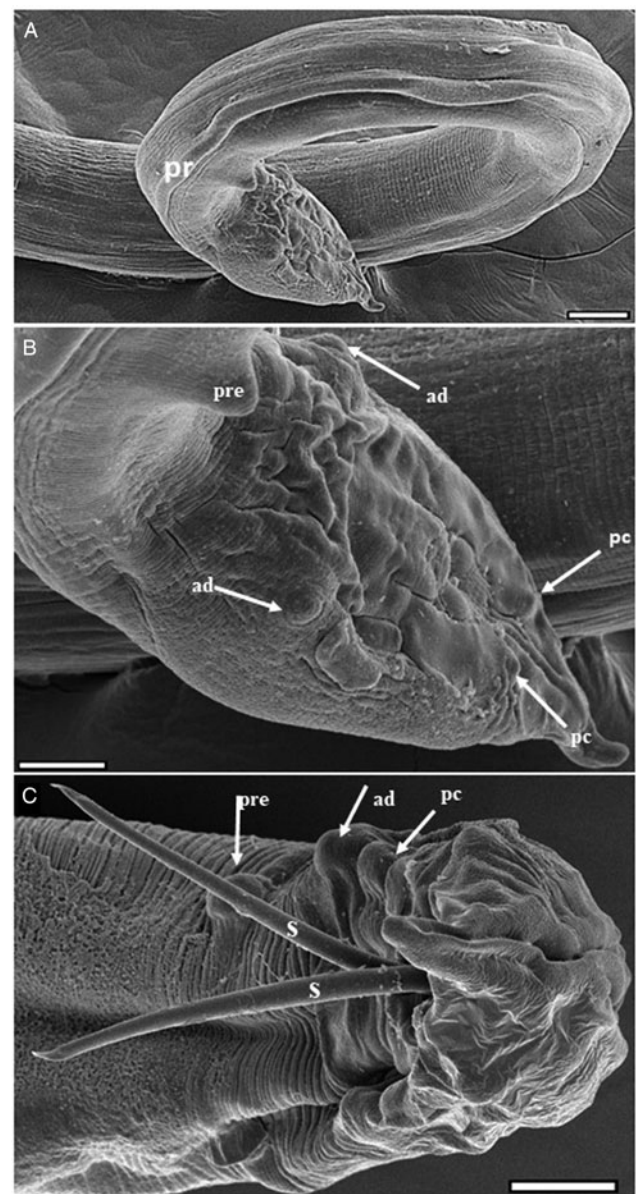


**Fig. 3.** Electron micrographs of *Echinocephalus spinosus* n. sp. parasite of spiral intestine of *Potamotrygon motoro*: (A–C) male posterior region (pr) with (B) showing details of precloacal (pre), adcloacal (ad), and postcloacal (pc) pappilae, and the two externalized spicules (s). Scale bars: A = 100 μm, (B, C) = 50 μm.

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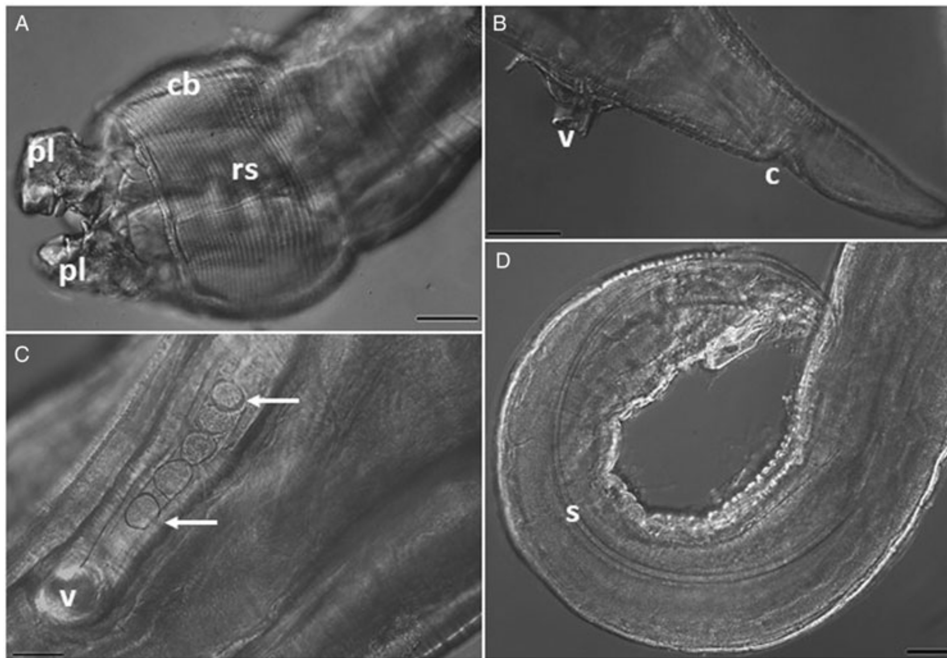


