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First molecular assessment on *Cosmocerca* spp. from Brazilian anurans and description of a new species of *Cosmocerca* (Ascaridomorpha: Cosmocercoidea) from the white-spotted humming frog *Chiasmocleis albopunctata* (Boettger, 1885) (Anura: Microhylidae)

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Abstract

Cosmocerca spp. are common nematode parasites of amphibians. We provide herein molecular data for two species of *Cosmocerca* and describe a new species, *Cosmocerca albopunctata* n. sp., using light microscopy and molecular data (cytochrome c oxidase subunit 1 – COI mtDNA). *Cosmocerca albopunctata* n. sp. can be easily distinguished from other congeneric species by the combination of characteristics such as body size, length of spicules and gubernaculum, and the arrangements and number of caudal papillae (7 + 1:1 + 1:6). The phylogenetic results based on the partial COI mtDNA sequences clustered the new species in a monophyletic clade along with the other sequences of *Cosmocerca* spp. Therefore, our results contribute to the knowledge about the species diversity and genetic data for *Cosmocerca* spp. in the Neotropical region.

Introduction

Nematodes of the genus *Cosmocerca* Diesing, 1861 (Ascaridomorpha: Cosmocercoidea) are cosmopolitan parasites found in the digestive tract of several amphibians (see Campião *et al.*, 2014; Bursey *et al.*, 2015; Sou *et al.*, 2018a, b; Chen *et al.*, 2020; Ni *et al.*, 2020; Harnoster *et al.*, 2022; Machado *et al.*, 2022) and some lizards (Bursey & Goldberg, 2004; Bursey *et al.*, 2015; Ávila & Silva, 2019). To date, 37 species of *Cosmocerca* have been reported worldwide (Bursey *et al.*, 2015; Sou *et al.*, 2018a; Ávila & Silva, 2019; Banerjee & Sou, 2020; Chen *et al.*, 2020; Ni *et al.*, 2020; Harnoster *et al.*, 2022) and only 11 occur in the Neotropical region: *Cosmocerca brasiliensis* Travassos, 1925; *Cosmocerca chilensis* Lent & Freitas, 1948; *Cosmocerca cruzi* Rodrigues & Fabio 1970; *Cosmocerca gymnophthalmicola* Ávila & Silva, 2019; *Cosmocerca parva* Travassos 1925; *Cosmocerca podicipinus* Baker & Vaucher, 1984; *Cosmocerca paraguayensis* Moravec & Kaiser, 1994; *Cosmocerca rara* Freitas & Vicente 1966; *Cosmocerca travasssosi* Rodrigues & Fabio, 1970; *Cosmocerca uruguayensis* Lent & Freitas, 1948; and *Cosmocerca vrcibradici* Bursey & Goldberg 2004 (Bursey *et al.*, 2015; Ávila & Silva, 2019).

Despite the increasing number of studies regarding the superfamily Cosmocercoidea in the last 10 years (Ross *et al.*, 2010; Jones *et al.*, 2011; Sato *et al.*, 2015; Tran *et al.*, 2015; Chen *et al.*, 2018, 2020, 2021a, b; Maity *et al.*, 2019; Sümer *et al.*, 2019; Liu *et al.*, 2020; Sata & Nakano, 2020; Harnoster *et al.*, 2022), the current knowledge about the molecular phylogeny of the family is still scarce (Chen *et al.*, 2021a). To date, molecular data for only 39 species of Cosmocercoidea, of which 23 species are of the subfamily Cosmocercinae (18S, 28S, ITS, 12S, Cox1 and Cox2), are available in the GenBank database. Among these sequences, only 10 *Cosmocerca* spp. have been accessed: *Cosmocerca ornata* Dujardin, 1845; *Cosmocerca simile* Chen, Zhang, Feng & Li, 2021; *Cosmocerca japonica* Yamaguti, 1938; *Cosmocerca longicauda* (Linstow, 1885); *Cosmocerca kalesari* Rizvi, Bursey & Bhutia, 2011; *Cosmocerca daly* Harnoster, du Preez & Svitin, 2022; *Cosmocerca monicae* Harnoster, du Preez & Svitin,

Table 1. Data for the anurans sampled from 2018 to 2020 in the municipalities of Mato Grosso (MT) and São Paulo (SP) states, Brazil, and surveyed for *Cosmocerca* species (NH = number of hosts).

