UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS
CÂMPUS DE BOTUCATU
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS - ZOOLOGIA

Helmintos parasitos de anuros brasileiros

Edna Paulino de Alcantara

Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (Zoologia) do Instituto de Biociências da Universidade Estadual Paulista – UNESP, Campus de Botucatu, São Paulo, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Zoologia).

Botucatu, SP
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Palavras-chave: Anfíbio; Biologia molecular; Helmintofauna; Parasitologia; Taxonomia.

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"Em algum lugar, alguma coisa incrível está esperando para ser descoberta."
(Carl Sagan)

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Sumário

SUMÁRIO

Resumo

RESUMO

O Brasil é o país com a maior riqueza e diversidade de anfíbios, sendo conhecidas atualmente 1.206 espécies válidas. A ordem anura apresenta a maior riqueza com 1.162 espécies. Este grupo taxonômico apresenta uma grande diversidade morfológica e comportamental, sendo considerado importante bioindicador ambiental de áreas prioritárias para conservação. No que diz respeito a parasitologia de animais silvestres, estes hospedeiros são excelentes modelos, pois apresentam uma grande diversidade de habitats e de alimentação. Os anuros são hospedeiros frequentes para diversas espécies de platelmintos, nematoides e acantocéfalos. Apesar disso, o estudo parasitológico desses organismos ainda é incipiente, sendo que aproximadamente 92% das espécies ainda não foram estudadas quanto a sua helmintofauna. Neste contexto, o presente trabalho teve como objetivo inventariar a diversidade de helmintos parasitos de anuros em oito estados brasileiros, considerando as análises morfológicas, moleculares e as relações filogenéticas desses parasitos. Durante os anos de 2017 a 2021 foram coletados helmintos de 50 espécies de anuros das famílias: Bufonidae, Hemiphractidae, Hylidae, Hylodidae, Leptodactylidae, Microhylidae e Phyllomedusidae. Foi coletado um total de 28.787 espécimes de helmintos. Destes, 25.172 espécimes representantes de 50 *taxa* do filo Nematoda, 2.671 espécimes de 10 *taxa* da classe Trematoda, 64 espécimes de dois *taxa* da classe Cestoda, 67 espécimes de um *taxon* do subclasse Acanthocephala e 16 espécimes pertencentes a um *taxon* da classe Monogenea. Até o presente momento, 15 novas espécies de helmintos foram registradas: 14 espécies de Nematoda e uma espécie de Trematoda. A diversidade de helmintos parasitas deste estudo evidencia a necessidade de se intensificar o estudo da diversidade e análises filogenéticas desses parasitas.

Palavras-chave: Anfíbio, Parasitologia**,** Helmintofauna, Taxonomia, Biologia Molecular

Abstract

ABSTRACT

Brazil is the country with the greatest richness and diversity of amphibians, with 1,206 valid species currently known. The anura order has the highest richness with 1,162 species. This taxonomic group presents a great morphological and behavioral diversity, being considered an important environmental bioindicator of priority areas for conservation. Regarding the parasitology of wild animals, these hosts are excellent models, as they present a great diversity of habitats and food. Anurans are frequent hosts for several species of flatworms, nematodes and acanthocephalans. Despite this, the parasitological study of these organisms is still incipient, and approximately 92% of the species have not yet been studied for their helminth fauna. In this context, the present work aimed to inventory the diversity of helminth parasites of anurans in eight Brazilian states, considering the morphological and molecular analyzes and the phylogenetic relationships of these parasites. From 2017 to 2021, helminths were collected from 50 frog species belonging to the families: Bufonidae, Hemiphractidae, Hylidae, Hylodidae, Leptodactylidae, Microhylidae, and Phyllomedusidae. A total of 28,787 helminth specimens were collected. Of these, 25,172 specimens representing 50 taxa of the phylum Nematoda, 2,671 specimens of 10 taxa of the class Trematoda, 64 specimens of two taxa of the class Cestoda, 67 specimens of a taxon of the subclass Acanthocephala and 16 specimens belonging to a taxon of the class Monogenea. To date, 15 new species of helminths have been recorded: 14 species of Nematoda and one species of Trematoda. The diversity of helminth parasites in this study highlights the need to intensify the study of the diversity and phylogenetic analysis of these parasites.

Keywords: Amphibian, Parasitology, Helminthofauna, Taxonomy, Molecular Biology

Introdução

INTRODUÇÃO

Biologia de anfíbios

O surgimento dos anfíbios percorre uma grande gama de modificações corporais nos peixes (Bergmann, 2016). De acordo com Frost *et al.* (2006) os anfíbios foram os primeiros vertebrados a colonizar o ambiente terrestre, os quais possuíam características intermediárias entre os peixes e os amniotas terrestres. Os primeiros vertebrados terrestres (tetrápodes) passaram a viver predominantemente na água em regiões bem rasas. Esses animais possuíam nadadeiras fortes que auxiliavam no deslocamento, dessa forma eles tinham condições de se aventurar por breves períodos fora da água, em busca de recursos até então não aproveitados pelos vertebrados, restritos ao ambiente aquático (peixes) (Bergmann, 2016).

Dentre esses tetrápodes primitivos, dois grupos tiveram grande importância: os Labirintodontes e os Lepospôndilos. A partir dos labirintodontes surgiram os Lissamphibia (anfíbios modernos) que se dividem em três grupos com base em seu modo de locomoção: Anura, Caudata e Gymnophiona (Apoda) (Kardong, 2011).

Os animais pertencentes à ordem Anura (sapos, rãs e pererecas) não possuem cauda ou ela é muito reduzida, praticamente vestigial. Os sapos têm a pele úmida, porém áspera, cheia de verrugas e glândulas paratoides, além de se deslocarem com saltos curtos ou caminhando lentamente, utilizando as quatro patas. As rãs têm a pele bem lisa e úmida, não possuem glândulas paratoides e se deslocam com grandes e ágeis saltos, enquanto as pererecas são facilmente distinguíveis pela presença de ventosas adesivas nas pontas dos dedos. As salamandras pertencentes à ordem Caudata possuem geralmente quatro patas, mesmo que de tamanho mais reduzido e possuem uma cauda longa, bem desenvolvida, além do mais movem-se por ondulações laterais. Já as cecílias ou cobras-cegas, que pertencem a ordem Gymnophiona não apresentam patas (Apoda = “a” que significa sem e “podos” que significa patas), tendo um corpo serpentiforme, e por terem um comportamento mais fossorial possuem os olhos muito reduzidos, ou mesmo, ausentes (Bernarde, 2012; Bergmann, 2016).

Os anfíbios possuem grande diversidade de modos reprodutivos e de locomoção, compartilhando características únicas, em que se destaca na maioria das espécies uma metamorfose no seu ciclo de vida. No caso dos gimnofionos não ocorre metamorfose, com desenvolvimento direto e do ovo saem pequeninas cobras-cegas. Os demais passam por

uma fase larval aquática, com respiração branquial, até chegar a uma fase adulta com respiração pulmonar (Duellman e Trueb, 1994) para viverem em terra firme. Porém, essa vida em terra firme é intimamente dependente da água, sendo que esses animais nunca se afastam demais dela (Bergmann, 2016).

Os anfíbios são animais ectotérmicos, os quais, não conseguem controlar a temperatura do seu corpo fisiologicamente e dependem diretamente do ambiente para conseguir aumentar ou diminuir sua temperatura corporal. Os anfíbios possuem glândulas que produzem muco para manter a pele úmida e, em alguns casos, produzem toxinas com função defensiva. Na grande maioria dos casos, os anfíbios têm o muco tóxico (Bergmann, 2016). A secreção da pele dos anfíbios anuros pode ter função nociva, adesiva e odorífera. Geralmente, os anuros que possuem cores brilhantes e chamativas, como no caso das espécies de Dendrobatidae, possuem toxinas nocivas na pele. Além da pele venenosa, é comum os anfíbios se defenderem através das técnicas de camuflagem, imobilização, tanatose (fingir de morto), descargas cloacais e inflando-se para parecerem maiores (Bernarde, 2012).

A reprodução dos anfíbios é uma das mais diversas entre todos os grupos de animais. Os anfíbios são dioicos, e embora predominantemente ovíparos, são conhecidas 74 variações no modo reprodutivo, que se dividem em três estratégias principais: ovos aquáticos, ovos terrestres e arborícolas não aquáticos e ovos retidos no oviduto. Os seus ovos estão envoltos por uma substância gelatinosa e sua pele é fina, altamente vascularizada e glandular (Bernarde, 2012).

A vocalização nos anfíbios é uma capacidade importante para muitas espécies. Ela é específica, diferenciando as espécies e permitindo que os casais se formem na época de acasalamento. São os machos que cantam, atraindo as fêmeas e formando então os pares. A vocalização é uma capacidade presente apenas nos anuros. As salamandras não possuem cordas vocais, mas emitem guinchos (com apenas uma exceção, *Dicamptodon* spp. possuem cordas vocais) (Storer et al., 2005). Apesar das vocalizações terem um maior foco reprodutivo, elas são utilizadas em diversas situações, existindo diferentes tipos de canto, como por exemplo cantos de corte, de encontro, de reciprocidade, de agonia, agressivos e de equívoco (quando um macho abraça outro pensando que é fêmea) (Bernarde, 2012). No entanto, ainda existem espécies que não dependem só da vocalização como meio de acasalamento. Algumas espécies utilizam sinais visuais movimentando as pernas para sinalizar aos seus parceiros, como por exemplo, as rãs do gênero *Hylodes*, típicas da Mata

Atlântica, as quais são conhecidas como sapos-sinaleiros devido esse tipo de comportamento (Bergmann, 2016). Apesar da vocalização ser um meio de acasalamento entre os anuros, no qual quanto mais alto um macho vocaliza, maior a chance de uma fêmea encontrá-lo e selecioná-lo para o acasalamento, a vocalização também aumenta a chance de predação (Pough et al., 2008). Os anfíbios anuros machos, após disputarem e conquistarem o direito de acasalar com uma fêmea, realizam o amplexo. O amplexo nada mais é do que um "abraço" em que o macho se prende à fêmea, que geralmente é maior que ele, e assim garantir que ele irá fecundar os ovos dela (Bergmann, 2016). Nos anuros, a fecundação é externa, à medida que os ovos são liberados pela fêmea, o macho libera o esperma fecundando os ovos. Já nas salamandras a fecundação é interna, fato que também é atribuído às cobras-cegas (Kardong, 2011).

Mundialmente são reconhecidas 8.482 espécies de anfíbios (Frost, 2022). O Brasil é o país com a maior riqueza e diversidade de anfíbios (Bernarde, 2012). Atualmente são conhecidas 1.206 espécies de anfíbios amplamente distribuídas, das quais 1.162 espécies pertencem à ordem Anura, cinco espécies à ordem Caudata e 39 espécies pertencentes à ordem Gymnophiona (Frost, 2022, Segalla et al., 2021).

Parasitismo em anuros

A ordem Anura é a mais diversa, são animais que apresentam uma grande diversidade morfológica e comportamental, sendo considerados importantes bioindicadores, e podem ser utilizados no estudo do funcionamento de ecossistemas e delineamento de áreas prioritárias para conservação (Figueiredo-de-Andrade e Silveira, 2016). Além do que, são excelentes modelos para o estudo de parasitologia, pois ocupam uma grande diversidade de habitats e de alimentação (Aho, 1990).