**Fig. 4.** Photomicrographs of *Echinocephalus spinosus* n. sp. parasite of spiral intestine of *Potamotrygon motoro*: (A) male anterior region showing pseudolabium (pl) and cephalic bulb (cb) with its rows of cephalic spines (rs); (B) and (C) female posterior region showing vulva (v) and anus (c) and eggs (white arrows); and (D) male posterior region showing one of the spicules (s). Scale bars: (A, D) = 100 μm, (B) = 200 μm, (C) = 50 μm.

corroborated by sequencing of the mitochondrial gene COI – GenBank accession number OP246209).

*Locality*: Tapajós River, municipality of Santarém, state of Pará, Brazil (geographical coordinates 2°26′23.30″S; 54°53′39,67″W, 2° 20′34.68″S; 54°53′27.69″W, and 2°23′34.41″S; 54°43′57.53″W).

*Types*: Deposited in the 'Museu de Diversidade Biologica-MDBio'-IB/UNICAMP: Holotype (male ZUEC NMA 31); Alotype (female ZUEC NMA 32); Paratypes (ZUEC NMA 33 – males and females).

*Hologenophores*, Deposited in the 'Museu de Diversidade Biologica- MDBio'-IB/UNICAMP, under the section number ZUEC NMA 34.

*Site of infection*: spiral valve–intestines.

*Etymology*: The Latin epithet *spinosus* is used as the specific name, based on the large number of rows of cephalic spines observed in this species.

*Representative DNA sequences*: Two sequences of partial SSU rDNA (GenBank accession number OP256110 and OP256156 and four sequences of COI mtDNA (GenBank accession number OP256158, OP256159, OP281694, and OP270220.

*ZooBank registration*: The Life Science Identifier for *E. spinosus* n. sp. is lsid:zoobank.org:pub:62FA0A09-87C8-45AC-A09F-0A55A5B436C7.

*Description*

*General*: Large nematodes with transversely striated cuticle, with rough posterior area for males (figs 2A, B, 3A and 5). Cephalic end with two large lateral pseudolabia with trilobed anterior portions (fig. 2A, C); each lobe carries two cuticular thickenings along the outer edges, which intertwine with those of the opposite pseudolabium; medial portion of each pseudolabium elongated dorsoventrally, with a pair of submedian papillae lateral and an amphids between them in the posterior region of the lobes (fig. 2C). Small triangular interlabia (one dorsal and one ventral) present between the pseudolabia. Posterodorsal and posteroventral region of each pseudolabium and interlabia lack cuticular serrations. Prominent cephalic bulb armed with 38–40 transverse rows of small, elongated spines; rows of spines close together but not overlapping; anterior and posterior rows composed of smaller spines than those in the middle portion (fig. 2A, B). Long oesophagus, 17–20% of body length, wider near its posterior end. Four cervical sacs present, extending posteriorly to about one to two-thirds of the length of the oesophagus. Well-developed deirids located symmetrically, immediately posterior the level of the nerve ring.

*Male (holotype) (based on five whole specimens and one tail fragment)*: Body 55–66 mm long by 0.6–0.9 mm maximum wide. Pseudolabia 154–184 long and 278–309 wide. Cephalic bulb 438–525 long and 605–646 wide, with 38–40 rows of cephalic spines. Nerve ring 0.8–1.0 mm from the anterior end, with 24–43 wide. Cervical sac 1.5–2.2 mm in length and 78–173 in maximum width. Oesophagus 6 to 11% of total body length, 3.6–6.2 mm in length; muscular oesophagus 1.6–3.4 mm in length, by 283–362 in width; glandular oesophagus 2.0–3.2 mm in length, by 378–535 in width. Similar spicules, equal in length, 2.7–3.6% of total body length, 1.3–1.4 mm long by 0.37 mm wide (figs 2, 3 and 5). V-shaped gubernaculum, 77.8 long. Caudal alae curved ventrally, surrounding the cloaca, 45.3 long, supporting nine pairs of caudal papillae; three precloacal pairs of unequal length, with the median pair larger and more lateral; one pair adcloacal; six postcloacal pairs, unequal in length, with the first pair extremely small at the extremity, third pair more lateral and largest, and sixth pair immediately posterior to the cloaca. Phasmids paired near tail end. Modified annulus on the ventral surface near the caudal wing, extending anteriorly. Tail 260 long (figs 3 and 5).