Species	Host	NH	Locality
Cosmocerca albopunctata sp. n.	*Chiasmocleis albopuntacta*	1	Santa Júlia farm, Gavião Peixoto, SP
Cosmocerca parva	*Leptodactylus podicipinus*	2	Carandá farm, Araraquara, SP and São Sebastião do Paraíso farm, Boa Esperança do Sul, SP
	Dendropsophus nanus	1	Santa Júlia farm, Gavião Peixoto, SP
Cosmocerca podicipinus	*Leptodactylus latrans*	2	Cocalinho, MT
	Boana caiapo	1	Cocalinho, MT
	Physalaemus centralis	1	Cocalinho, MT
	Pseudis platensis	1	Cocalinho, MT

2022; *Cosmocerca makhadoensis* Harnoster, du Preez & Svitin, 2022; *Cosmocerca* sp.1; and *Cosmocerca* sp.2. Notwithstanding, none of these is from the Neotropical region.

During a helminthological survey of Brazilian anurans in the São Paulo and Mato Grosso states, some Cosmocercinae nematodes were collected. By using light microscopy and molecular analyses of the mitochondrial DNA (cytochrome c oxidase subunit 1 – COI mtDNA), we were able to accurately characterize and determine the systematic position of those nematodes. This study reports the first molecular assessment of *C. podicipinus* and *C. parva* and describes a new species of *Cosmocerca*.

Materials and methods

Nematode identification and DNA extraction

Cosmocerca specimens from nine anurans' hosts from Mato Grosso and São Paulo states were collected (table 1). The frogs were killed with 2% lidocaine hydrochloride and the internal organs were removed, dissected, and analysed under a stereomicroscope. Nematodes were collected from the intestines of the anurans and stored directly in 96% ethanol. Each nematode found was cut and the anterior part was used for molecular analysis and the posterior one was used for morphological studies. For the species identification, the morphology of the posterior end of the nematodes was compared with all published *Cosmocerca* spp.

The posterior end of the nematode specimens was cleared with lactophenol, mounted in temporary slides, and analysed under a microscope with a computerized image analysis system (Qwin Lite 3.1, Leica Microsystems, Wetzlar, Germany). Morphological measurements are given in micrometres and presented as range with values for holotype indicated in parentheses (only for the description of the new species).

Drawings were made using a camera lucida attached to a Leica DMLS microscope with phase contrast optics. The anterior end of 11 males and one female of the analysed worms were separated for molecular analyses.

After morphological identification, DNA extractions were performed using the anterior end of the specimens with DNeasy® Blood & Tissue Kit (QIAGEN) according to the manufacturer's protocol. The partial COI mtDNA was amplified by polymerase chain reaction (PCR) using the primers: COIF (5′-TTTTTTG GTCATCCTGAGGTTTAT-3′) and COIR (5′-ACATAATGAAA ATGACTAACAAC-3′) (Lazarova *et al.*, 2006). The cycling conditions followed Chen *et al.* (2018).

The PCR reactions were performed using 3 µl of DNA extract, 1 µl of each primer, 7.5 µl or 8.5 µl of ultrapure water (Sigma-Aldrich, United Kingdom), and 12.5 µl Master Mix MyFiTM Mix Bioline®, with a final volume of 25 µl. PCR products were subjected to gel electrophoresis at 80 V in a 1.5% agarose gel, stained with Gel Red, and observed using an ultraviolet transilluminator. The products of interest were purified by adding 2 µl of ExoSAP-IT® (Affymetrix, Santa Clara, CA, USA) to 5 µl of PCR product according to the manufacturer's recommendations. Amplicons were sequenced using PCR primers on a 3500 Genetic Analyzer capillary sequencer (Applied Biosystems) using BigDye Terminator Cycle Sequencing Ready Reaction Kit v.3.1 (Applied Biosystems) according to the manufacturer's recommendations.

The newly generated sequences were assembled and edited using Sequencher v.5.2.4 (Gene Codes, Ann Arbor, MI, USA), and subjected to the Basic Local Alignment Search Tool algorithm available in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov) for preliminary identification.

Phylogenetic analyses

The newly generated sequences were aligned along with other Cosmocercidae sequences retrieved from the GenBank database. *Falcaustra sinensis* Liu, Zhang & Zhang, 2011 [MF113223] and *Falcaustra* sp. [MN729572] were used as the outgroup for the alignment.