Os anuros são hospedeiros frequentes para diversas espécies de platelmintos, nematoides e acantocéfalos. Os trabalhos iniciais disponíveis datam desde o século XVII (Ver Fernandes e Kohn, 2014) e, desde então, o conhecimento das espécies de helmintos parasitas de anuros brasileiros tem sido incrementado recentemente com a descrição de novas espécies (Nascimento et al., 2013; Feitosa et al., 2015; Araujo-Filho et al., 2015; Kuzmin et al., 2016; Pereira et al., 2017; Larrat et al., 2018; dos Santos et al., 2018; Santos et al., 2019; Willkens et al., 2019; Felix-Nascimento et al., 2020; Morais et al., 2020; Alcantara et al., 2021a, b; Tavares-Costa et al., 2022) e registros de novas localidade e de

novos hospedeiros (Toledo et al., 2015, Teles et al., 2014, 2015, 2017, 2018a, b; Campião et al., 2014, 2015, 2016a, 2016b; Aguiar et al., 2014, 2015a, 2015b; Toledo et al., 2015, 2017; Martins-Sobrinho et al., 2017; Graça et al., 2017; Lins et al., 2017; Sena et al., 2018; Alcantara et al., 2018, 2020; Maia-Carneiro et al., 2018; Silva et al., 2018; 2019; 2020; Oliveira et al., 2019, Sousa et al., 2020; Parra et al., 2020; Henzel et al., 2020; Madelaire et al., 2020; Rebêlo et al., 2020; Silva-Neta et al., 2020; Mascarenhas et al., 2021; Vieira et al., 2021; Machado et al., 2022). Apesar disso, é fato que os helmintos parasitas destes animais ainda carecem de descrições taxonômicas adequadas, além do que o conhecimento dessa fauna no Brasil ainda é escasso.

Parasitos são considerados importantes componentes da biodiversidade global, sendo os helmintos o grupo mais diverso dentre os metazoários parasitos de vertebrados (Poulin e Morand, 2004; Mouritsen e Poulin, 2005). Os helmintos parasitas constituem uma diversidade oculta (Poulin, 1998), que além de sofrer influência do ambiente por serem os primeiros a serem prejudicados devido ao desgaste energético de seu hospedeiro (Gibb e Hochuli, 2002, Laurance et al., 2002), também podem atuar como reguladores da população hospedeira em meio ao dinamismo ambiental (Poulin, 1995, 1997, 1998). Estes podem ser parasitas de várias classes de vertebrados e compreendem quatro grupos principais: Trematoda, Cestoda, Nematoda e Acanthocephala.

O estudo da fauna parasitária, apesar de ser bastante incipiente, é extremamente importante, contribuindo com o conhecimento da biodiversidade e com o entendimento da relação parasita-hospedeiro (Brooks e Hoberg, 2001; Poulin e Morand, 2004). Além disso, os parasitas podem estar relacionados a muitos aspectos biológicos de seus hospedeiros, assim como a qualidade do ambiente, podendo ser utilizados como indicadores de perturbação do ecossistema (Galli et al., 2001). Os helmintos parasitas vivem no interior de vertebrados e invertebrados parasitando o sistema cardiovascular, respiratório, digestório, nervoso, excretor, urinário, reprodutor e cavidades (Travassos, 1950). Muitos helmintos têm ciclos de vida complexos, que requerem um período de desenvolvimento em um ou mais hospedeiros intermediários e normalmente fase larval de vida livre (Anderson, 2000).

A identificação de espécies baseada em características morfológicas é biologicamente insatisfatória (De Queiros, 2005; Baum e Donoghue, 1995). Existem grandes problemas em aplicar o conceito morfológico para delimitar espécies, como por exemplo a existência de espécies crípticas, “Populações naturais morfologicamente idênticas ou muito semelhantes, mas reprodutivamente isoladas” (e.g. espécies do gênero

Rhabdias); espécies politípicas, “espécies com variedades de formas” e as espécies com plasticidade fenotípica, “espécies com ampla variação morfológica relacionada ao seu hospedeiro” (e.g. *Mesocoelium monas*) (Mayr, 1963; Bickford et al., 2007; Tkach et al., 2014).

Segundo Poulin *et al.* (2019), o uso de informação genética é fundamental na taxonomia sistemática de parasitas, especialmente para descrições de novas espécies, uma vez que essa ferramenta permite que problemas taxonômicos entre alguns grupos sejam resolvidos por meio de filogenias moleculares, descrição de padrões de fluxo gênico entre espécies e populações e, principalmente, para compreensão da diversidade em grupos de parasitas complexos (Tkach et al., 2014; Müller et al., 2018). Com a implementação de técnicas moleculares o número de novas espécies descritas aumentou, revelando a existência de espécies crípticas, não detectadas por abordagens morfológicas tradicionais (Derycke et al., 2010; Nadler e Pérez-Ponce de León, 2011; Jorge et al., 2013; Ristau et al., 2013).

Dessa forma a taxonomia clássica tem sido incrementada, atualmente, com dados da biologia molecular, ou seja, o uso de caracteres morfológicos e moleculares, com isso tem gerado novos e relevantes dados sobre a fauna de helmintos de anuros (Müller et al., 2018, 2021; Willkens et al., 2019, 2021; Morais et al., 2020; Queiroz et al., 2021, Tavares-Costa et al., 2022). Apesar do aumento no número de estudos sobre a diversidade de helmintos parasitos de anuros, bem como a utilização de ferramentas moleculares, a fauna parasitária dos anuros brasileiros ainda é pouco investigada. Muitas espécies ainda necessitam de revisões taxonômicas e análises moleculares, uma vez que, com o avanço dessa metodologia, é notável o aumento de descobertas de novas espécies e reorganizações filogenéticas desses organismos.

Objetivos

OBJETIVOS

Objetivo geral

Inventariar a helmintofauna associada a anuros de oito estados brasileiros, com o uso da taxonomia integrativa através de análises morfológicas e moleculares.

Objetivos específicos

I. Realizar a taxonomia baseada em caracterização morfológica e molecular de parasitos de anfíbios;
II. Promover inventários estruturados e descrições de espécies de helmintos.
III. Realizar análise filogenética (filogenia molecular) de possíveis novas espécies de helmintos;

Material e Métodos

MATERIAL E MÉTODOS

Autorizações

O estudo foi realizado com autorização do Sistema de Autorização e Informação em Biodiversidade (SISBIO) (nº do processo 61940) e também do Comitê de Ética na Utilização Animal da Universidade Estadual Paulista "Júlio de Mesquita Filho" (CEUA - UNESP) (nº do protocolo 1061). O presente estudo conta com a ajuda do Prof. Dr. André Luiz Quagliatto Santos pesquisador e professor da Universidade Federal de Uberlândia e do Prof. Dr. Lucas Forti professor e pesquisador da Universidade Federal da Bahia.

Caracterização das áreas de estudo

O presente estudo foi desenvolvido em 21 municípios distribuídos entre os Estados de Ceará, Goiás, Mato Grosso, Mato Grosso do Sul, Paraná, Rio Grande do Sul, Santa Catarina e São Paulo (Figura 1, Tabela 1).

Estado do Ceará

Situado ao norte da região Nordeste, o estado do Ceará é caracterizado pelo complexo de vegetação composta de Mata úmida (Floresta Subperenifólia Tropical Plúvio-Nebular), Mata seca (Floresta Subcaducifólia Tropical Pluvial), Cerrado, Cerradão (Floresta Subcaducifólia Tropical xeromorfa) e Carrasco, ambientes esses que compõem uma matriz de Caatinga (Borges-Nojosa e Caramaschi, 2003). As coordenadas dos locais de coleta estão descritas na Tabela 1.

Estado de Goiás

Situado na região Centro-Oeste, o estado de Goiás é caracterizado pelo bioma Cerrado, cuja vegetação é marcada por árvores e arbustos tortuosos, cascas grossas e raízes profundas. O local onde a coleta foi realizada faz parte da Bacia Tocantins-Araguaia, e margeia o Rio Vermelho que deságua no Rio Araguaia, região localizada entre as cidades Aruanã e Britânia. As coordenadas dos locais de coleta estão descritas na Tabela 1.

Estado de Mato Grosso

As coletas foram realizadas na região que se localiza na divisa com o Estado de Goiás (Leste), cujo bioma é de transição entre Cerrado e Pantanal. As coordenadas do local de coleta estão descritas na Tabela 1.

Estado do Mato Grosso do Sul

As coletas foram realizadas na Reserva Particular de Patrimônio Natural Cisalpina, Brasilândia, Mato Grosso do Sul, onde o bioma predominante é o Pantanal, além do que se trata da área de confluência dos rios Verde e Paraná, no município de Brasilândia/MS. É uma extensa várzea inundada periodicamente na época das chuvas, com um complexo sistema de lagoas, córregos e canais interligados entre si e ao canal do rio Paraná. As coordenadas do local de coleta estão descritas na Tabela 1.

Estado de São Paulo

Nesse estado, onde predomina o bioma Mata Atlântica, foram coletados anuros procedentes de quatro Parques Estaduais (Parque Estadual Carlos Botelho; Parque Estadual Intervales; Parque Estadual da Caverna do Diabo e Parque Estadual do Turístico do Alto Ribeira) e também foram realizadas coletas na Fazenda São Sebastião do Paraíso e proximidades. As coordenadas dos locais de coleta estão descritas na Tabela 1.

Estado do Paraná

As coletas foram realizadas em três Parques Estaduais (Parque Estadual do Rio Guarani; Parque Nacional do Iguaçu e Parque Estadual do Turvo). As áreas de coletas estão inseridas no bioma Mata Atlântica, composta por floresta estacional semidecidual e a floresta ombrófila mista. As coordenadas dos locais de coleta estão descritas na Tabela 1.

Estado de Santa Catarina

As coletas foram realizadas no município São Miguel do Oeste. A área de estudo é caracterizada pela floresta ombrófila mista que faz parte do bioma Mata Atlântica. As coordenadas dos locais de coleta estão descritas na Tabela 1.

Estado do Rio Grande do Sul

Rio Grande do Sul é caracterizado por dois importantes biomas brasileiros: a Mata Atlântica e o Pampa. As coletas foram realizadas no município de Frederico Westphalen,

onde o seu território é composto 100% pelo bioma Mata Atlântica, com floresta ombrófila. As coordenadas dos locais de coleta estão descritas na Tabela 1.