*Female (holotype) (based on five whole specimens and two fragmented tails)*: Body 55–85 mm long by 0.9–1.4 mm

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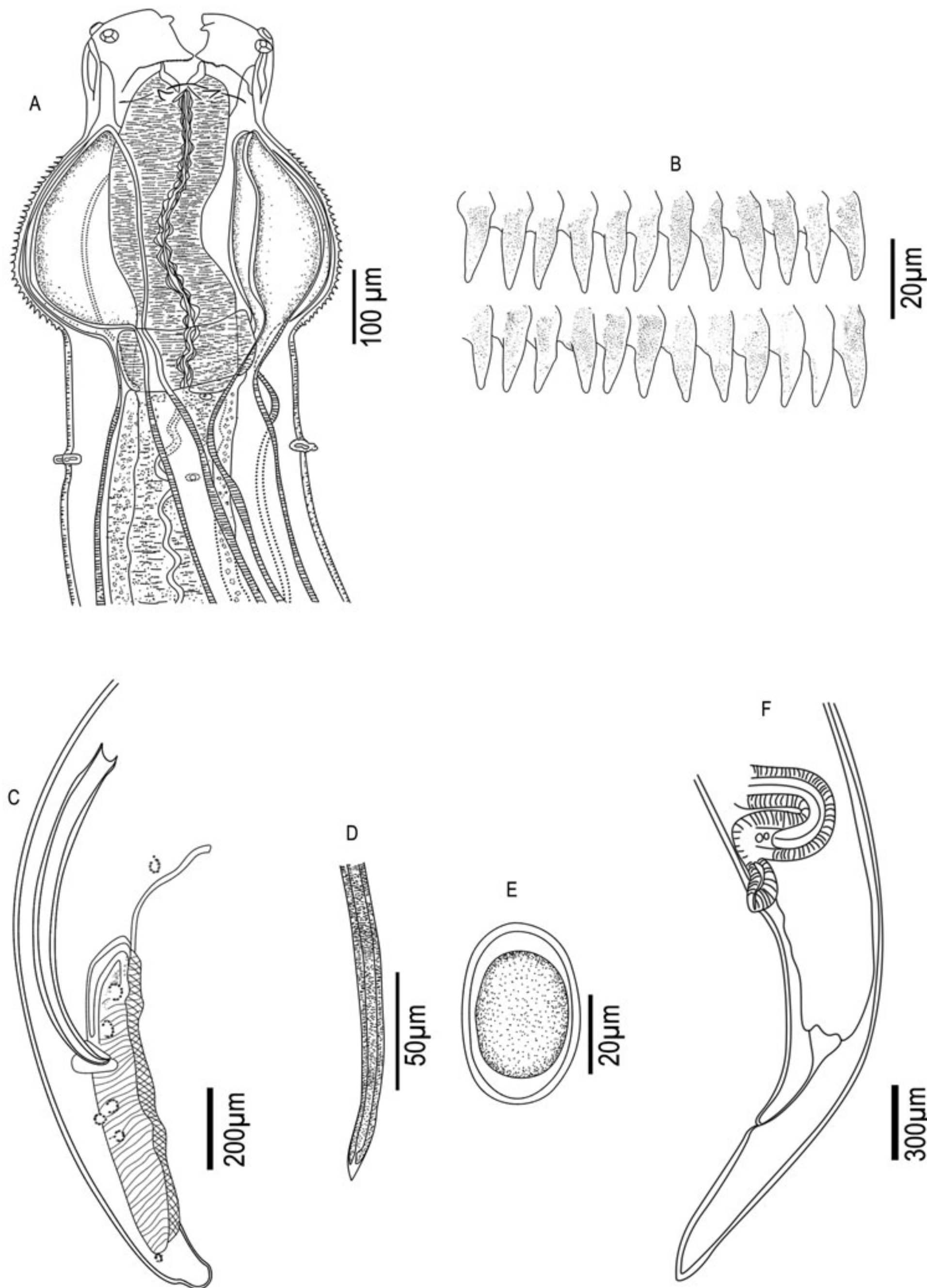