The alignment was performed using the default parameters of the algorithm Muscle (Edgar, 2004) implemented on Geneious 7.1.3 (Kearse *et al.*, 2012). The best-fitting model of nucleotide substitution for the dataset was Hasegawa–Kishino–Yano + G + I, selected in the JModelTest software (Posada, 2008) using the Akaike information criterion.

Phylogenetic trees were obtained using maximum likelihood (ML) and Bayesian inference (BI). The BI was performed with MrBayes 3.2 (Ronquist *et al.*, 2012) at the online platform CIPRES. The Markov chain Monte Carlo was run with 50,000,000 generations saving one tree every 1000 generations, with a burn-in of 25% of the trees. Only nodes with posterior probabilities greater than 90% were considered well supported. Phylogenetic analysis using ML was run in RAxML (Guindon & Gascuel, 2003) at the online platform CIPRES with 1000 bootstrap replicates. Only nodes with bootstrap values greater than 70% were considered well supported. The ML and BI trees were visualized and edited in FigTree v. 1.3.1 software (Rambaut, 2009).

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The COI mtDNA genetic divergence between sequences was calculated using the Kimura 2-parameter model with 1000 bootstrap replicates in MEGA 7 software (Kimura, 1980).

Results

Cosmocerca parva Travassos 1925

Three male specimens of *C. parva* were found in the large intestine of two *Leptodactylus podicipinus* (Cope, 1862) and one *Dendropsophus nanus* (Boulenger, 1889) from the São Paulo state.

Numerous somatic papillae lined up in two ventral, two subventral, two dorsal and two subdorsal rows. Caudal alae absent. Narrow lateral alae present spicules equal to 80.3–108.5 long, weakly sclerotized, gubernaculum well sclerotized, Y-shaped, 90.4–133.5 long. Cloacal papillae arranged in 5:3 + 1:7, as follows: five pairs of plectanes, each one with two complete rosettes and a relatively inconspicuous underlying sclerotized support in the precloacal region with three pairs of rosette papillae in the adcloacal region and one large unpaired papilla in the anterior lip of cloaca; and seven pairs of postclocal (six ventral pairs and one lateral) papillae. Tail 218.9 long, ending in a small spike.

Cosmocerca podicipinus Baker & Vaucher, 1984

Seven specimens (5 males, 2 females) of *C. podicipinus* were found in the large intestine of a *Boana caiapo* Pinheiro, Cintra, Valdujo, Silva, Martins, Silva & Garcia, 2018, two *Leptodactylus latrans* (Steffen, 1815), one *Physalaemus* centralis (Bokermann, 1962) and one *Pseudis platensis* Gallardo, 1961 from Mato Grosso state.

Numerous somatic papillae lined up in two ventral, two subventral, two dorsal and two subdorsal rows. Caudal alae absent. Narrow lateral alae present. Posterior end of body distinctly ventrally curved. Spicules small and equal, well sclerotized, with distal end pointed, 73.1–109.6 long, gubernaculum small and conical, well sclerotized, 96.2–124.5 long. Cloacal papillae arranged in 5:3 + 1:6, as follows: five pairs of plectanes, each with two complete rosettes of punctations directed perpendicular to the body surface and a relatively inconspicuous underlying sclerotized support that is not fused to other plectanes in the precloacal region; three pairs of broad and flat papillae which are commonly surrounded by a small rosette of punctations in the adcloacal region and one large unpaired papilla in the anterior lip of cloaca; and six pairs of postclocal, large, and simple papillae (distinguishable from somatic papillae). Tail 139.2–225.6 long, ending in a small spike.

Cosmocerca albopunctata n. sp. Alcantara and Silva

Small-sized, whitish nematodes. Body cylindrical, maximum width at about mid-body. Cuticle with fine transverse striations (figs 1–3). Numerous somatic papillae lined up in two ventral, two subventral, two dorsal and two subdorsal rows. Caudal alae absent. Narrow lateral alae present. Oesophagus divided into anterior pharynx, cylindrical corpus, isthmus and terminal posterior bulb with valves. Nerve ring located at about 1/2 of oesophageal length. Excretory pore posterior to oesophageal bulb. Deirids not observed. Tail conical, with pointed tip.

