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Description of *Cystodiscus elachistocleis* sp. nov. (Cnidaria: Myxosporea) parasitizing the gallbladder of *Elachistocleis cesarii* from Brazil, based on morphological and molecular analyses

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Abstract. Numerous pseudoplasmodia containing myxospores belonging to the genus *Cystodiscus* were found in the gallbladder of *Elachistocleis cesarii* from Mato Grosso State, Brazil. Herein, we describe *Cystodiscus elachistocleis* sp. nov., using morphological and small subunit ribosomal DNA sequences. The mature myxospores were ellipsoid to ovoid, measuring in average 10.6 (9.8–11.2) µm in length and 6.2 (5.6–6.6) µm in width. Polar capsules were pyriform and equal in size measuring in average 3.6 (2.8–4.3) µm in length and 2.6 (2.2–3.1) µm in width. Polar filaments had 2–4 coils. The myxospores had 3–5 transverse ridges. The phylogenetic analysis showed *Cystodiscus elachistocleis* sp. nov. as a sister species of *Cystodiscus* cf. *immersus* 1, in a subclade formed by species that parasitize the amphibians gallbladder. This is the first species of *Cystodiscus* described parasitizing a species of *Elachistocleis* and the third species of *Cystodiscus* described in Brazil.

Keywords. *Elachistocleis*, frog, phylogeny, Microhylidae, Myxidiidae.

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Introduction

Myxozoans are cnidarians parasites with a complex life cycle (Lom & Dyková 2006). Lutz (1889) proposed the genus *Cystodiscus* Lutz, 1889 to accommodate *Cystodiscus immersus* Lutz, 1889 based on a large disc-like appearance found in the amphibious gallbladder. The genus was abandoned and classified as a synonym of *Myxidium* Bütschli, 1882; however, Hartigan *et al.* (2012), performing phylogenetic molecular analyses, reerected the genus *Cystodiscus* as a monophyletic group instead of the polyphyletic nature of *Myxidium*. They have a global distribution and are endoparasites of a great diversity of amphibian hosts (Hartigan *et al.* 2012a, 2012b, 2016).

Cystodiscus spp. could be pathogenic but some hosts do not show pathological signs and release myxospores over time without any impact on the health of their hosts (Hartigan *et al.* 2012b). In pathogenic cases, the hosts may show inflammation in the nervous tissue leading to behavioral changes and even spontaneous death. Also, in cases of liver diseases, they can cause inflammation and hyperplasia, affecting the metamorphosis of tadpoles, as well as metabolism and immune function (Hartigan *et al.* 2012b). Due to its pathogenic potential, the parasite can have an important ecological impact on the conservation of amphibians (Hartigan *et al.* 2012c).

Microhylidae Günther, 1858 is one of the most diverse families of extant amphibians, distributed across most of the tropics (Frost 2020). Three subfamilies of microhylids are recognized: Adelastinae Peloso, Frost, Richards, Rodrigues, Donnellan, Matsui, Raxworthy, Biju, Lemmon, Lemmon & Wheeler, 2016, Gastrophryninae Fitzinger, 1843, and Otophryninae Wassersug & Pyburn, 1987 (de Sá *et al.* 2012; Peloso *et al.* 2014). *Elachistocleis* Parker, 1927 (subfamily Gastrophryninae) is a genus of microhylid frogs widespread distributed with 19 species, most of which occur east of the Andes in South America (Frost 2020; Sánchez-Nivicela *et al.* 2020). In Brazil, 12 species of *Elachistocleis* were reported (Segalla *et al.* 2019). *Elachistocleis cesarii* (Miranda-Ribeiro, 1920), an endemic species in Brazil, occurs from southeastern to central Brazil in eastern São Paulo, south-central Minas Gerais, Goiás, and Mato Grosso States (Toledo *et al.* 2010; Frost 2020). This species has sexual dimorphism and females are larger than males. They have a fossorial habit, spending most of the year sheltered under the ground, and have a marked pattern of seasonal activity (Toledo *et al.* 2010). This species is not on the International Union for Conservation of Nature (IUCN) red list of endangered animals.