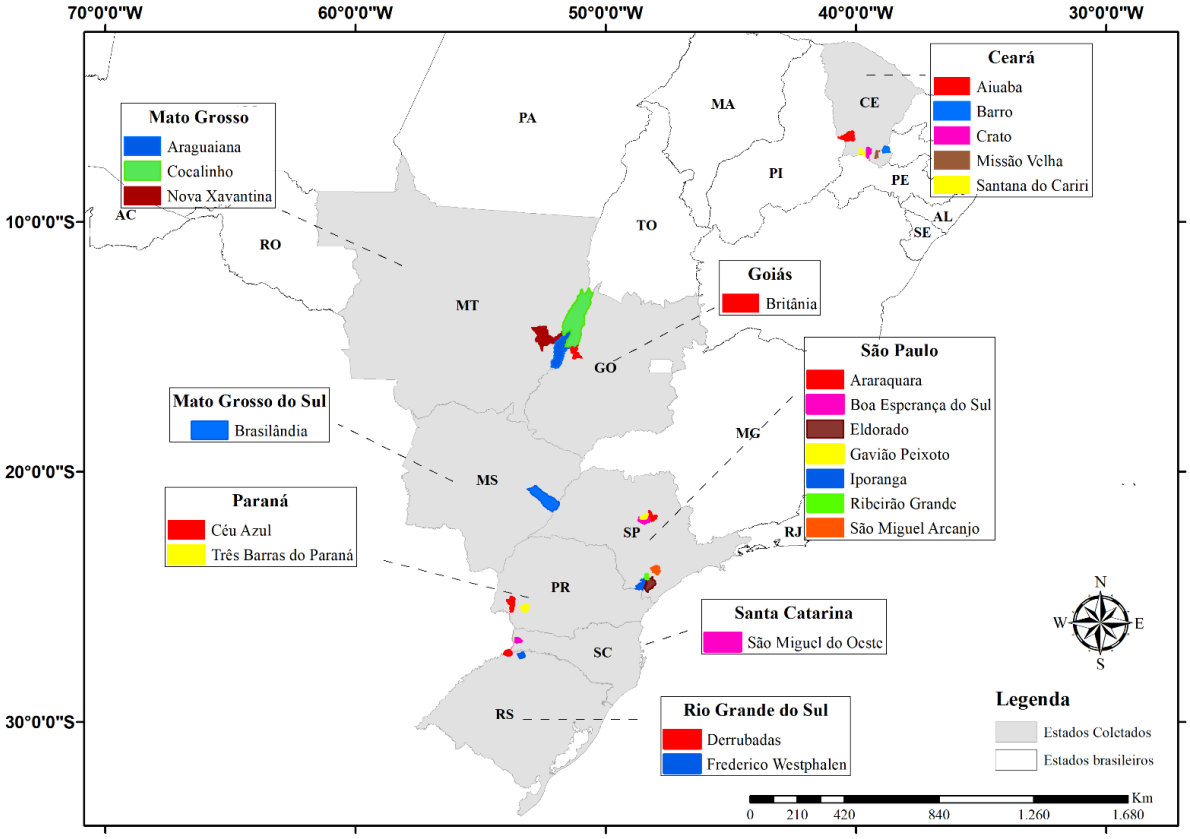

Figura 1. Mapa das localidades com todos os municípios que foram amostrados para a helmintofauna de anuros.

Tabela 1: Lista de Localidades amostradas com suas respectivas coordenadas geográficas. CE = Ceará; SP = São Paulo; PR = Paraná; RS = Rio Grande do Sul; ST = Santa Catarina; MS = Mato Grosso do Sul; MT = Mato grosso; GO = Goiás.

Localidade	Município	Estado	Coordenadas
Chapada do Araripe	Crato	CE	7º16'47.0"S, 39º26'17.7"O
Pontal de Santa Cruz	Santana do Cariri	CE	7º12'37.8"S, 39º44'00.8"O
Distrito de Cuncas	Barro	CE	7º10'36"S, 38º46'54"O
Estação Ecológica de Aiuaba	Aiuaba	CE	6°40'24.2"S, 40°12'49.6"O
Floresta Petrificada	Missão Velha	CE	7°15'58.8"S, 39°04'56.9"O
Parque Estadual Carlos Botelho	São Miguel Arcanjo	SP	24°3'44.34"S, 47°59'32.41"O
Parque Estadual Turístico do Alto Ribeira	Iporanga	SP	24°32'0.1"S, 48°41'57.34"O
Parque Estadual Caverna do Diabo	Eldorado	SP	24°38'10.78"S, 48°24'12.39"O
Parque Estadual Intervales	Ribeirão Grande	SP	24°16'28.21"S, 48°25'2.44"O
Faz. São Sebastião do Paraíso	Boa Esperança do Sul	SP	21°51'36.83"S, 48°27'3.56"O
Fazenda Santa Júlia	Gavião Peixoto	SP	21°50'43.74"S, 48°28'43.43"O
Fazenda Carandá	Araraquara	SP	21°52'30.28"S, 48°13'50.78"O
Pesqueiro - Faz. São Sebastião do Paraíso	Gavião Peixoto	SP	21°50'41.83"S, 48°28'10.24"O
Parque Nacional do Iguaçu	Céu Azul	PR	25°9'4.03"S, 53°50'28.77"O
Parque Estadual Rio Guarani	Três Barras	PR	25°26'42.87"S, 53°9'37.87"O
Parque Estadual do Turvo	Derrubadas	RS	27°8'31.68"S, 53°52'39.35"O
Frederico Westphalen	Frederico Westphalen	RS	27°21'43.36"S, 53°24'38.32"O
São Miguel do Oeste	São Miguel do Oeste	SC	26°45'36.10"S, 53°31'30.47"O
RPPN Cisalpina	Brasilândia	MS	21°15'34.36"S, 51°54'18.96"O
Riacho cano RPPN Cisalpina	Brasilândia	MS	21°16'0.49"S, 51°54'7.65"O
Lagoa buraco 2 RPPN Cisalpina	Brasilândia	MS	21°11'18.00"S, 51°54'12.40"O
Estrada rio Paraná RPPN Cisalpina	Brasilândia	MS	21°13'38.20"S, 51°52'37.10"O
Ao redor da AIQ 2, RPPN Cisalpina	Brasilândia	MS	21°17'30.60"S, 51°54'50.30"O
Estrada Rio Bom Jardim RPPN Cisalpina	Brasilândia	MS	21°17'20.98"S, 51°55'2.76"O
Estrada Rhinella RPPN Cisalpina	Brasilândia	MS	21°17'41.10"S, 51°55'11.60"O
Rio novo RPPN Cisalpina	Brasilândia	MS	21°19'53.47"S, 51°56'39.61"O
Estrada Beira Rio RPPN Cisalpina	Brasilândia	MS	21°16'40.23"S, 51°55'30.20"O
Rancho do Japonês	Araguaiana	MT	14°35'49.1"S, 51°42'55.71"O
Alojamento CEPEMA	Cocalinho	MT	14°31'40.16"S, 51°41'34.07"O
Rio Pindaíba. Rancho do Japonês	Araguaiana	MT	14°35'47"S, 51°43'9.59"O
Riacho 2	Araguaiana	MT	14°32'51.38"S, 51°40'47.96"O
Lagoa 2. Rancho do Japonês	Araguaiana	MT	14°35'53.44"S, 51°42'56.43"O
Fazenda Boa Esperança - Lago Jacaré	Cocalinho	MT	14°46'44.82"S, 51°32'50.86"O
Fazenda Boa Esperança	Cocalinho	MT	14°46'32.27"S, 51°32'50.29"O
Riacho	Nova Xavantina	MT	14°31'48.03"S, 51°41'43.88"O
Riacho 1	Araguaiana	MT	14°32'13.49"S, 51°40'38.81"O
Faz. Amado Batista	Cocalinho	MT	14°28'39.27"S, 51°36'31.95"O
Banhado estrada (lago Pseudis)	Cocalinho	MT	14°45'56.81"S, 51°32'18.29"O
Estrada	Araguaiana	MT	14°35'5.29"S, 51°41'19.15"O

Continuação da Tabela 1

Estrada	Araguaiana	MT	14°33'16.46"S, 51°40'52.44"O
Fazenda Sertaneja Retiro	Cocalinho	MT	14°26'19.98"S, 51°35'20.17"O

Coleta dos hospedeiros

Entre o período de agosto de 2017 a fevereiro de 2021, 494 espécimes de anuros foram coletados. As áreas de amostragem foram investigadas utilizando-se os métodos de busca ativa (Corn e Bury, 1990) e encontros ocasionais. As coletas foram realizadas nos períodos noturno e diurno, nas estações seca e chuvosa.

Após a captura os anuros foram eutanasiados com solução de tiopental sódico, em seguida tiveram seus comprimento-rostro-cloacal (CRC) aferido utilizando-se um paquímetro (precisão de 0,01 mm) e sua massa foi obtida com o uso de balanças Pesola®. Em seguida tiveram todos os órgãos internos e a cavidade celomática avaliados quanto a presença de helmintos e, posteriormente, foram fixados em solução de formol 10%, preservados em etanol 70%, e tombados na coleção Herpetológica da Universidade Federal do Ceará - CHUFC e noMuseu de Zoologia "prof. Adão José Cardoso," Universidade Estadual de Campinas (Unicamp), Campinas, São Paulo, Brazil .

Coleta, preparo e identificação dos helmintos

Os anuros foram necropsiados avaliando os respectivos sítios de infecção quanto à presença de helmintos parasitas: trato gastrointestinal, sistema respiratório, urinário, fígado, vesícula biliar e inspeção da cavidade abdominal. Todos os órgãos foram separados em placas de Petri contendo solução salina 0,85% de NaCl ou água e examinados em microscópio estereoscópico. Para cada espécie de parasita encontrado foi registrado o sítio de infecção e a quantidade de espécimes e, posteriormente, acondicionados em frascos contendo solução de álcool etílico 70% e em álcool 96% para análises moleculares. Os helmintos coletados foram fixados seguindo a metodologia adequada para cada grupo (Amato et al., 1991).

Nematoda

Os nematoides coletados foram fixados em solução de álcool-formol-ácido acético (AFA) à quente (Amato et al., 1991) e foram acondicionados em frascos individuais para

análises posteriores. Para a identificação, esses nematoides foram submetidos à clarificação pelo lactofenol de Aman (Andrade, 2000) e montados temporariamente entre lâmina e lamínula para identificação sob o microscópio a partir da observação dos caracteres taxonômicos (Figura 2). As identificações foram baseadas nos trabalhos de Yamaguti (1961), Vicente *et al.* (1991), além de artigos recentes de descrição de espécies.