Male (3 mature specimens, 1 entire and two posterior ends): Body 6250 long; maximum width 380.1. Oesophagus 304.6 long, pharynx + corpus + isthmus 223.5 long; oesophageal bulb 81.1 × 57.1. Nerve ring 233.5 and excretory pore 533.3 from anterior extremity, respectively. Lateral alae extending from oesophageal bulb to cloaca region. Posterior end of body distinctly ventrally curved. Spicules small and equal, well sclerotized, with distal end pointed, 89.6–99.8 (89.6) long. Gubernaculum small and conical, well sclerotized 119.7–157.8 (155.4) long. Cloacal papillae arranged in 7 + 1:1 + 1:6, as follows: seven pairs of plectanes and one unpaired small rosette between the cloaca and the first pair of plectane in the precloacal region; plectane consisting of central papilla with two rows of 10 cuticular tubercles on underlying sclerotized segments; one large unpaired papilla in the anterior lip of anus and one large unpaired papilla in the posterior lip of anus; and six pairs of postclocal, large, and simple papillae (four ventral pairs, one lateral pair, and one dorsal pair). Tail 191.4–266.6 (253.7) long, ending in a small spike.

Female: Unknown.

Taxonomic summary

Type host: White-spotted humming frog *Chiasmocleis albopunctata* (Boettger, 1885) (Anura: Microhylidae, Gastrophryninae).

Type locality: Santa Júlia farm, municipality of Gavião Peixoto (21°50′43.74″S; 48°28′43.43″W), São Paulo state, Brazil.

Site of infection: Large intestine.

Level of infection: Three specimens of *C. albopunctata* n. sp.

Type specimens: Holotype and two paratypes (Helminthological Collection of the Oswaldo Cruz Institute (CHIOC) of Rio de Janeiro. The accession number of the holotype CHIOC number 39361a (male) and accession numbers of paratype CHIOC number 39361b-c (two male)).

Etymology: The epithet *albopunctata* is derived from the name of the host

of the new species.

GenBank Accession number: (accession numbers: OP153854 and OP153856).

Molecular characterization

The three newly generated COI mtDNA sequences of *C. parva* were 413, 426 and 428 base pairs (bp) in length, and showed no genetic divergence among them. The seven newly generated COI mtDNA sequences of *C. podicipinus* were 400, 414, 415, 419, 423, 424 and 426 bp in length, and ranged from 98.2%–100% of similarity among them. The two newly generated COI mtDNA sequences of *C. albopunctata* n. sp. were 426 and 427 bp in length and presented 99.1% of similarity between them (supplementary table 1).

The similarity matrix of COI mtDNA sequences from *Cosmocerca* species showed that the lowest genetic distance (pairwise distance) in *C. parva* was 11.4%–12.3% compared with *C. podicipinus*, and the greatest genetic distance was 23.8%–25.7% compared with *C. albopunctata* n. sp. Pairwise comparison of *C. podicipinus* with *C. albopunctata* n. sp. displayed 25.3%–28.6% of nucleotide divergence (supplementary table 1). The COI mtDNA sequences of *Cosmocerca* spp. are deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) (accession numbers: OP153854-OP153865).

Both phylogenetic reconstructions inferred by ML and BI methods recovered all sequences of *Cosmocerca* in a monophyletic clade (pp = 0.61; bootstrap: 83), with the sequences of *Cosmocercoides* as their sister group (pp = 1; bootstrap: 100). *Cosmocerca* spp. sequences were divided into two supported clades: one comprising all sequences of *C. podicipinus* and *C. parva* (pp = 1; bootstrap: 100); and another with the sequences