**Fig. 5.** Schematic drawing of *Echinocephalus spinosus*. n. sp. parasite of the spiral intestine of *Potamotrygon motoro*: (A) ventral view of the anterior region showing cephalic bulb and cervical zone of male. Note the muscular and glandular esophagus and cervical sacs; (B) pattern and structure of spines in the middle region of the cephalic bulb; (C) lateral view of the male caudal end; (D) spicule with hyaline tips; (E) egg; and (F) lateral view of female posterior extremity showing the vulva.

maximum wide. Pseudolabia 154–236 in length by 296–378 in width. Cephalic bulb 475–677 long by 587–803 wide, with 38–40 rows of spines. Nerve ring 0.7–1.2 mm from the anterior end, with 37–78 in width. Cervical sacs 1.9–2.8 mm in length by 60–267 in width. Oesophagus 7–11% of total body length, 5.4–6.6 mm in length; oesophageal muscle 2.8–3.6 mm in length by 166–394 in width; glandular oesophagus 2.3–3.4 mm in length by 315–551 in width. Vulva opening 779 and 1088 from the rear end. Didelphic uterus, prodelphic. Eggs with thin, smooth shell, and oval, with 38.1–41.2 long and 32.5–34.1 wide. Tail 365.3 long (figs 2, 4 and 5).

### Molecular analyses and phylogenetic study

The rDNA sequencing of specimens of *E. spinosus*. n. sp. resulted in representative DNA sequences: two sequences of partial SSU rDNA, 465 and 555 bp-long; and four sequences of COI mtDNA, 638, 654, 552, and 616 bp-long. The Basic Local Alignment Search Tool search performed with these sequences

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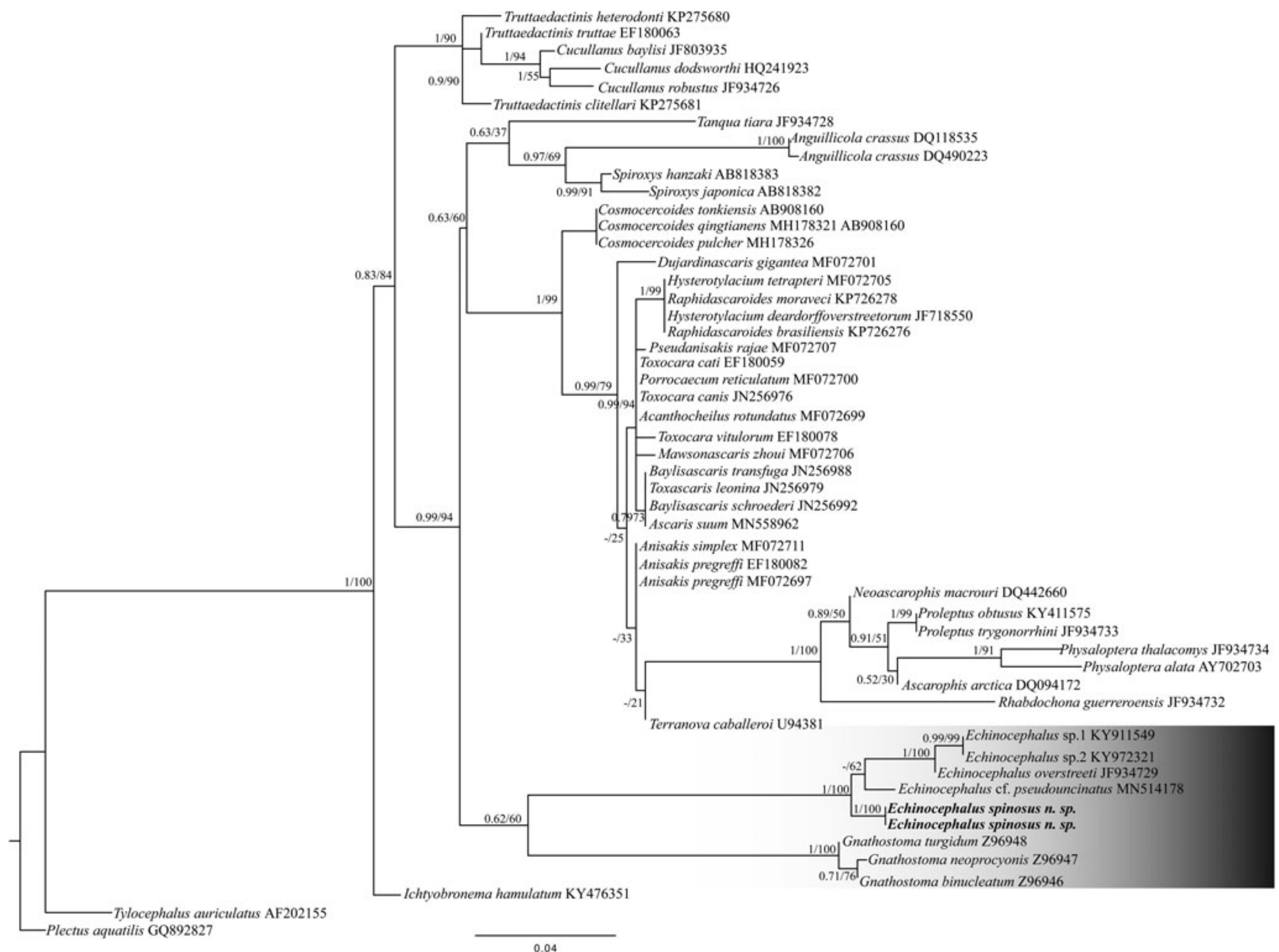