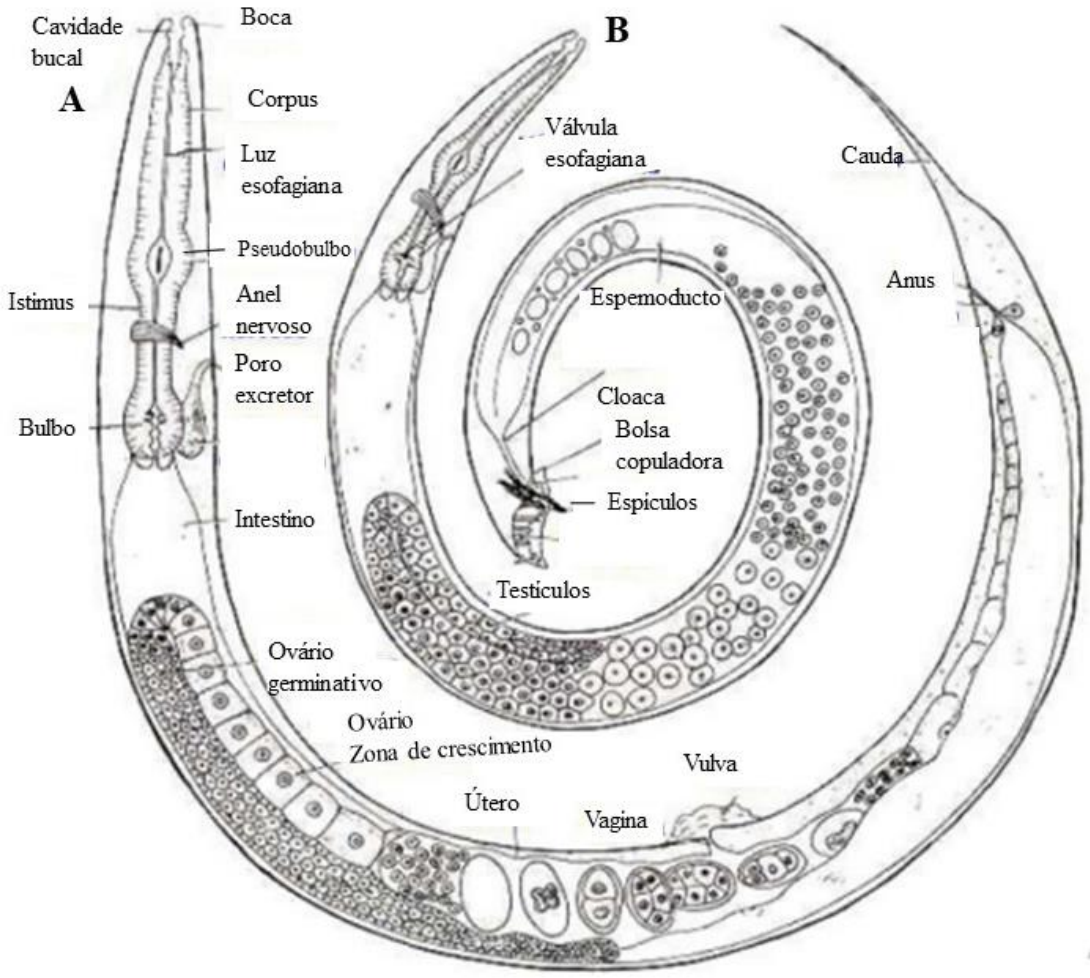

Figura 2. Esquema da morfologia geral de nematoides representado por uma fêmea (**A**) e um macho (**B**). (Adaptado Hirschmann, 1960).

Digenea

Os digenéticos coletados foram fixados após compressão entre lâmina e lamínula em solução aquecida de AFA (Amato et al., 1991) e em seguida acondicionados em frascos individuais. Para a identificação, os digenéticos foram corados pela técnica do carmim clorídrico (Andrade, 2000; Rey, 2001), submetidos a uma série alcoólica (70%, 80%, 90% e 100%) e em seguida montados temporariamente entre lâmina e lamínula em eugenol

(Andrade, 2000; Rey, 2001). Após a montagem, os digenéticos foram analisados sob microscópio de luz para a observação das estruturas de valor sistemático (Figura 3). Para a identificação das espécies de digenéticos foram utilizados os trabalhos de Travassos et al., (1969), Yamaguti (1971), Fernandes e Kohn (2014), além de outras bibliografias mais recentes que se fizeram necessárias.

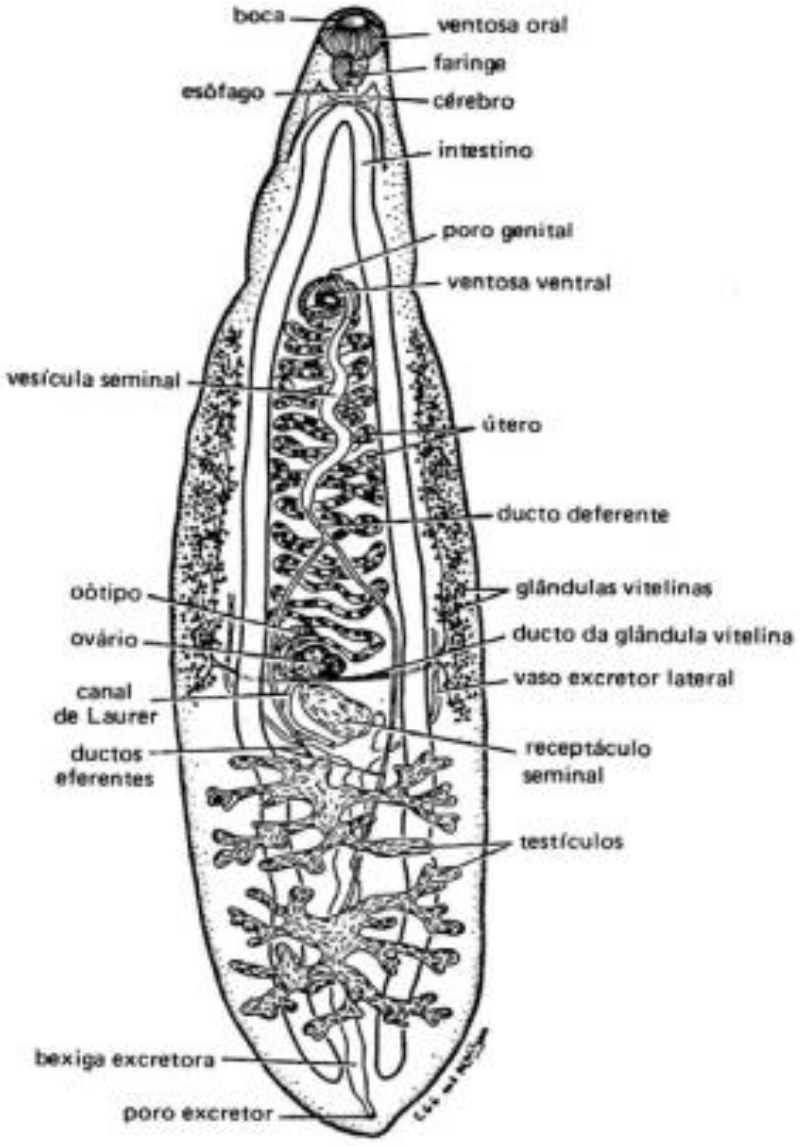

Figura 3. Esquema da morfologia geral de digenéticos mostrando os órgãos de fixação e sistemas digestivo, excretor e reprodutivo. Adaptado de Noble e Noble (1982).

Cestoda

Os cestoides foram fixados em solução aquecida de AFA, sem compressão, corados pela técnica do carmim clorídrico (Andrade, 2000; Rey, 2001), submetidos a uma série alcoólica (70%, 80%, 90% e 100%) e em seguida montados temporariamente entre lâmina e lamínula em eugenol. Após a montagem, foram analisados sob microscópio de luz para a observação dos caracteres morfológicos (Figura 4). A identificação foi baseada nos trabalhos de Yamaguti (1959) e Schmidt (1986), além de outras bibliografias mais recentes que se fizeram necessárias.

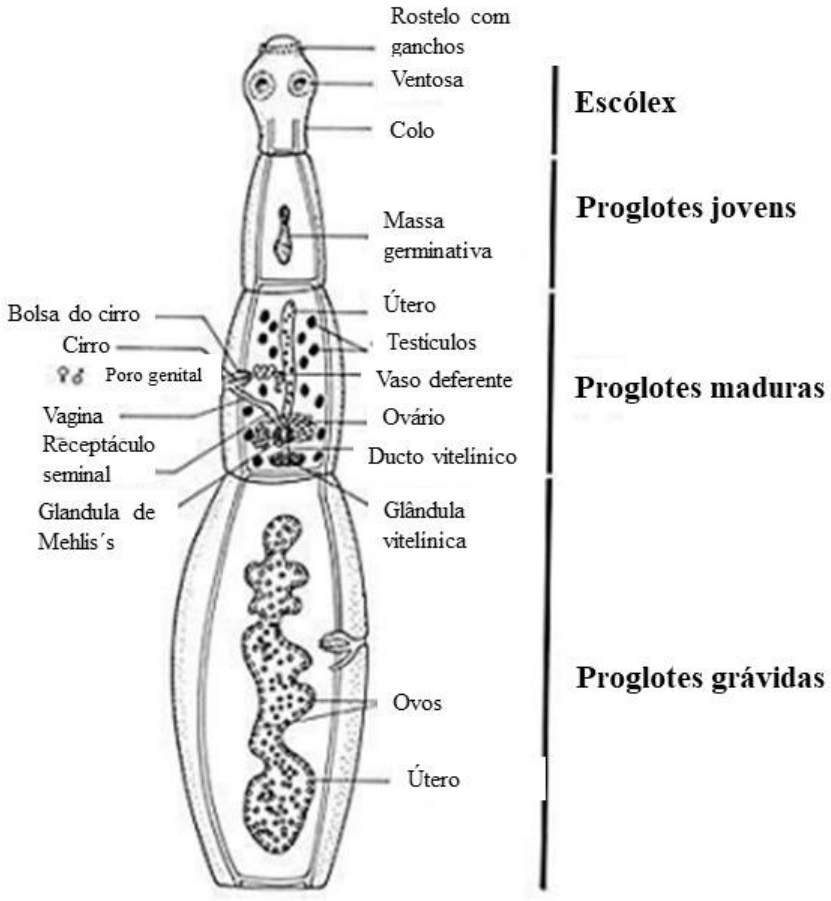

Figura 4. Esquema da morfologia geral de cestoides mostrando os órgãos de fixação e sistema reprodutivo. Adaptado de Moglan e Popescu (2009)

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Monogenea

Os monogenéticos coletados foram submetidos à fixação após compressão entre lâmina e lamínula e fixados em solução de AFA (Amato et al., 1991) e em seguida acondicionados em frascos individuais. Para a identificação, os monogenéticos foram corados pela técnica do carmim clorídrico (Andrade, 2000; Rey, 2001), submetidos a uma série

alcoólica (70%, 80%, 90% e 100%) e em seguida montados temporariamente entre lâmina e lamínula em eugenol (Andrade, 2000; Rey, 2001). Após a montagem, os monogenéticos foram analisados sob microscópio de luz para a observação dos caracteres morfológicos (Figura 5). Para a identificação das espécies de monogenéticos foram utilizados o trabalho de Cohen (2013) e bibliografias mais recentes que se fizeram necessárias.