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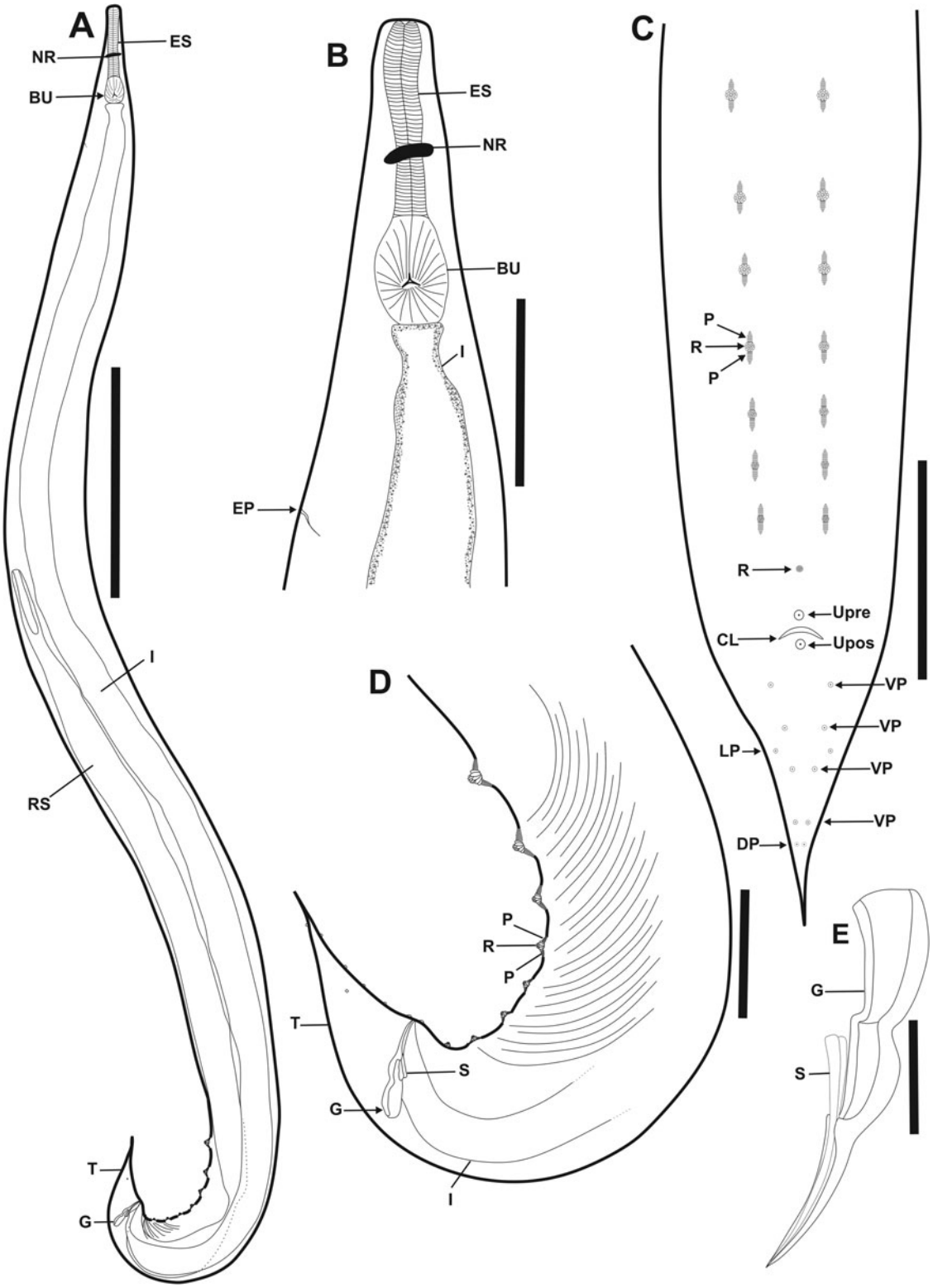

Fig. 1. Male of *Cosmocerca albopunctata* n. sp.: (A) body, lateral view; (B) anterior end, lateral view; (C) posterior end, ventral view; (D) posterior end, lateral view; and (E) detail of spicules and gubernaculum. B = oesophageal bulb; CL = cloaca; DP = dorsal papilla pair; EP = excretory pore; ES = oesophagus; G = gubernaculum; I = intestine; LP = lateral papilla pair; NR = nerve ring; P = plectane; R = rosette; RS = reproductive system; S = spicule; T = tail; VP = ventral papilla pair; Upos = unpaired postclocal papilla; and Upre = unpaired preclocal papilla. Scale bars: 1 mm (A); 200 μm (B–D); and 20 μm (E).

of *C. albopunctata* n. sp., *C. ornata*, *C. simile, Cosmocerca* sp. (MT108305) and *C. japonica* (pp = 0.98; bootstrap: 87) (fig. 4). In this last clade, the sequences of *C. japonica* were positioned into two separate clades, with low support by both analyses.