During a study on the biodiversity of anuran parasites in Mato Grosso State, Brazil, pseudoplasmodia containing myxospores morphologically consistent with *Cystodiscus* were observed in the gallbladder of *E. cesarii*. The present study aims to describe this new species of *Cystodiscus* parasitizing *E. cesarii* from Brazil, based on morphological and molecular analyses, thus increasing the knowledge of Brazilian anurans parasites.

Material and methods

Anuran collection and morphological analysis

In February 2020, one female anuran, *E. cesarii* measuring 39 mm snout-vent length and weight of 5.8 g, was captured for a major project of biodiversity of parasites in amphibians (FAPESP 2018/09623-4; FAPESP 2018/00754-9). A new sampling effort was carried out at different times within the major project, but it was not successful in collecting more specimens of *E. cesarii*. The specimen was collected on a dirt road in the municipality of Araguaiana, Mato Grosso State, Brazil (14°35′5.29″ S, 51°41′19.15″ W). The host was deposited in the Herpetologica Collection of the Regional University of Ceará (Num. CHUFC A9761).

The anuran was killed using 50 mg/kg of sodium thiopental (Thiopentax®), a commercial anesthetic administered intracerebrally, following the guidelines of Sebben (2007) and the Animal Use Ethics Committee (IBAMA license 60640-1; CEUA-UNESP 1061). The coelom was opened via a midventral longitudinal incision and the gall bladder was examined for cysts or signs of myxosporean infection. Gallbladder contents were removed by puncturing the bladder wall and pipetting out the contents (Hartigan *et al.* 2016). The fresh smears containing pseudoplasmodia were evaluated with a differential interference contrast microscope (Leica DMLB 5000, Leica Microsystems, Wetzlar, Germany) at 1000 × magnification. The morphological measurements of myxospores freshly preserved followed the recommendations of Lom & Arthur (1989). Measurements of 30 myxospores were obtained and presented as mean ± standard deviation.

Molecular analysis

Two pseudoplasmodia from the gallbladder were fixed in absolute ethanol and used for the molecular analyses. The access to the genetic data was authorized by the Brazilian Ministry of Environment (Sisgen AA4666FA). The two isolated pseudoplasmodia were collected from the bile material with fine-tipped sterile needles. DNA isolation was carried out following the animal tissue protocol of the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). The partial SSU rDNA gene was amplified with PCR using general eukaryotic and myxozoan primers (Table 1).

Amplification was performed in a Bio-Rad Mycycler (Bio-Rad Laboratories Pty Ltd., Gladesville, Australia), with initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 55°C for 45 s, 72°C for 2 min and a final extension at 72°C for 7 min. PCR reactions were performed in 25 µl of solution containing 3 µl of extracted DNA and 1 µl of each PCR primer at 10 pmol, using PCR Ready-to-Go beads (Pure TaqTMReady-to-GoTM beads, GE Healthcare, Chicago, USA). The solution consisted of stabilizers, BSA, dATP, dCTP, dGTP, dTTP, ± 2.5 units of puReTaq DNA polymerase and reaction buffer. Each bead was reconstituted to a final volume of 25 µl. PCR products were analysed by electrophoresis on 1% agarose gel stained with GelRed and visualised under UV light. The products of the PCR reaction for the SSU rDNA gene were purified and precipitation reaction by Ethanol/EDTA/Sodium Acetate according to the protocol suggested by the manufacturer was performed, and then sequenced with a BigDye® Terminator ver. 3.1 Cycle Sequencing Kit (Applied). Automatic sequencing by capillary electrophoresis was performed on the ABI3730xl DNA Analyzer (Applied Biosystems).