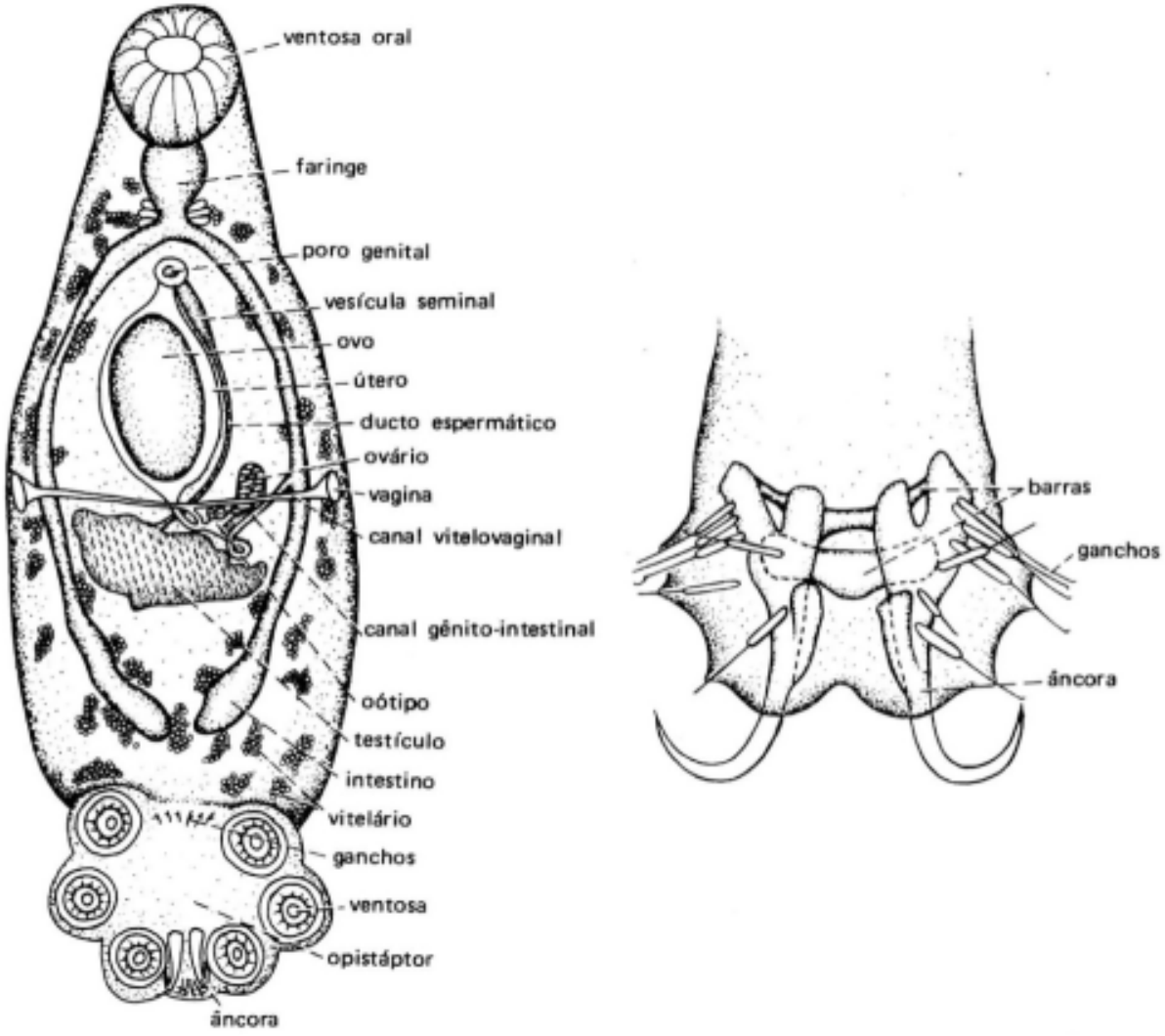


Figura 5. Esquema da morfologia geral de monogenea mostrando os órgãos de fixação e sistema reprodutivo. Adaptado de Smyth (1976).

Acanthocephala

Cistacantos de acantocéfalos foram coletados e colocados em placa de Petri contendo água destilada e então mantidos sob refrigeração até a completa exposição da probóscide. Uma vez obtida a exposição da probóscide, foram submetidos à fixação empregando-se o AFA (Amato et al., 1991). As amostras dos acantocéfalos foram coradas pela técnica do

carmim clorídrico submetidos a uma bateria crescente de álcool etílico (70%, 80%, 90% e 100%) e em seguida foram montados temporariamente entre lâmina e lamínula em meio de eugenol. Posteriormente, foram analisados sob microscópio de luz para a observação dos caracteres morfológicos (Figura 6). A identificação foi baseada no trabalho de Yamaguti (1963), além de outras bibliografias mais recentes que se fizeram necessárias.

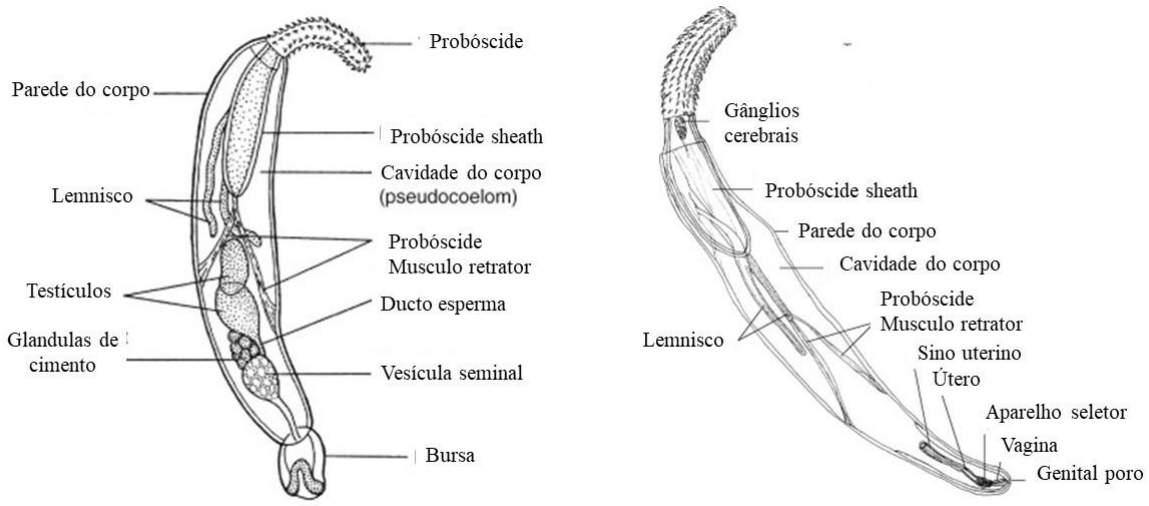


Figura 6. Esquema da morfologia geral de Acantocéfalos. Adaptado de Margulis e Chapman (2009).

Os dados morfométricos e fotomicrografias dos helmintos foram obtidos em sistema computadorizado de análise de imagens LAS V3 (Suíte Leica Application V3; Leica Microsystems, Wetzlar, Alemanha), em microscópio Leica com contraste diferencial de interferência (DIC), no Laboratório de Parasitologia de Animais Silvestres, do Setor de Parasitologia do Instituto de Biociências, UNESP-Botucatu. Para cada espécie foram aferidas suas medidas em micrometro (µm), exceto quando indicado outra unidade de medida, sendo apresentada para cada estrutura sua variação. As possíveis novas espécies de helmintos, foram desenhadas em câmara clara adaptada em microscópio óptico DMLS (Leica).

Para auxiliar na identificação das espécies de helmintos, alguns parasitos foram processados em microscopia eletrônica de varredura (MEV). O material foi fixado em glutaraldeído 2,5% em tampão fosfato 0,1 M pH 7.3 (24h) e pós-fixado em tetróxido de ósmio 1% (2h) no mesmo tampão. A desidratação foi realizada em sequência crescente de soluções de álcool etílico e a secagem realizada por meio de ponto crítico em CPD 020 (Balzer Union), com CO_2 líquido. Os espécimes foram colocados em suporte adequado e recobertos com

camada de 20 nm ouro em aparelho MED 010 (Balzer Union). A análise foi realizada em MEV-SEM 515 da Philips.

Os helmintos serão depositados na Coleção Helmintológica do Instituto de Biociências (CHIBB) da Universidade Estadual Paulista (Unesp), Câmpus Botucatu. Alguns helmintos foram e serão depositados na coleção Helmintológica do Instituto Oswaldo Cruz (CHIOC).

Análises moleculares e filogenética

O DNA genômico foi extraído através do Kit comercial DNeasy® Blood&Tissue (Qiagen) de acordo com o protocolo do fabricante, com volume final de 30 µl. Os fragmentos de DNA foram amplificados usando a menor (18S) e a maior (28S) subunidade do gene ribossomal, além do ITS (Chilton et al., 2006) e o gene mitocondrial COI com a região Barcoding (Folmer et al., 1994).

As reações de amplificação consistiram no volume final de 25 µl com 3 µl do DNA extraído, 0,5 ou 1,0 µl de cada primer, 7,5 ou 8,5 µl de água ultrapura (Sigma-Aldrich, Reino Unido) e 12,5 µl de Master Mix MyFiTM Mix Bioline®. Os produtos de PCR (2 µl) foram aplicados em gel de agarose (1%) usando o gel Red e Loading Buffer para confirmar os tamanhos dos amplicons e a quantidade dos fragmentos. Os produtos foram purificados usando o kit QIAquick PCR Purification (Qiagen) e o sequenciamento automatizado foi realizado diretamente em produtos de PCR dos espécimes com o kit BigDye v.3.1 Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA, USA). Sequências foram aplicadas em corridas no analisador da Applied Biosystems ABI 3500 DNA. Os primers de amplificação utilizados para cada gene ou região e as condições especificas de ciclo de PCR estão descriminados na Tabela 2.

A identidade das sequências foi verificada através do BLAST (Basic Local Alignment Search Tool) e sequências contíguas foram montadas e editadas usando o programa Sequencher v. 5.2.4 (Gene Codes, Ann Arbor, MI). Sequências serão enviadas para o Banco de Dados GenBank após o aceite dos manuscritos. O alinhamento foi feito pelo Muscle implementado no Geneious versão 7.1.3 (Kearse et al., 2012).

Para a reconstrução filogenética foram usados os métodos de Máxima Verossimilhança (Maximum Likelihood) (ML) usando o programa RAxML-HPC v.8 rodado na ferramenta XSEDE contido no portal CIPRES Science (www.phylo.org) (Miller et al., 2010) e Inferência Bayesiana (BI) usando o programa MrBayes v. 3.2.7 rodados na ferramenta XSEDE contido no portal CIPRES Science (www.phylo.org) (Miller et al., 2010).

Os comandos e configurações específicas para cada tipo de análise estão discriminados nos capítulos correspondentes. Para os métodos de confiabilidade foram usados o bootstrap para a análise de Máxima Verossimilhança e probabilidade "a posteriori" para a análise Bayesiana. O programa Mega 5.05 (Tamura et al., 2011) foi usado para a realização das análises de distâncias genéticas usando o método Neighbor Joining. As árvores resultantes de cada análise foram visualizadas usando o programa FigTree v.1.3.1 (Rambaut, 2009) e editadas no programa CorelDraw X6.

Tabela 2: Primers de amplificação utilizados para cada gene, condições do ciclo e sus respectivas referências.