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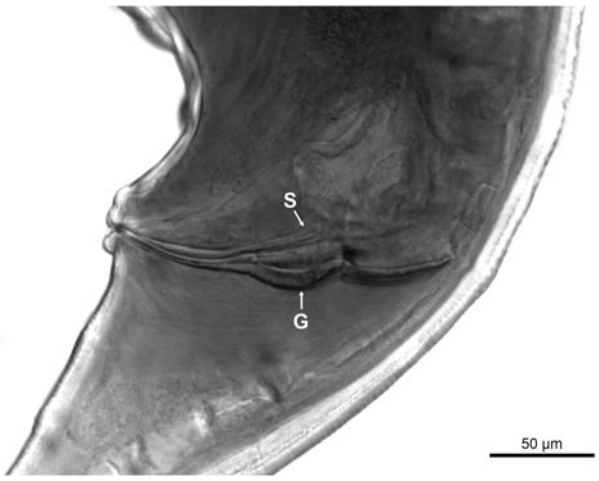

Fig. 2. Enlarged view of the cloacal region of *Cosmocerca albopunctata* n. sp. showing spicules (S) and gubernaculum (G).

Discussion

Cosmocerca is characterized by the presence of two spicules, usually weakly sclerotized or rudimentary (Gibbons, 2010), absence of tail alae, presence of rosette papillae and plectanes in males, and presence of somatic papillae and two prodelphic ovaries in females. The recognition of these combined characteristics allowed us to allocate our specimens as *Cosmocerca*.

Cosmocerca albopunctata n. sp. can be easily distinguished from its congeneric species by a combination of characters, such as the body size, length of spicules and gubernaculum, and the number and arrangements of the caudal papillae (7 + 1:1 + 1:6). In addition, in the new species, the position of the excretory pore is post-bulbar. Bursey *et al.* (2015) have reported this type of arrangement in *Cosmocerca longispicula* Moravec and Kaiser, 1994 from the Panamanian region, *C. longicauda* (Linstow, 1885) from the Palaearctic region and *Cosmocerca leytensis* Bursey *et al.*, 2015 from the Oriental region. However, according to these authors, the excretory pore in the anterior ends of males and females of *Cosmocerca* spp. are rarely illustrated in a species description, which makes it difficult to affirm if this characteristic is considered a common feature in *Cosmocerca* spp.

To date, 37 species of *Cosmocerca* have been reported worldwide. Among these species, *C. parva*, *C. uruguayensis*, *C. vrcibradici*, *C. commutata* (Diesing, 1851) and *C. longispicula* present seven pairs of plectanes (Bursey *et al.*, 2015) as in *C. albopunctata* n. sp.

Among the above-mentioned species, *C. parva* was originally described from *Hylodes nasus* (Lichtenstein, 1823) (=*Helosia nasus*), from the municipality of Angra dos Reis, Rio de Janeiro state, Brazil (Travassos, 1925). This species has been reported from dendrobatids, leptodactylids, bufonids, hylids, microhylids, hylodids, odontophrynid, craugastorids and phyllomedusids in Argentina, Brazil, Colombia, Guiana, Paraguay and Peru (Campião *et al.*, 2014). *Cosmocerca parva* is distinguished from the new species by the presence of two to four pairs of papillae which are commonly surrounded by one or two small rosettes of punctations in the adcloacal region (Mordeglia & Digiani, 1998), while in the *C. albopunctata* n. sp. they are absent.

Cosmocerca uruguayensis Lent & Freitas, 1948 was originally described from *Odontophrynus americanus* (Duméril and Bibron, 1841) (= *Ceratophrys americana*) in Montevideo, Uruguay (Lent & Freitas, 1948) and it has also been reported in Venezuela (Campião *et al.*, 2014). *Cosmocerca uruguayensis* could be easily distinguished from the new species by the absence of lateral alae, while in *C. albopunctata* n. sp. this characteristic is present.

Cosmocerca vrcibradici was originally described from the intestine of the lizard *Cercosaura eigenmanni* (Griffin, 1917) (= *Prionodactylus eigenmanni*) in Rondônia state, Brazil (Bursey & Goldberg, 2004). The species has already been registered in *Alopoglossus angulatus* Linnaeus, 1758, *Alopoglossus atriventris* Duellman, 1973, *Norops fuscoauratus* D'Orbignyi, 1837, *Cercosaura oshaughnessyi* (Boulenger, 1885) and *Uranoscodon superciliosus* Linnaeus, 1758 (Ávila & Silva, 2010). *Cosmocerca vrcibradici* differs from *C. albopunctata* n. sp. by possessing larger spicules (171–183 vs. 98.2–99.8) (Bursey & Goldberg, 2004).