The partial sequences obtained were assembled and edited using a Sequencher™ ver. 5.2.4 (Gene Codes, Ann Arbor, MI) to obtain a consensus sequence. The newly generated partial sequences of SSU rDNA were aligned using Geneious ver. 7.1.3 (Kearse *et al.* 2012) with the ClustalW algorithm (Larkin *et al.* 2007) and default settings with related genetic sequences that appeared on Blastn search (Table 2). The Bayesian inference (BI) and Maximum-Likelihood (ML) analyses were performed using MrBayes ver. 3.1.2 (Ronquist & Huelsenbeck 2003) Markov Chain Monte Carlo (MCMC) chains were run for 10 million generations and the log-likelihood scores were plotted. The 'burn-in' was set to 30%. PhyML 3.1 (Guindon *et al.* 2010) software was used to perform ML analysis, using bootstrap confidence calculated with 1000 replications and GTR+I+G evolutionary model which was chosen by jModeltest (Posada 2008) as the best model for this analysis. Phylogenetic trees were generated and edited in FigTree ver. 1.4 (Rambaut 2012). *Chloromyxum trilineatum* Sekyia, Rosyadi, Zhang & Sato, 2019 (LC417364) was used as an out group. The aligned sequences of myxosporean parasites were compared using a pair-wise distance (p-distance) matrix (Table 3).

Table 1. Primers used for the amplification and sequencing of the SSU rDNA of *Cystodiscus elachistocleis* sp. nov. found parasitizing the gallbladder of *Elachistocleis cesarii* (Miranda-Ribeiro, 1920) from Araguaiana, Mato Grosso State, Brazil.

Primer	Sequence 5'-3'	Paired with	Reference
Erib1	ACCTGGTTGATCCT	Act1r	Barta *et al.* (1997)
Act1r	AATTTCACCTCTCGCTGCCA	Erib1	Hallett & Diamant (2001)
Myxgen4F	GTGCCTTGAATAAATCAGAG	Erib10	Diamant *et al.* (2004)
Erib10	CTTCCGCAGGTTCACCTACGG	Myxgen4F	Barta *et al.* (1997)
MX5	CTGCGGACGGCTCAGTAAATCAGT	MX3	Andree *et al.* (1999)
MX3	CCAGGACATCTTAGGGCATCACAGA	MX5	Andree *et al.* (1999)

Institutional abbreviations

INPA = Instituto Nacional de Pesquisas da Amazônia

Results

Taxonomy

Phylum Cnidaria Hatschek, 1888
Unranked subphylum Myxozoa Grassé, 1970
Class Myxosporea Bütschli, 1881
Order Bivalvulida Shulman, 1959
Family Myxidiidae Thélohan, 1982
Genus *Cystodiscus* Lutz, 1889

Cystodiscus elachistocleis sp. nov.
urn:lsid:zoobank.org:act:E2AE8907-E225-4804-A2F9-E3920AD6298D
Figs 1–3

Type host

Elachistocleis cesarii (Miranda-Ribeiro, 1920).

Site of infection

Gallbladder.

Etymology

The species epithet is derived from the genus *Elachistocleis* of the host.

Material examined

Hapantotypes

BRAZIL • 10+ myxospores; Mato Grosso State, Araguaiana municipality; 14°35′5.29″ S, 51°41′19.15″ W; glycerogelatin slide; GenBank MZ645740-MZ645741; INPA79.

Description

Numerous pseudoplasmodia were found free in the bile. Pseudoplasmodia (Fig. 1C) of approximately 2 mm were rounded in shape, appeared to be formed by a 'gelatinous' substance, and were floating in the host's bile containing several myxospores in its interior. The morphology of myxospores found

Table 2. Morphometric comparison of mature myxospores of *Cystodiscus elachistocleis* sp. nov. (INPA79) with *Cystodiscus* spp. Abbreviations: SSL = myxospore length; SW = myxospore width; PL = polar capsule length; PW = polar capsule width; TR = transverse ridges; PT = polar filament turns. All dimensions are given in µm.