Gene/região	Sequencia 5'-3'	Condições do ciclo	Referência	Helminto
Ribossomal				
18S				
	#18S-F CGCGAATRGCTCATTACAACAGC #18S-R GGGCGGTATCTGATCGCC	94°C/5min, 94°C/30s, 55°C/30s, 72°C/30s, 72°C/1min 10s, 72°C/7min (30 ciclos)	Floyd et al., (2005)	Cosmocercidae
	Ces 1 CCAGCAGCCGCGGTAACTCCA Ces 2 CCCCCGCCTGTCTCTTTTGAT	94ºC/4min, 94ºC/30s, 60ºC/30s, 72ºC/30s, 72ºC/5min (35 ciclos)	Skeriková et al., (2001)	Cestoda
ITS	#93 TTGAACCGGGTAAAAGTCG #94 TTAGTTTCTTTTCCTCCGCT	94°C/ 3min, 94°C/30s, 54°C/30s, 72°C/60s, 72°C/7min (35 ciclos)	Dare et al., (2008)	*Rhabdias* spp.
28S	#500 ACTTTGAAGAGAGAGTTCAAGAG #501 TCGGAAGGAACCAGCTACTA	94°C/3min, 94°C/30s, 54°C/30s, 72°C/60s, 72°C/7min (35 ciclos)	Dare et al., (2008)	*Rhabdias* spp.
	#28S_F AGCGGAGGAAAAGAAACTAA #28Sb ATCCGTGTTTCAAGACGGG	94°C/5min, 94°C/30s, 55°C/30s, 72°C/30s, 72°C/1min 10s, 72°C/7min (30 ciclos)	Nadler e Hudspeth (1998)	Cosmocercidae
	U178 GCACCCGCTGAAYTTAAG L1642 CCAGCGCCATCCATTTTCA	95°C/3min, 94°C/30s, 56°C/30s, 72°C/1min 30s, 72°C/4min (34 ciclos)	Mendoza-Palmero et al., (2015)	Platyhelminthes

	Sequencia 5'-3'	Condições do ciclo	Referência	Helminto
Cont. da tabela 3				
	#28Srd1a CCCSSGTAATTTAAGCATATTA #28Sb TCGGAAGGAACCAGCTAC	95°C/3min, 95°C/30s, 54°C/30s, 72°C/1im 72°C for 10 min (40 ciclos)	Whiting (2002)	*Parapharyngodon* spp.
Mitocondrial				
COI	Rhabdias COI1F GGKTTTTTTATGGGTAAYGGTC Rhabdias COIR GCNCCAGCYAANACWGGNAAAG	94°C/3min, 94°C/30s, 47°C/30s, 72°C/1min, 72°C/7min (35 ciclos)	Müller et al., 2018	*Rhabdias* spp.
	COIF TTTTTTGGTCATCCTGAGGTTTAT COIR ACATAATGAAAATGACTAACAAC	94°C/5min, 94°C/30s, 55°C/30s, 72°C/70s, 72°C/7min (30 ciclos)	Lazarova et al. 2006; Chen et al. 2018	Cosmocercidae
	JB3 TTTTTTGGGCATCCTGAGGTTTAT JB4 TAAAGAAAGAACATAATGAAAATG	94°C/5min, 92°C/30s, 47°C/45s, 72°C/1:30s, 72°C/10min (40 ciclos)	Morgan e Blair (1998); Razo-Mendivil et al., (2008)	Trematoda
	Nem F1_t1 tgtaaaacgacggccagtCRACWGTWAATCAYAARAA TATTGG Nem R1_t1 caggaaacagctatgactAAACTTCWGGRTGACCAAA AAATCA	94°C/40s, 45°C/40s, 72°C/1 min, 94°C/40s, 51°C/40s, 72 °C/1min, 72°C/5min (35 ciclos)	Prosser et al., (2013)	*Oswaldocruzia* spp.
	Nem160F TTRGTRGGRKRTCDTTKTC Nem646R AGCYCCMGCYAAWACAGG	95°C/3min, 95°C/30s, 45-55°C/30s, 72°C/30s, 72°C/10min (35 ciclos)	de Sousa et al., (2018)	*Parapharyngodon* spp.

Resultado geral

RESULTADO GERAL

Foram necropsiadas 494 espécimes de anuros, pertencentes a 50 espécies de sete famílias: Bufonidae, Hemiphractidae, Hylidae, Hylodidae, Leptodactylidae, Microhylidae e Phyllomedusidae (Tabela 3) e, destes, 300 espécimes estavam parasitados por alguma espécie de helminto. A espécie hospedeira mais representativa foi *Crossodactylus caramaschii* (56), seguida por *Leptodactylus macrosternum* (55) e *Scinax fuscovarius* (54).

Considerando todas as espécies de anuros analisadas, foi coletado o total de 28.787 espécimes de helmintos. Destes, 25.172 espécimes representantes de 50 *taxa* do filo Nematoda, 2.671 espécimes de 10 *taxa* da classe Trematoda, 64 espécimes de dois *taxa* da classe Cestoda, 67 espécimes de um *taxon* da subclasse Acanthocephala e 16 espécimes pertencentes a um *taxon* da classe Monogenea (Figura 7 e 8). Até o presente momento, 15 novas espécies de helmintos foram registradas: 14 espécies de Nematoda e uma espécie de Trematoda e 14 possíveis novas espécies.

Tabela 3. Espécies de anuros analisados em oito estados brasileiros. N = número de hospedeiros necropsiados; P = número de espécimes que estavam parasitados; Nem. = número de espécimes de nematoides encontrados, Dig. = número de espécimes de digenéticos encontrados, Cest. = número de espécimes de cestoides encontrados, Acant. = número de espécimes de cistacantos de acantocéfalos encontrados e Monog. = número de espécimes de monogenéticos encontrados.

Família / Espécie hospedeira	Estado	N	P	Nem.	Dig.	Cest.	Acant.	Monog.
Bufonidae Gray, 1825 (5 spp.)								
Rhinella diptycha (Cope, 1862)	SP; GO; MS; MT;CE	41	28	1352	2519	0	3	0
Rhinella granulosa (Spix, 1824)	CE	2	2	162	5	9	0	0
Rhinella mirandaribeiroi (Gallardo, 1965)	MT	5	2	1	5	7	0	0
Rhinella ocellata (Günther, 1858)	MT	5	2	2	0	0	0	0
Hemiphractidae Peters, 1862 (1 spp.)								
Gastrotheca microdiscus (Andersson in Lönnberg & Andersson, 1910)	SP	4	04	19	0	0	0	0
Hylidae Rafinesque, 1815 (18 spp.)								
Boana albopunctata (Spix, 1824)	SP	1	0	0	0	0	0	0
Boana caiapo Pinheiro, Cintra, Valdujo, Silva, Martins, Silva & Garcia, 2018	MT	3	2	5		0	0	0
Boana cf. *raniceps*	MT; MS	4	2	90	1	0	0	0
Boana faber (Wied, 1821)	MT	1	1	0	0	2	0	0
Boana lundii (Burmeister, 1856)	SP	2	1	1	0	0	0	0
Boana raniceps (Cope, 1862)	CE; GO; MS; MT	35	16	85	2	0	9	0
Corythomantis greeningi Boulenger, 1896	CE	10	10	250	0	0	0	0
Dendropsophus aff. *microcephalus*	GO	1	1	1	0	0	0	0
Dendropsophus anataliasiasi (Bokermann, 1972)	MT	4	0	0	0	0	0	0
Dendropsophus araguaya (Napoli & Caramaschi, 1998)	MT	2	0	0	0	0	0	0
Dendropsophus nanus (Boulenger, 1889)	SP; MT	5	2	2	0	0	0	0
Dendropsophus soaresi (Caramaschi & Jim, 1983*)*	CE	1	1	0	0	0	0	0
Osteocephalus taurinus Steindachner, 1862	MT	6	4	181	0	0	0	0
Pseudis platensis Gallardo, 1961	MS; MT	9	7	18	46	6	0	0
Scinax fuscovarius (A. Lutz, 1925)	SP; MT	54	16	64	0	0	0	0
Scinax x-signatus (Spix, 1824)	SP; CE	14	8	251	0	0	0	0

Continuação da Tabela 1	**Estado**	**N**	**P**	**Nem.**	**Dig.**	**Cest.**	**Acant.**	**Monog.**
Scinax sp.	MS; SP	3	2	0	22	0	1	0
Trachycephalus typhonius (Linnaeus, 1758)	GO; MT; SP	7	6	107	0	0	0	0
Hylodidae Günther, 1858 (3 spp.)								
Crossodactylus caramaschii Bastos & Pombal, 1995	SP	56	54	367	0	33	6	0
Crossodactylus schmidti Gallardo, 1961	PR; RS; SC	27	15	76	12	0	0	0
Hylodes heyeri Haddad, Pombal & Bastos, 1996	SP	2	02	12	0	0	0	0
Leptodactylidae Werner, 1896 (1838) (18 spp.)								
Adenomera cf. *saci*	MT	6	1	2	0	0	0	0
Adenomera juikitam Carvalho & Giaretta, 2013	CE	4	0	0	0	0	0	0
Leptodactylus brevipes Cope, 1887	GO; MT	2	1	2	0	0	0	0
Leptodactylus fuscus (Schneider, 1799)	SP	4	1	102	0	0	0	0
Leptodactylus labyrinthicus (Spix, 1824)	SP; MT	17	16	8550	24	7	7	0
Leptodactylus macrosternum Miranda-Ribeiro, 1926	GO; MS; MT	55	36	3744	5	0	6	0
Leptodactylus mystaceus (Spix, 1824)	SP	9	9	198	0	0	0	0
Leptodactylus podicipinus (Cope, 1862)	SP; GO; MS; MT	15	9	104	1	0	3	0
Leptodactylus pustulatus (Peters, 1870)	GO; MT	6	6	1	29	0	2	0
Leptodactylus vastus A. Lutz, 1930	CE	2	2	212	0	0	3	16
Leptodactylus sp.	SP; MT	7	5	3299	0	0	0	0
Physalaemus centralis Bokermann, 1962	MT	2	1	4	0	0	0	0
Physalaemus cuvieri Fitzinger, 1826	CE; SP	7	2	32	0	0	0	0
Physalaemus marmoratus (Reinhardt & Lütken, 1862)	MS	1	1	1	0	0	0	0
Physalaemus nattereri (Steindachner, 1863)	MS; MT	9	3	0	0	0	3	0
Physalaemus sp.	MT	1	1	1	0	0	0	0
Pseudopaludicola mystacalis (Cope, 1887)	MT; GO	31	7	6	0	0	4	0
Microhylidae Günther, 1858 (1843) (4 spp.)								0
Chiasmocleis albopunctata (Boettger, 1885)	SP	2	1	10	0	0	0	0
Dermatonotus muelleri (Boettger, 1885)	MS	7	7	5603	0	0	20	0
Elachistocleis cesarii (Miranda Ribeiro, 1920)	MS; MT	2	2	4	0	0	0	0
Continuação da Tabela 1	**Estado**	**N**	**P**	**Nem.**	**Dig.**	**Cest.**	**Acant.**	**Monog.**

Phyllomedusidae Günther, 1858 (1 spp.)								
Pithecopus gonzagai (Caramaschi, 2006)	CE	1	1	251	0	0	0	0
Total de espécimes		**494**	**300**	**25.172**	**2.671**	**64**	**67**	**16**

Legenda: CE = Ceará; GO = Goiás, MT = Mato Grosso; MS = Mato Grosso do Sul; PR = Paraná; RS = Rio Grande do Sul ; SP = São Paulo; ST = Santa Catarina.