Cosmocerca commutata was originally described from the intestine of *Bufotes viridis* (Laurenti, 1768) (= *Bufo viridis*) (Diesing, 1851). This species is commonly found in frogs (see Koyun *et al.*, 2013). *Cosmocerca commutata* differs from *C. albopunctata* n. sp. by possessing larger spicules (180 vs. 98.2–99.8) and gubernaculum (186–213 vs. 119.7–157.8) (Bursey *et al.*, 2015).

Cosmocerca longispicula was originally described from the intestine of *Colostethus* sp. from France (Moravec & Kaiser, 1994).

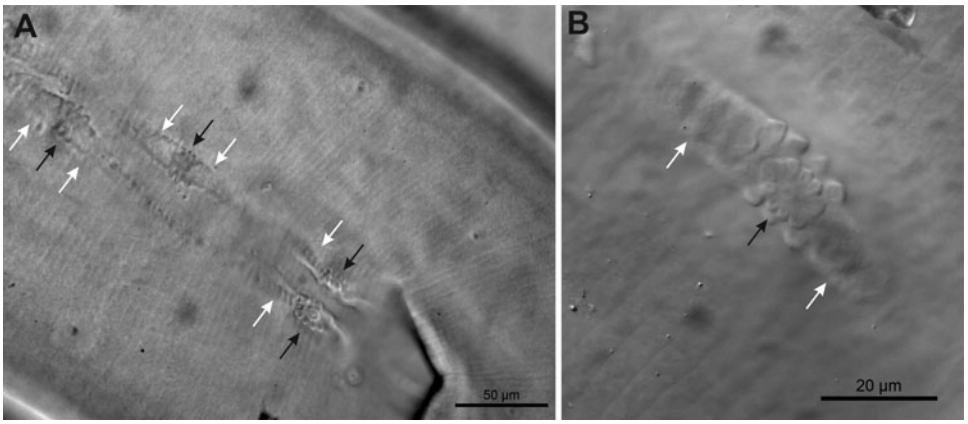

Fig. 3. (A) Part of the precloacal region of the male of *Cosmocerca albopunctata* n. sp. showing rosettes (black arrows) and plectanes (white arrows); and (B) detail of preclocal rosette (black arrow) and plectanes (white arrow).

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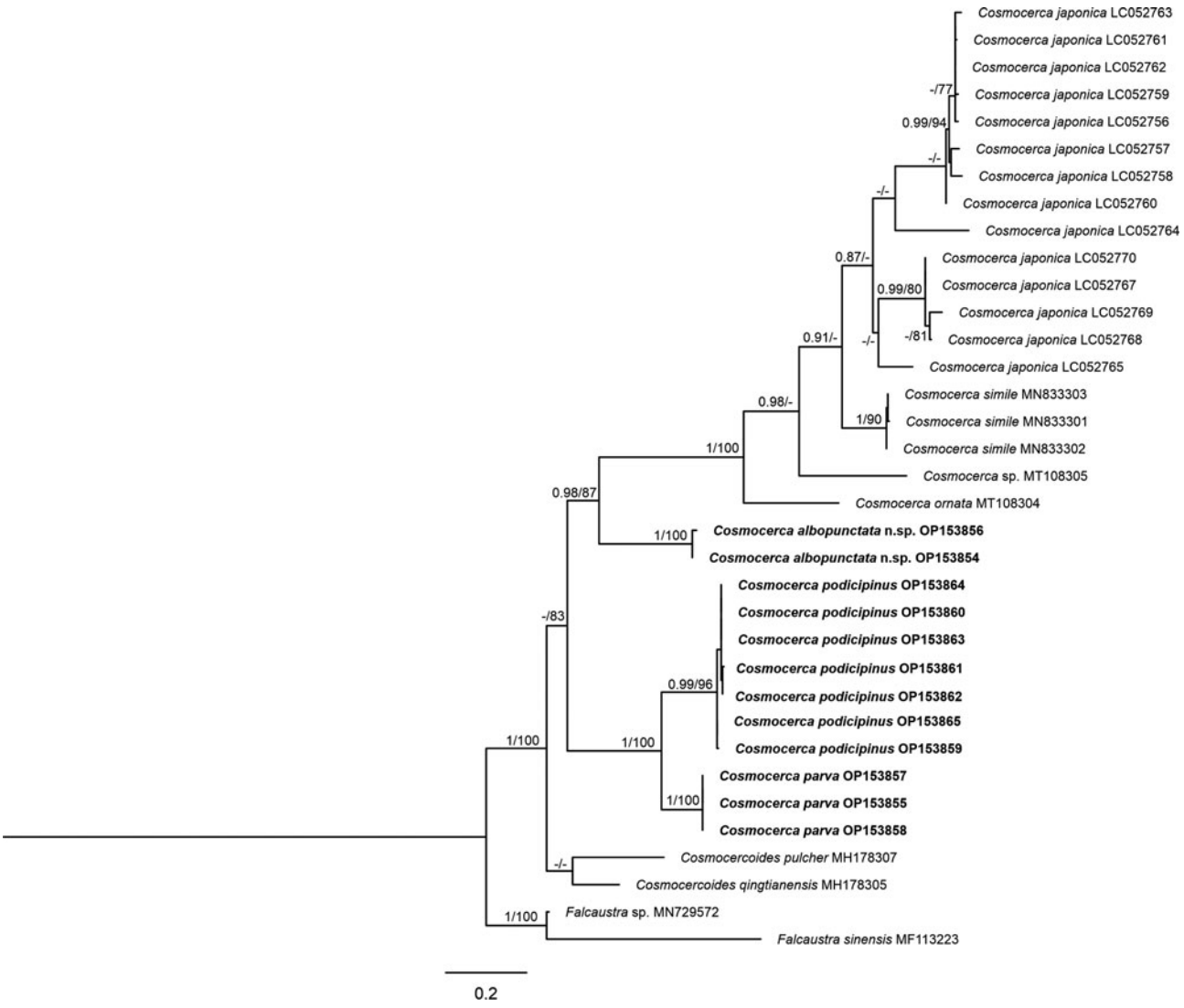