Species	SL	SW	PL	PW	TR	PT	Site of infection	Type host	Country	Reference
C. elachistocleis sp. nov.	10.6 ± 0.3 (9.8–11.2)	6.2 ± 0.5 (5.6–6.6)	3.6 ± 0.4 (2.8–4.3)	2.6 ± 0.3 (2.2–3.1)	3–5	2–4	gallbladder	*Elachistocleis cesarii* (Miranda-Ribeiro, 1920)	Brazil	Present study
C. immersus Lutz, 1889	11.8–13.3	7.5–8.6	3.5–4.2	–	7–9	–	gallbladder	*Rhinella marina* (Linnaeus, 1758)	Brazil	Kudo & Sprague (1940)
C. lyndoyense Carini, 1932	11.0–12.0	7.5–8.0	4.0 (in diameter)	–	–	–	gallbladder	*Rhinella marina* (Linnaeus, 1758)	Brazil	Carini (1932)
C. melleni (Jirku, Bolek, Whipps, Janovy, Kent & Modry, 2006)	12.3 (12.0–13.5)	7.6 (7.0–9.0)	5.2 (4.8–5.5)	4.2 (3.8–4.5)	2–5	6–7	gallbladder	*Pseudacris triseriata* Wied-Neuwied, 1838	USA	Jirku *et al.* (2006)
C. serotinus (Kudo & Sprague, 1940)	16.0–18.0	9.0	5.0–5.5 (in diameter)	–	10–13	3–5	gallbladder	*Rana pipiens* Schreber, 1782	USA	Kudo & Sprague (1940)
C. typhonius (Gray, 1993)	10.9 (9.8–12.2)	7.2 (5.7–8.9)	3.8 (2.5–5.5)	3.6 (3.3–5.2)	9–11	4–5	gallbladder	*Bufo margaritifer* (Laurenti, 1768)	Peru	Gray (1993)
C. haldari (Sarkar, 1982)	10.8 (10.0–12.0)	6.7 (6.5–7.0)	3.6 (3.0–4.0)	–	–	–	gallbladder	*Hyla arborea* Linnaeus, 1758	India	Sarkar (1982)
C. lesminteri (Delvinquier, Markus & Passmore, 1992)	12.5 (9.5–15.0)	6.5 (5.7–8.0)	–	–	1–2	–	gallbladder	*Tomopterna krugerensis* Passmore & Carruthers, 1975	South Africa	Delvinquer *et al.* (2012)
C. axonis Hartigan, Fiala, Dyková, Rose, Phalen & Šlapeta, 2012	14.1 (13.0–15.5)	8.5 (8.0–10.5)	3.8 (3.0–5.0)	3.7 (3.0–5.0)	5–12	4–5	gallbladder and bile ducts	*Litoria raniformis* Keferstein, 1867	Australia	Hartigan *et al.* (2012c)
C. australis Hartigan, Fiala, Dyková, Rose, Phalen & Šlapeta, 2012	16.0 (15.0–18.0)	8.7 (8.0–10.0)	5.3 (5.0–6.0)	4.8 (4.0–5.5)	5–11	5–6	gallbladder and bile ducts	*Limnodynastes peronii* Duméril & Bibron, 1841	Australia	Hartigan *et al.* (2012c)

Table 3. The similarity in SSU rDNA sequences of *Cystodiscus* spp. The upper triangle shows the percentage of nucleotide similarity, while the lower triangle shows the actual nucleotide difference.

	Host isolate	1	2	3	4	5	6
1	*Cystodiscus elachistocleis* sp. nov. MZ645740		97.3	94.1	94.9	94.0	91.4
2	*Cystodiscus* cf. *immersus* HQ822162	24		95.1	96.2	94.9	93.0
3	*Cystodiscus* cf. *immersus* 2 HQ822159	52	43		84.6	93.8	91.4
4	*Cystodiscus melleni* DQ003031	89	34	48		93.1	92.3
5	*Cystodiscus australis* HQ822149	53	45	55	61		91.0
6	*Cystodiscus axonis* HQ822165	76	62	76	68	80	

in the gallbaldder of *E. cesarii* corresponded to the genus *Cystodiscus*. The myxospores (Figs 1A–B, 2) were ellipsoid to ovoid. The two myxospore valves were joined by a raised ridge that appeared straight or slightly curved (S-shaped in sutural view) along the medial axis of the myxospore. Transverse depressions on the surface of the myxospores appeared connected to the sutural ridge depression. Their measurements presented as mean ± SD (range) were: myxospore length 10.6 ± 0.3 (9.8–11.2) µm, myxospore width 6.2 ± 0.5 (5.6–6.6) µm. The polar capsules were pyriform and equal in size, situated each one in a myxospore extremity and measured 3.6 ± 0.4 (2.8–4.3) µm in length and 2.6 ± 0.3 (2.2–3.1) µm in width. The polar filament present within the polar capsule had 2–4 coils. The myxospores showed 3–5 transverse ridges and a binucleated sporoplasm. Filiform polar appendages were not observed.