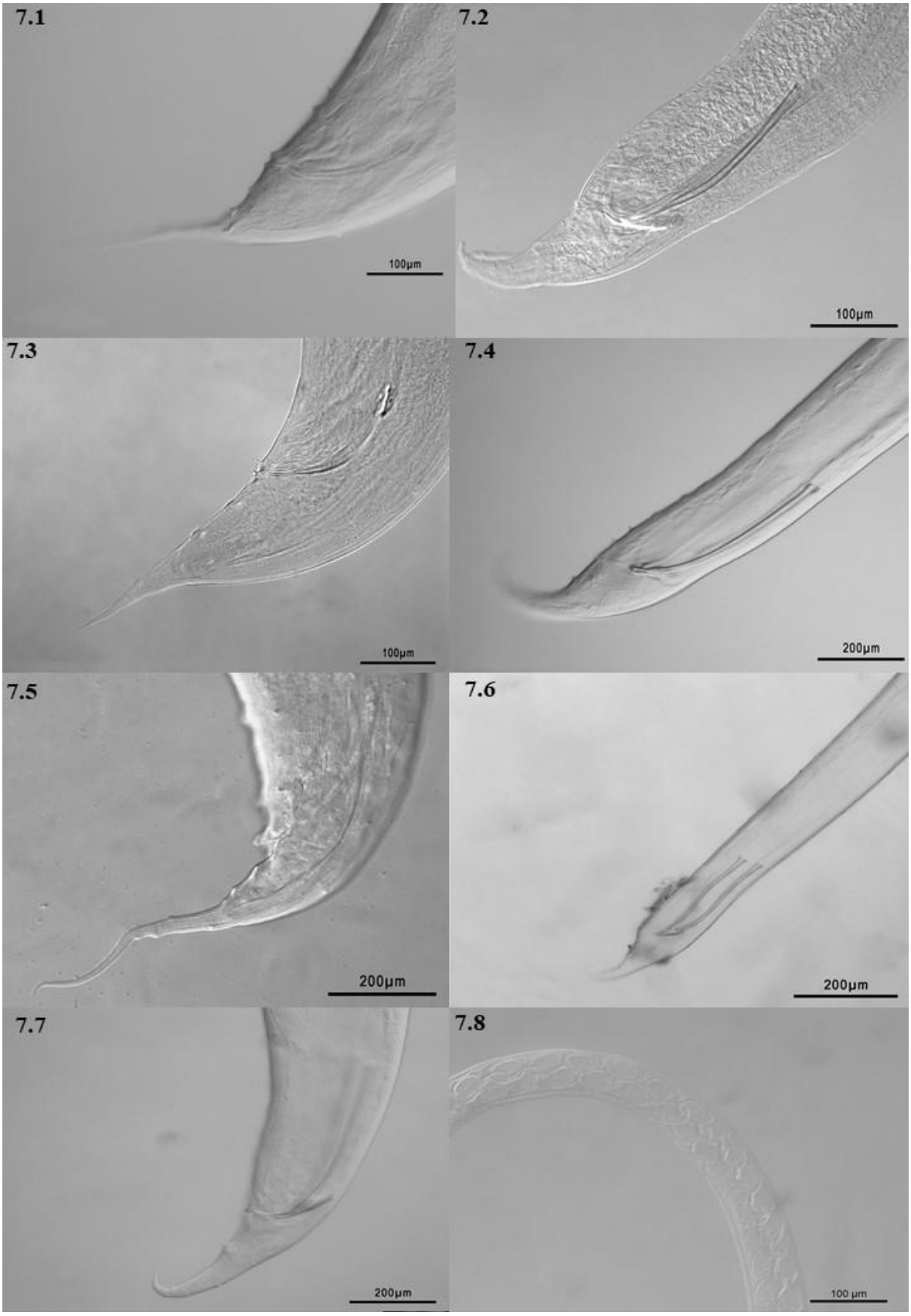
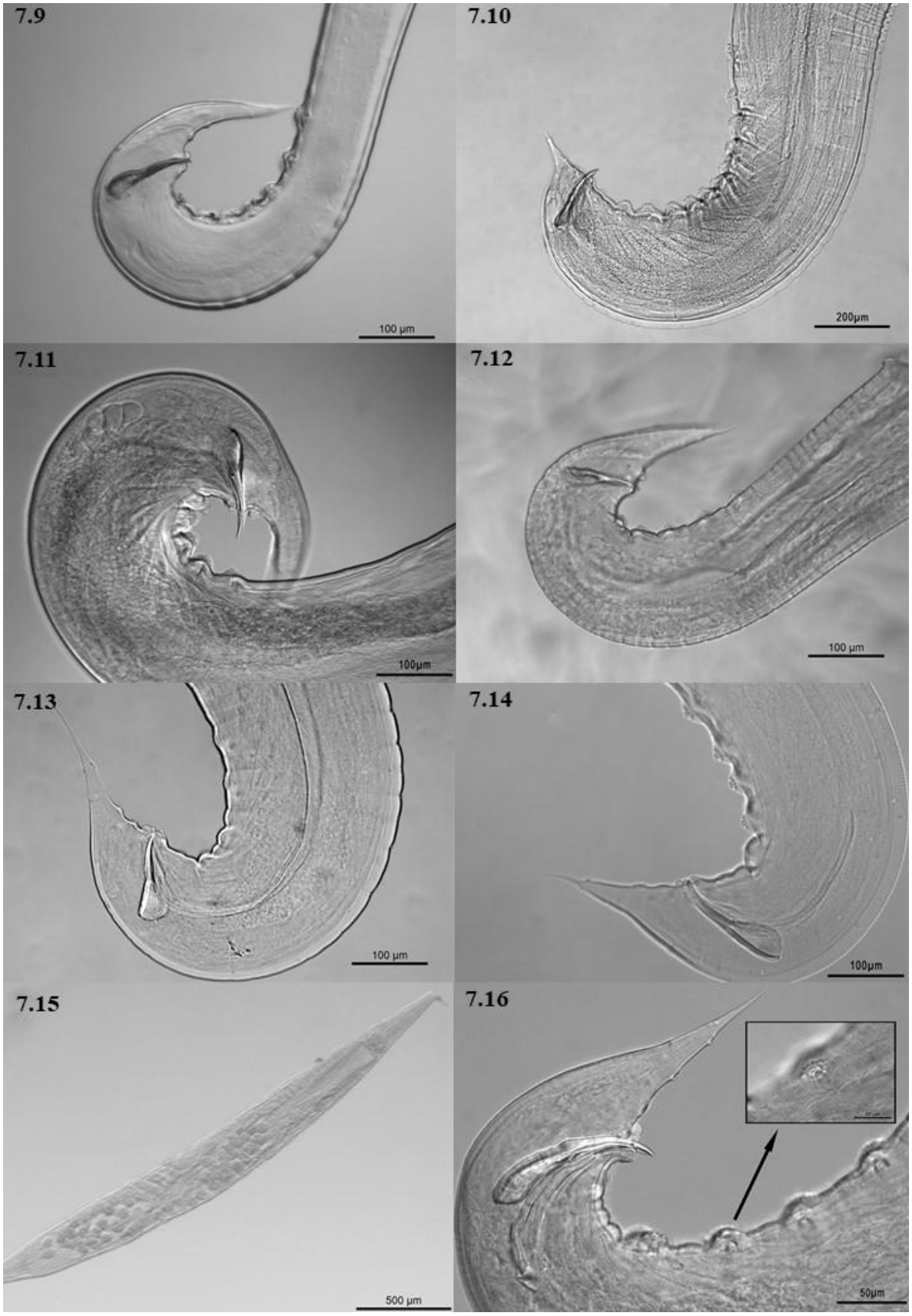
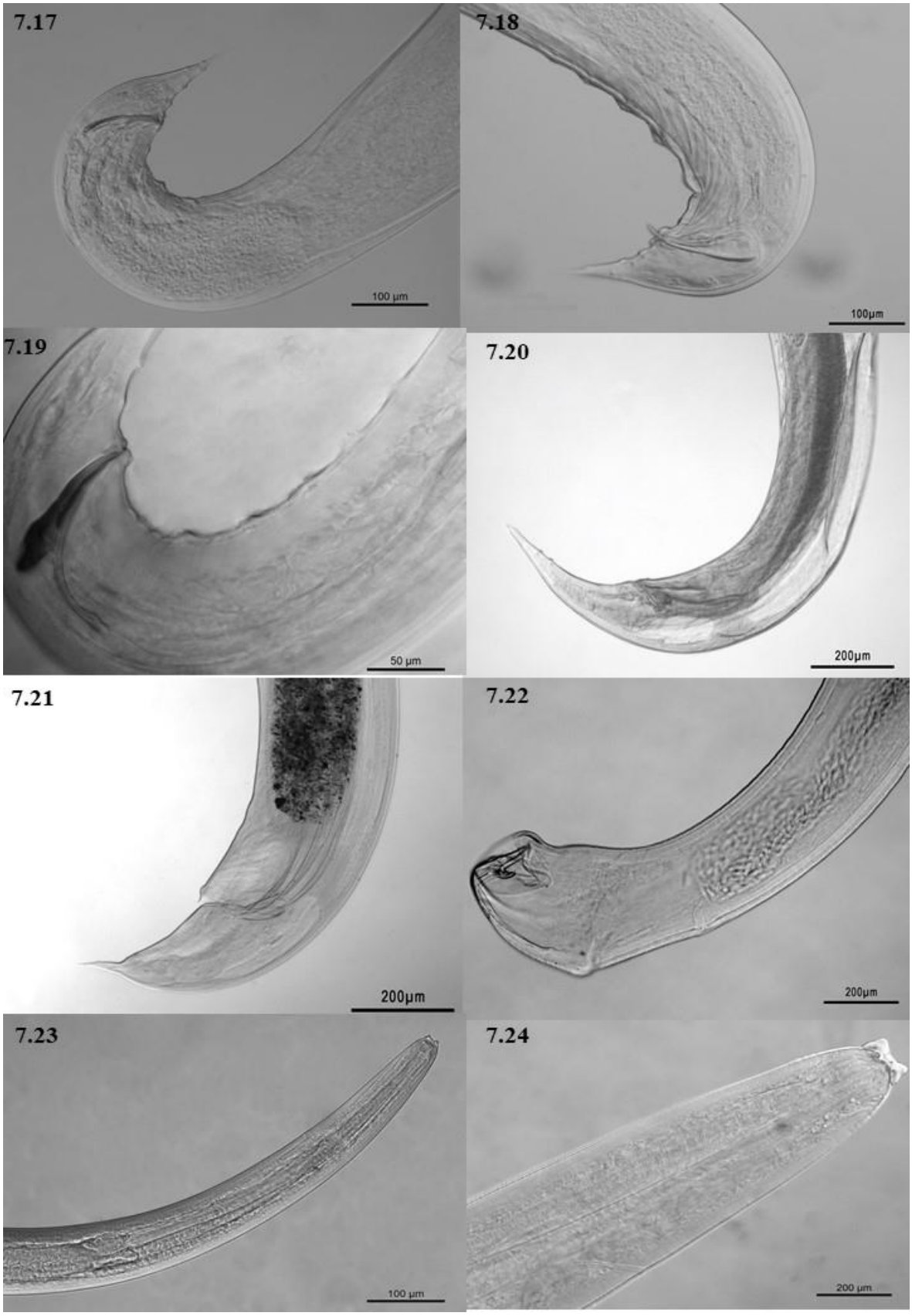