**Fig. 6.** Maximum likelihood (ML) topology based on partial small subunit ribosomal ribonucleic acid sequences of Nematoda (Spirurina). GenBank accession numbers are indicated next to species names. Numbers above nodes represent supported nodes by posterior probabilities for Bayesian analyses and bootstrap for ML analyses, respectively (posterior probabilities >0.90 and bootstrap scores >70). Branch length scale bar indicates number of substitutions per site.

did not match any other nematode available in GenBank. Genetic divergence analysis based on SSU rDNA sequences ranged from 1% to 18%, and the divergence among *Echinocephalus* species varied from 1% to 3% (supplementary table S2). When the COI was used, the genetic divergence varied from 1% to 59%. The divergences between specimens of *E. spinosus*. n. sp. and the gnathostomatids *Gnathostoma spinigerum* AB037132 and *Gnathostoma binucleatum* AB037131 were 20% to 23%, and 1% among specimens of *E. spinosus* n. sp. (supplementary table S3).

The analysis of the alignment of the SSU rDNA sequences comprising 471 bp characters and 26 sequences (supplementary table S1) in the DAMBE revealed lower ISS values than critical Iss assuming a symmetrical topology (Iss.cAsym) and no saturation in either transitions and transversions in both asymmetrical (Iss.cSym) values, revealing the lack of a saturation signal in the matrix. Both BI and ML phylogenetic inferences converged in similar topologies and recovered the *Echinocephalus* lineage as the sister of *Gnathostoma*. In the *Echinocephalus* lineage, the sequence of *E. spinosus*. n. sp. arises as a sister of that of *Echinocephalus* cf. *pseudouncinatus* (MN514178), which was obtained from larvae stage parasitizing the mollusc *Atrina maura* from Mexico (Karagiorchis *et al.*, 2022) (fig. 6). The divergence time analysis revealed that *E. spinosus* n. sp. diverged from its ancestor at around 26.54 Ma, with a confidence interval of 31.54–21.54 Ma (fig. 7).

## Discussion

Within the gnathostomatids, as far as we know, the occurrence of adult stages of species of the genus *Echinocephalus* are restricted to elasmobranch hosts, mainly rays/stingrays (Moravec & Justine, 2021). Only *E. diazi* and *E. daileyi* have so far been described in freshwater hosts, both from potamotrygonin stingrays from the north of South America. However, there is a lack of consensus over the real freshwater occurrence of *E. diazi*, as it was initially described from a species identified as *P. hystrix,* caught in the estuarine Lake Maracaibo (Troncy, 1970), and subsequently twice reported infecting the marine ray *S. schmardae* (Deardorff *et al.*, 1981), a myliobatiform now recognized in the subfamily Styracurinae in Potamotrygonidae (Carvalho *et al.*, 2016). This suggests that *E. diazi* is a marine, or at least, marine/brackish organism. Thus, Deardorff *et al.* (1981), when describing *E. daileyi* from the Amazon and Orinoco River basins, attested to it being the first member of the genus *Echinocephalus* reported to infect stingrays inhabiting a freshwater environment, *sensu stricto*. Following this