Remarks

Cystodiscus elachistocleis sp. nov. was morphometrically compared to all *Cystodiscus* spp. described worldwide (Table 2). The species that most resembled *C. elachistocleis* sp. nov. was *C. haldari* (Sarkar, 1982), which showed similarity in all measurements available for comparison. However, the geographical distance (Brazil vs India) and the different type host (*E. cesarii* vs *Hyla arborea* (Linnaeus, 1758)) allow us to separate the two species. Furthermore, *C. haldari* does not present any comparative values such as the number of turns of the polar filament and the number of transverse ridges, which could differentiate the two species. *Cystodiscus thyponius* Gray (1993) also presented measures of length and width of myxospores similar to those found in *C. elachistocleis* sp. nov. However, differences were observed in the polar capsule width (2.6 ± 0.3 (2.2–3.1) vs 3.6 (3.3–5.2)) and the number of transverse ridges (3–5 vs 9–11).

Regarding the *Cystodiscus* spp. described from Brazil, *C. elachistocleis* sp. nov. was morphometrically compared with two species parasitizing the gallbladder of *Rhinella marina* (Linnaeus, 1758). *Cystodiscus immersus*, Kudo & Sprague (1940) presented a longer body length than that found in *C. elachistocleis* sp. nov. (11.8–13.3 vs 10.6 ± 0.3 (9.8–11.2)), in addition to a greater number of transverse ridges (7–9 vs 3–5). *Cystodiscus lyndoyense* Carini, 1932 showed the length of the body (11.0–12.0 vs 10.6 ± 0.3 (9.8–11.2)) and the body width (7.5–8.0 vs 6.2 ± 0.5 (5.6–6.6)) longer than that found for *C. elachistocleis* sp. nov. The other *Cystodiscus* spp. already described presented a body length longer than that observed in *C. elachistocleis* sp. nov.

Molecular analyses

Fragments of 1730-bp and 1916-bp of the SSU rDNA gene were generated. The fragments showed 100% similarity when aligned. The BLAST search of the sequences did not reveal a direct match with myxozoan sequences available in GenBank. The genetically closest species was *C. immersus*, which exhibited a similarity of 97.3%, and a difference of 24 out of 885 nucleotides (Table 3).

A well-supported phylogenetic tree divided into three main groups was obtained (Fig. 3). A monophyletic group composed of *Sphaeromyxa* Thélohan, 1892 spp. that parasitizes fish, a polyphyletic group composed of *Zschokkella* Auerbach, 1909 and *Myxidium* Bütschli, 1882 spp. that infect fish, and