Fig. 4. Maximum likelihood (ML) topology based on COI mtDNA sequences of Cosmocercidea, showing the phylogenetic position of *Cosmocerca* spp. from Brazilian anurans. Numbers above nodes represent posterior probabilities values for Bayesian analyses followed by bootstrap values for ML analyses (posterior probabilities >0.90 and bootstrap values >70 were considered well supported). The branch length scale bar indicates the number of substitutions per site.

Cosmocerca albopunctata n. sp. can be easily distinguished from *C. longispicula* by possessing smaller spicules (98.2–99.8 vs. 294–300) and larger tail compared to *C. longispicula* (191.4–266.6 vs. 135–144) (Moravec & Kaiser, 1994). This geographical location of *C. longispucula* reinforces the differentiation of these species.

The phylogenetic results based on the partial COI mtDNA sequences supported the new species, *C. parva* and *C. podicipinus* as members of *Cosmocerca*, thus corroborating Chen *et al.* (2020). Moreover, all sequences of each species were clustered together in supported clades, corroborating their current taxonomic assignation. Our phylogenetic analyses are also consistent with the morphological data for the analysed species. Although *C. japonica* is not a major focus of our study, the sequences of this species were positioned into two separate unsupported clades (fig. 4), indicating that these sequences might need further evaluation of their current taxonomic status.

The interspecific nucleotide divergence detected in COI mtDNA between *C. albopunctata* n. sp. and its congeners are: *C. simile* (23%–26.1%); *C. ornata* (28.8–29%); *Cosmocerca* sp. (MT108305) (30.2–31.7%); and *C. japonica* (27.1%–35.8%). Chen *et al.* (2020) observed a lower percentage of interspecific nucleotide divergence between *Cosmocerca* species (10.2–15.5%). Therefore, our results are following the expected divergence range previously found in the literature for the family Cosmocercidae, corroborating *C. albopunctata* n. sp. as a different species from its congeners.

The molecular identification of the cosmocercoid nematodes remains very limited (Chen *et al.*, 2020). The genetic data for *Cosmocerca* spp. generated here are very important for further studies of the DNA-based taxonomy, population and phylogenetics of the superfamily Cosmocercoidea (Chen *et al.*, 2021a). Therefore, our results contribute to the knowledge about the species diversity and genetic data for *Cosmocerca* and serve as preliminary information on Neotropical cosmocercids, that should be accessed in future studies to help unravel phylogenetic relationships in cosmocercids.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X22000517.

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Conflicts of interest. None.

Ethical standards. All applicable international, national and/or institutional guidelines for the ethical handling of animals were followed (ICMBio SISBIO #60640-1; CEUA-UNESP 1061; SISGEN).

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