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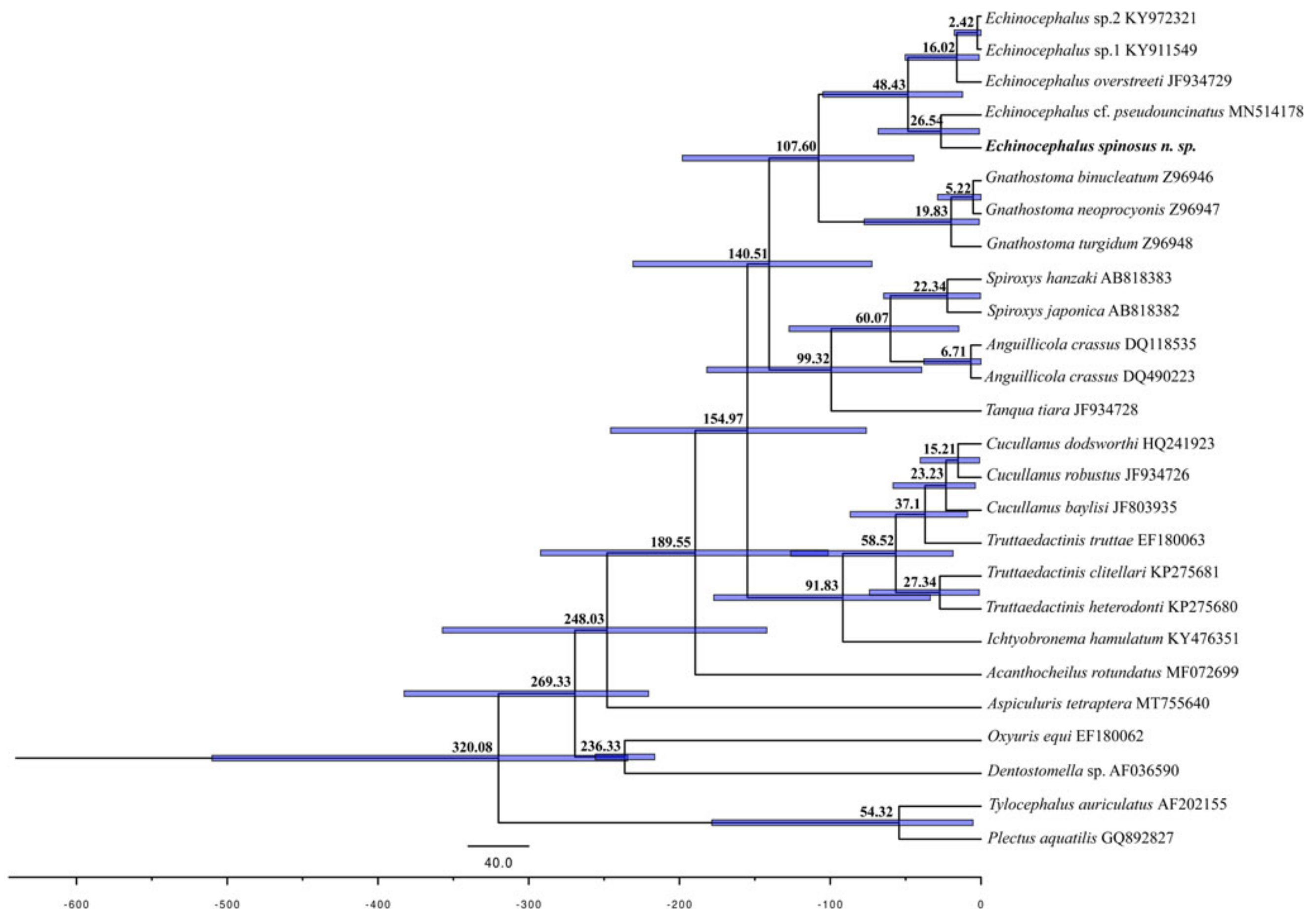


**Fig. 7.** Estimated divergence time for nematode species. The blue bars in the nodes indicate 95% higher posterior density (HPD) of the posterior Bayesian distribution of molecular time estimates. The geological time scale is in accordance with the International Chronostratigraphic Chart (http://www.stratigraphy.org, v2020/01).

conception, *E. spinosus* n. sp., now described infecting *P. motoro* from the Tapajós River, is only the second *Echinocephalus* species found parasitizing a stingray habiting a *sensu stricto* freshwater environment, also in the north of South America.