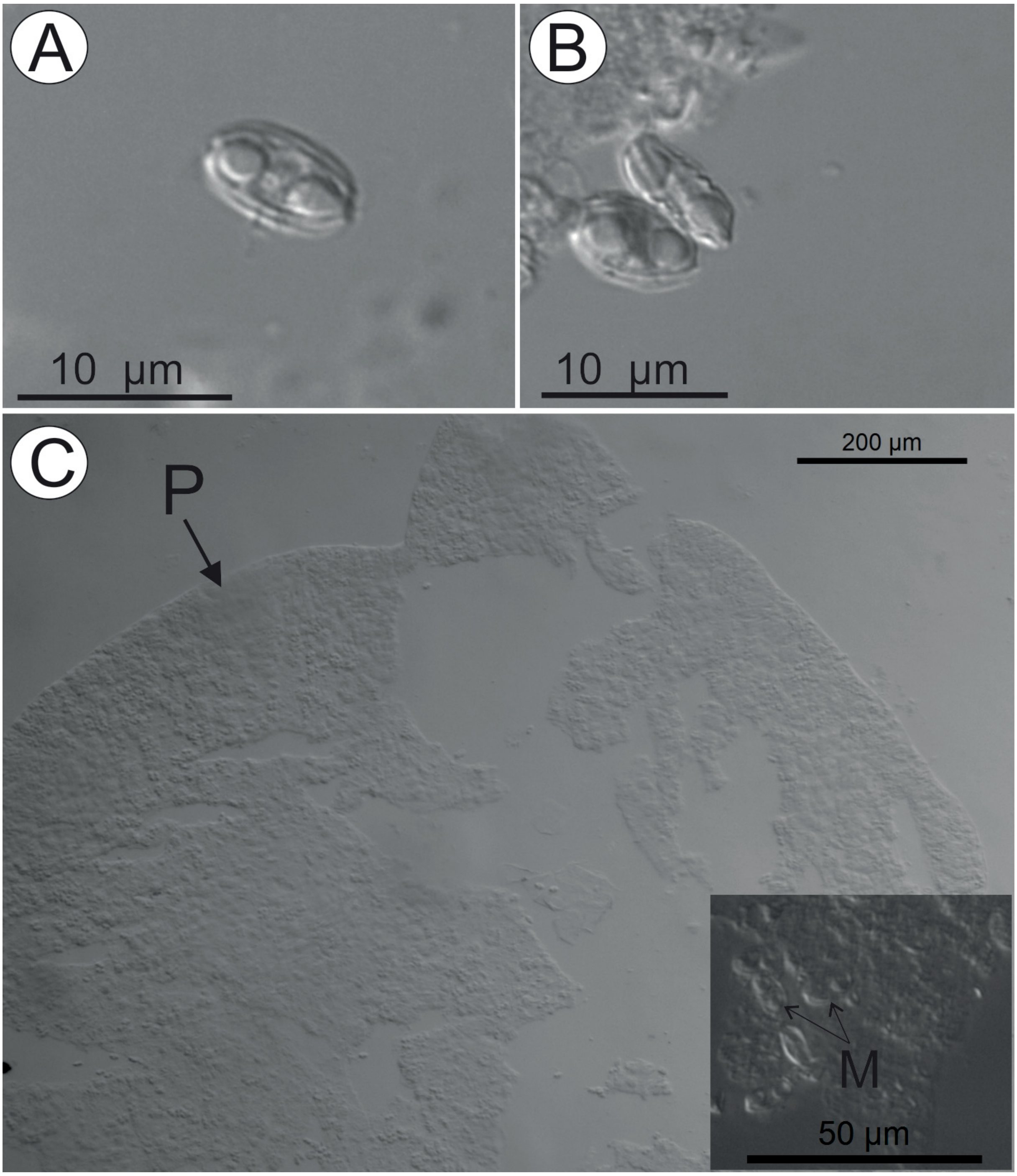

Fig. 1. Myxospores and pseudoplasmodia of *Cystodiscus elachistocleis* sp. nov. (INPA79) found parasitizing the gallbladder of *Elachistocleis cesarii* (Miranda-Ribeiro, 1920) from Araguaiana, Mato Grosso State, Brazil. **A**. Front view of *C. elachistocleis* sp. nov. **B**. Front and side view of *C. elachistocleis* sp. nov. **C**. Pseudoplasmodium (P) containing several myxospores of *C. elachistocleis* sp. nov. highlights myxospores (M) through the pseudoplasmodium-forming tissue.

finally a monophyletic group composed of *Cystodiscus* spp. that parasitize amphibians. Still, there is a small monophyletic group composed of *Myxidium* ssp. found in reptiles, fish, and birds. *Cystodiscus elachistocleis* sp. nov. appears as a sister species of *Cystodiscus* cf. *immersus* 1, in a subclade formed by species that parasitize the amphibians gallbladder.

Discussion

Considering the myxospore morphology, the morphometric data, and SSU rDNA gene partial sequence obtained in the present study, we described *C. elachistocleis* sp. nov. This finding contributes to our knowledge of the biodiversity of *Cystodiscus* in amphibians from Brazil. To our knowledge, *C. elachistocleis* sp. nov. is the first record of a myxozoan species parasitizing *E. cesarii* and the third species of *Cystodiscus* described from Brazil.