Based on the keys proposed by Moravec & Justine (2021) and Saad *et al.* (2022), *E. spinosus* n. sp. and *E. daileyi* differ in the number of transverse rows of spines in the cephalic bulb, with 30–32 for *E. daileyi,* and 38–40 for the new species, and in spicule length, with 1.4–2.4 mm in *E. daileyi* and 1.3–1.9 mm in *E. spinosus* n. sp. When compared to *E. diazi*, *E. spinosus* n. sp. differs in the number of transverse rows of spines in the cephalic bulb (respectively, 25 and 38–40 rows), in having shorter spicules (respectively, 2 and 1.3–1.9 mm), and in number of pairs of pre-anal papillae (two and three, respectively). Regarding the species described infecting marine hosts, besides of the difference of environment (marine *vs.* freshwater), they differ from *E. spinosus* n. sp. by the number of transverse rows of spines in the cephalic bulb, length of the spicules or number of pairs of caudal papillae (Moravec & Justine, 2021; Saad *et al.*, 2022).

Accurate identification of species is essential for studies of diversity (Blasco-Costa *et al.*, 2014), and molecular analyses can provide important insights in this area. Thus, in addition to morphology, we also provided molecular analysis based on SSU rDNA (fig. 6) and COI (supplementary fig. S1, supplementary table S3) sequences of *E. spinosus* n. sp. These data showed that the interspecific nucleotide variation between species of *Echinocephalus* varied from 1% to 3% for the SSU rDNA (supplementary table S2). Unfortunately, there were no *Echinocephalus* spp. sequences available for the COI gene to compare our data to (supplementary fig. S1). Previous molecular phylogenetic studies using the SSU ribosomal gene have demonstrated a high degree of sequence similarity among a subset of the *Echinocephalus* species (van Megen *et al.*, 2009; Abdel-Ghaffar *et al.*, 2013). Abdel-Ghaffar *et al.* (2013) used the portion of the SSU gene + internal transcribed spacer (ITS) and verified *Echinocephalus carpiae* Abdel-Ghaffar *et al.*, 2013), described from larvae obtained from *Cyprinus carpio* from Egypt, had divergence of 7% to *E. overstreeti.* Later, based on SSU rDNA Karagiorchis *et al.* (2022) demonstrated that the difference was of only 3.1%, and proposed *E. carpiae* as a junior synonym of *E. overstreeti.* As expected, the SSU rDNA gene is more conserved than the ITS region, and presented lower divergence, being robust for the separation of species, and several authors have used it to distinguish species and infer phylogenies of nematodes (Dare *et al.*, 2008; Dubey & Shine, 2008; Langford & Janovy, 2013; Karagiorchis *et al.*, 2022).

Phylogenetic analyses carried out by Laetsch *et al.* (2012) showed *E. overstreeti* emerging as a sister lineage of *Anguillicola* spp. (Anguillicolidae). Nonetheless, when adding four SSU rDNA sequences of *Echinocephalus* species downloaded from GenBank, plus that provided in the present study, our analyses

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recovered a lineage of gnathostomatids with *Echinocephalus* species arising as a sister of *Gnathostoma* spp. However, another gnathostomatid, *Tanqua tiara* von Linstow, 1879, appears as a sister species of the lineage composed of the *Anguillicola* and *Spiroxys* species (Anguillicoliidae), corroborating the absence of the monophyly of gnathostomatids observed by Laetsch *et al.* (2012) (fig. 6). This profile seems to be reflected in the radiation of these nematode lineages into distinct groups of definitive hosts – that is, mammals (*Gnathostoma*), squamates (*Tanqua*), and marine and freshwater elasmobranchs (*Echinocephalus*), as well as the diversity of their intermediate and paratenic hosts (Laetsch *et al.*, 2012).

Our phylogeny using COI also recovered *E. spinosus* n. sp. and *Gnathostoma* spp. as being closely related, but without good resolution and bootstrap and posterior probability support (supplementary fig. S1). These results may be related to the high genetic divergence of this marker (supplementary table S3) and the lack of other related sequences, specially of the gnathotomatid genera available in the GenBank database. As proposed by Laetsch *et al.* (2012), future studies providing new COI sequences of gnathostomatids and of other related nematodes may bring further insights into this marker in the study of its evolution.

To the best of our knowledge, there are no reports of freshwater *Echinocephalus* species in other continents. Despite this being only the second description of an *Echinocephalus* species parasitizing Neotropical potamotrygonins (the first was *E. daileyi*), it can be hypothesized that the radiation of this gnathostomatid lineage in South America followed *sensu stricto* freshwater stingray. This hypothesis is endorsed by the report of *Echinocephalus* sp., not identified at a species taxonomic level, parasitizing the largespot river stingray *P. falkneri* from the upper Paraná River basin, in the south of South America (Lacerda *et al.*, 2009).

Compared to bony fishes, which have comparable species richness in marine and freshwater habitats (Levêque *et al.*, 2008), few cartilaginous fish species – mainly freshwater stingrays – were able to colonize permanently freshwater environments (Nelson *et al.*, 2016). According to Kirchhoff *et al.* (2017), stingrays colonized freshwater environments independently at least four times, and marine incursions had a pivotal role in their adaptation to this habitat. Neotropical potamotrygonins, meanwhile, are exclusively freshwater stingray, occurring in the main South American watersheds, with the greatest diversity in the Amazon (Carvalho *et al.*, 2003; Silva & Loboda, 2019; Fontenelle *et al.*, 2021; Loboda *et al.*, 2021).

During its geological history, South America has been the setting for several marine incursions (Räsänen *et al.*, 1995; Lundberg *et al.*, 1998), which were the scenario for the emergence of organisms of marine derived lineages, now endemic to freshwater habitats of this continent (i.e. anchovies, drum fish, stingrays, dolphins, manatees, sponges, crustaceans, molluscs and parasites) (Nuttall, 1990; Cassens *et al.*, 2000; Hamilton *et al.*, 2001; Boeger & Kritsky, 2003; Bloom & Lovejoy, 2017; Cavalcanti *et al.*, 2019; Adriano *et al.*, 2021; Oliveira *et al.*, 2021; Zatti *et al.*, 2022). In this context of marine derived lineages, several studies have hypothesized the emergence of South American stingrays (Lovejoy *et al.*, 1998, 2006; Carvalho *et al.*, 2004; Adnet *et al.*, 2014; Bloom & Lovejoy, 2017; Kirchhoff *et al.*, 2017). Fontenelle *et al.* (2021) generated a time-calibrated phylogeny including 35 Neotropical freshwater stingrays and showed that the divergence between the freshwater potamotrygonins and the marine Styracurinae was estimated at 26.4 Ma (ranging 32.1–20.6 Ma). These data place the origin of the potamotrygonins into the late Oligocene to early Miocene, when the western Amazon was dominated by the Pebas wetlands – an epicontinental marine/freshwater system covering more than one million km$^2$, from where the diversification of potamotrygonins would have occurred (Fontenelle *et al.*, 2021). This time estimation is corroborated by the recent find of stingray teeth fossils from the Oligocene–Miocene deposits, from Contamana, Peru (Chabain *et al.*, 2017), the age of which matches the molecular results presented by Fontenelle *et al.* (2021).

Corroborating the late Oligocene to early Miocene origin of potamotrygonins, our time-calibrated phylogenetic analysis revealed the divergence between the freshwater *E. spinosus* n. sp. and its ancestor to be 26.5 Ma (ranging 31.5–21.5 Ma), placing the freshwater *Echinocephalus* lineage origin into the same geological scenario revealed by Fontenelle *et al.* (2021) for potamotrygonins. Associated with the results of Chabain *et al.* (2017) and Fontenelle *et al.* (2021), our data indicate that the Pebas wetlands seems to have provided adequate conditions for the transitions from marine to freshwater life of this host–parasite complex. Later, the co-radiation of stingrays/*Echinocephalus* occurred in the continent. Studies on gnathostomatid parasites of South American freshwater stingrays from different watersheds could result in fascinating new insights into the co-radiation/co-speciation of this host–parasite complex in the context of the geological history of South America.

In conclusion, the present study demonstrates that combining morphological-light and scanning electron microscopy, and DNA based phylogenetic analyses are suitable for the diagnosis of gnathostomatids. The time-calibrated phylogeny analysis provided support for a scenario of the co-emergence of freshwater potamotrygonins/*Echinocephalus* during a geological time that corresponds to the period of occurrence of the Pebas wetlands in the western Amazon.

Associated with recent biogeographical studies into potamotrygonins, the discovery of new *sensu stricto* freshwater *Echinocephalus* species suggests that the origin of the freshwater potamotrygonins/*Echinocephalus* was followed by the co-radiation of this host–parasite complex across the continent, opening avenues for further evolutionary studies involving this fascinating and complex host–parasite–geological history.

**Authors' contributions.** M.I.M. and L.L.C, conceived the initial idea for the study and undertook sampling, performed morphological, and molecular analysis . M.I.M., M.S.B.O. and E.A.A., performed phylogenetic analyses and time estimation . M.I.M. and L.L.C., participated in the writing of the draft manuscript confection. Project administration, supervision, funding acquisition, revision and the organization of the manuscript was conducted by E.A.A. All authors read the manuscript and gave final approval for publication.

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**Ethical standards.** None.

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