Brazil is one of the most species-rich areas for amphibians globally (Segalla *et al.* 2017). However, amphibians are also considered the most threatened vertebrate group with many species facing extinction (Wake & Vredenburg 2008; Rebouças *et al.* 2021). Amphibians also are hosts of a variety of endoparasites and ectoparasites, which in some cases influence their fitness, behavior, feeding, reproduction, and fertility (Barta & Desser 1984; Lainson *et al.* 2003; Muñoz-Leal *et al.* 2017). For this study, only one specimen of *E. cesarii* was collected despite the extensive sampling effort performed. This could be due to the low presence of the host at the collection site or its ecology, which makes collecting it more difficult than other species. Despite this, several studies describe new myxozoans from a single infected host (Hartigan *et al.* 2012a; Chen *et al.* 2020; Mathews *et al.* 2021).

Regarding myxozoan parasites, there are only two species of *Cystodiscus* described on Brazilian anurans, *C. immersus*, and *C. lyndoyense*. However, the morphological and morphometric data differ from the species in the present study. The specificity and the host organ combined with myxospore morphology and molecular data are essential for a determination of a new myxozoan species. Following this taxonomic strategy, we described a new species of *Cystodiscus*, a gallbladder parasite of *E. cesarii*.

The phylogenetic analysis performed here supports previous work that suggests that *Cystodiscus* are amphibian gallbladder species (Hartigan *et al.* 2012a). The monophyletic subclade composed of *Cystodiscus* spp. was well supported. The low values of posterior probability found in some nodes within the subclade can be explained by the low number of described species that have their partial

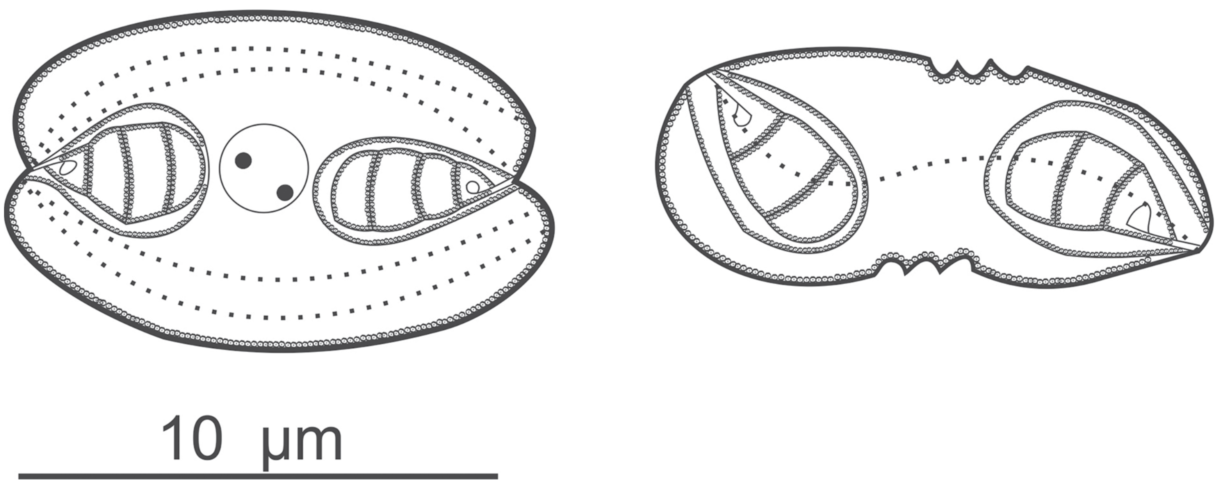

Fig. 2. Schematic drawing of *Cystodiscus elachistocleis* sp. nov. (INPA79) found parasitizing the gallbladder of *Elachistocleis cesarii* (Miranda-Ribeiro, 1920) in front and side view.

sequences of the SSU rDNA gene available. *Cystodiscus* cf. *immersus* 1 and *Cystodiscus* cf. *immersus* 2 appear with different sister species in the subclade, indicating that they are two distinct species.

The genus *Cystodiscus* is globally distributed and may have ecological implications outside of Brazil (Hartigan *et al.* 2012c). These parasites are associated with inflammation of nervous tissue and hepatic disease in several threatened or common frog species (Hartigan *et al.* 2012b). Although the situation of *E. cesarii* in the list of endangered species is 'least concern - LC', a parasite that causes serious pathologies in these amphibians could further decrease the population of amphibians in Brazil, something that has been happening gradually (Subirá *et al.* 2012; Ceballos *et al.* 2020). Despite this, in the present study, no pathologies were observed in the gallbladder or any other host organ associated with parasitism by *Cystodiscus elachistocleis* sp. nov.

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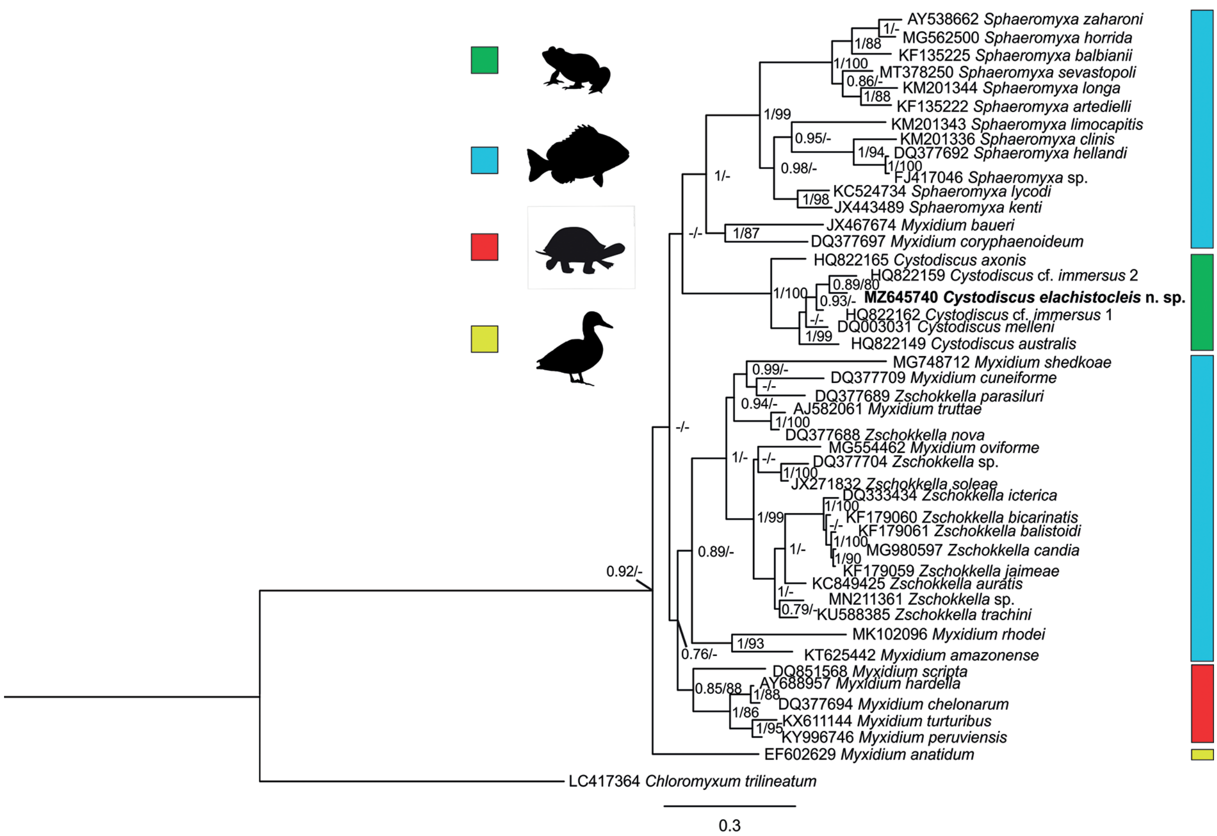

Fig. 3. Phylogenetic tree of Bayesian analysis based on partial SSU rDNA partial sequences showing the position of *Cystodiscus elachistocleis* sp. nov. (INPA79) among genetically similar species. Node numbers represent the Bayesian posterior probabilities and bootstrap (BI/ML). Values less than 0.7 are represented by dashes. The scale bar represents the number of substitutions per site.

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