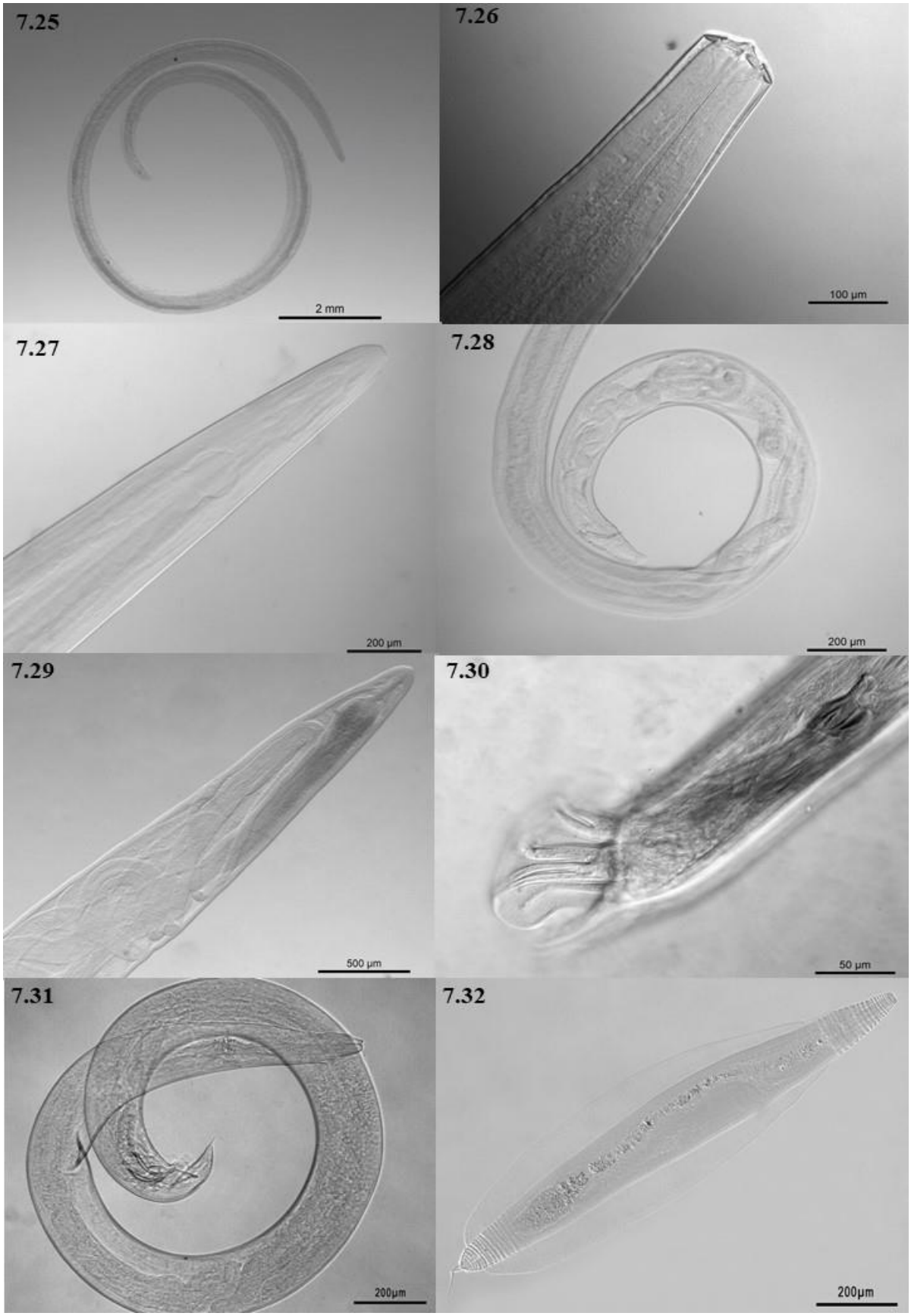
Figura 7. Espécies de nematoides registrados em anuros brasileiros. 7.1 *Aplectana crossodactyli*; 7.2 *Aplectana hylambatis;* 7.3 *Aplectana longa;* 7.4 *Aplectana membranosa;* 7.5 *Aplectana* n. sp.; 7.6 *Aplectana* sp.1*;* 7.7 *Aplectana* sp.2*;* 7.8 *Capillaria* sp.



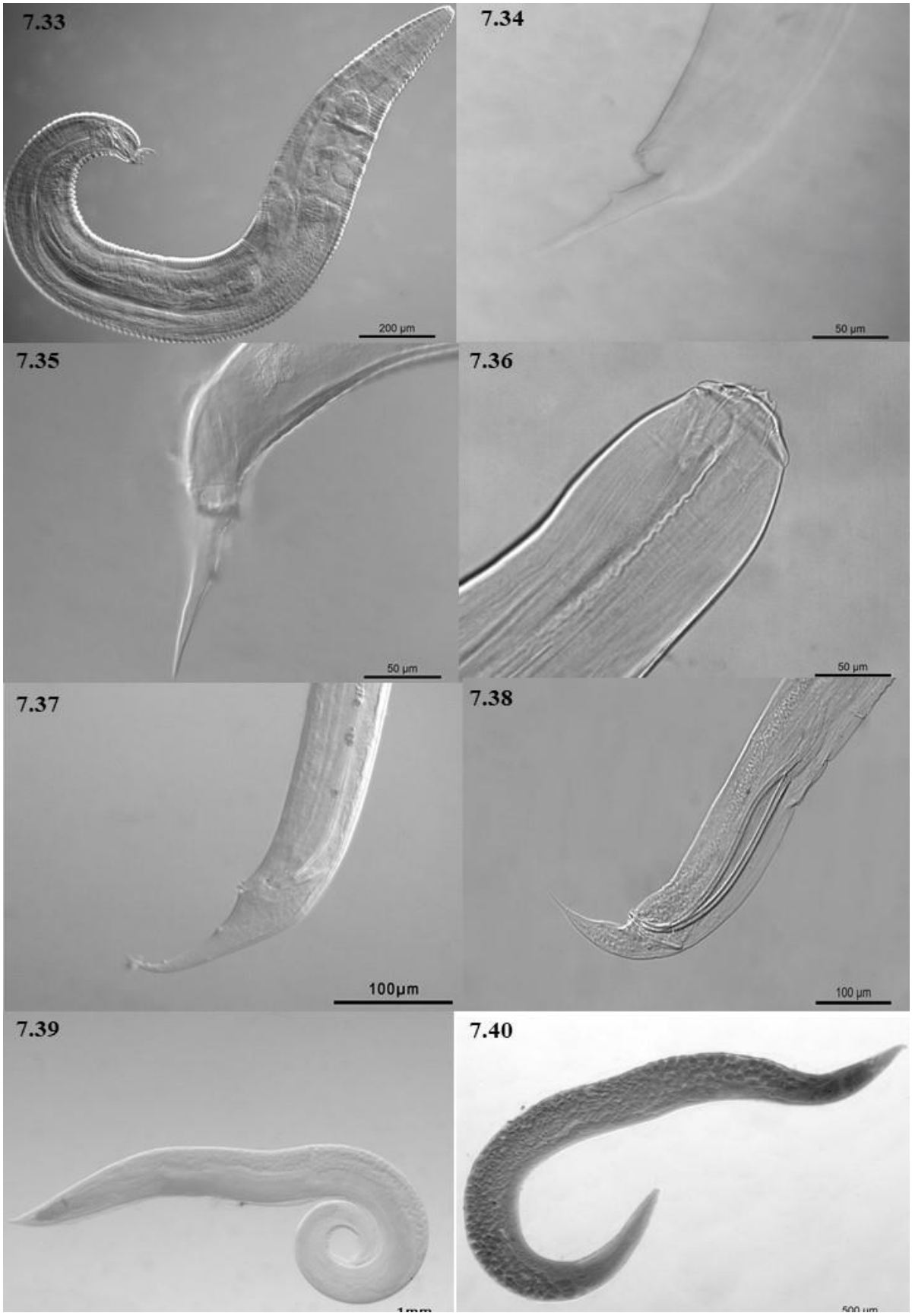
Continuação da ***Figura 7***. 7.9 *Cosmocerca* s. sp.*;* 7.10 *Cosmocerca brasiliensis;* 7.11 *Cosmocerca parva;* 7.12 *Cosmocerca podicipinus*; 7.13 *Cosmocerca* sp.1*;* 7. 14 *Cosmocerca* sp.2*;* 7.15 Cosmocercídeo*;* 7.16 *Cosmocercoides sauria.*



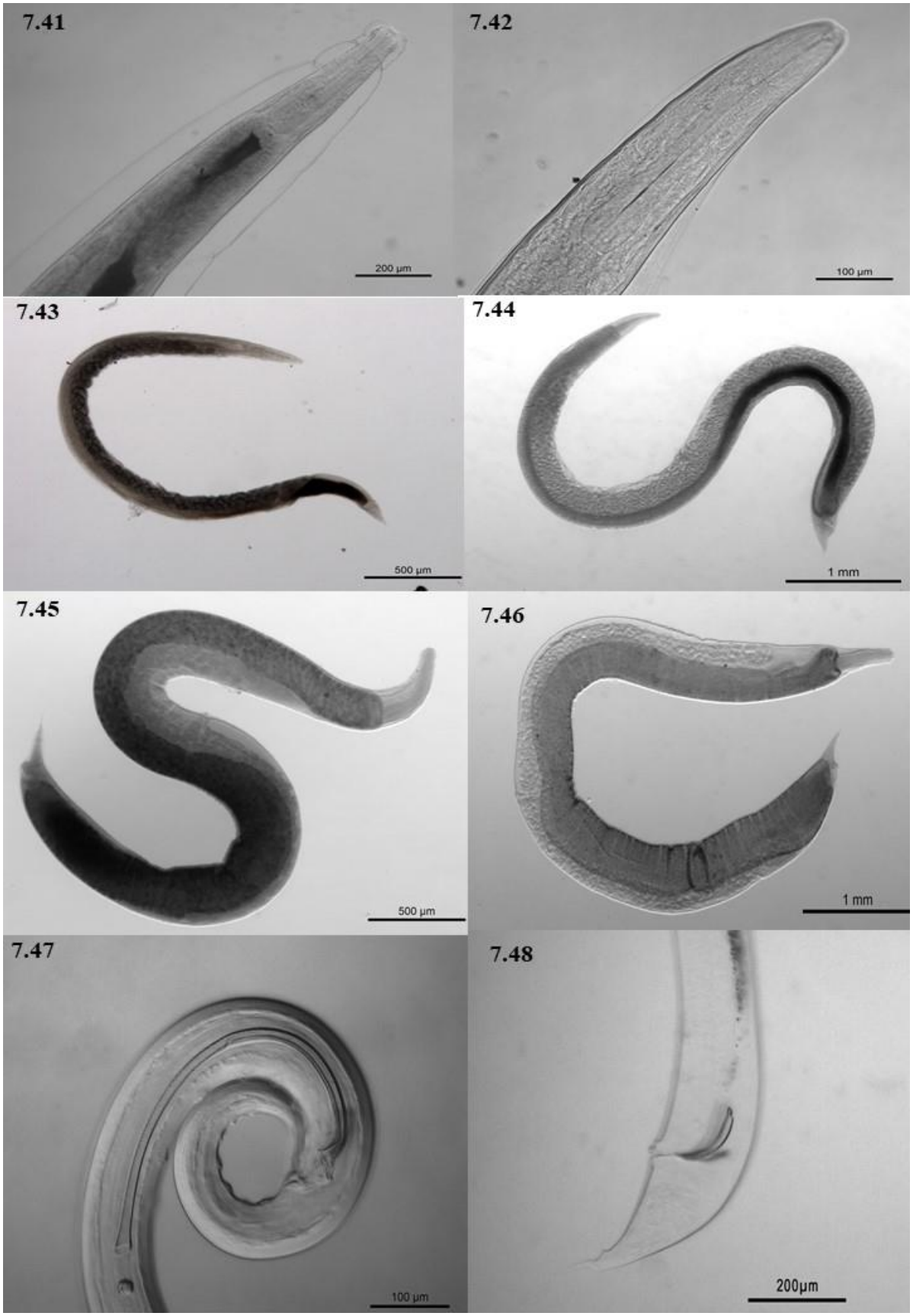
Continuação da ***Figura 7****.* 7.17 *Cosmocercoides* n. sp.1.*;* 7.18 *Cosmocercoides* n. sp.2*;* 7. 19 *Cosmocercoides* n. sp.3*;* 7.20 *Falcaustra belemensis;* 7.21 *Falcaustra* sp.*;* 7. 22 *Hedruris mucronifer;* 7.23 Larva de *Brevimulticaecum* sp.*;* 7. 24 Larva de filarídeo.



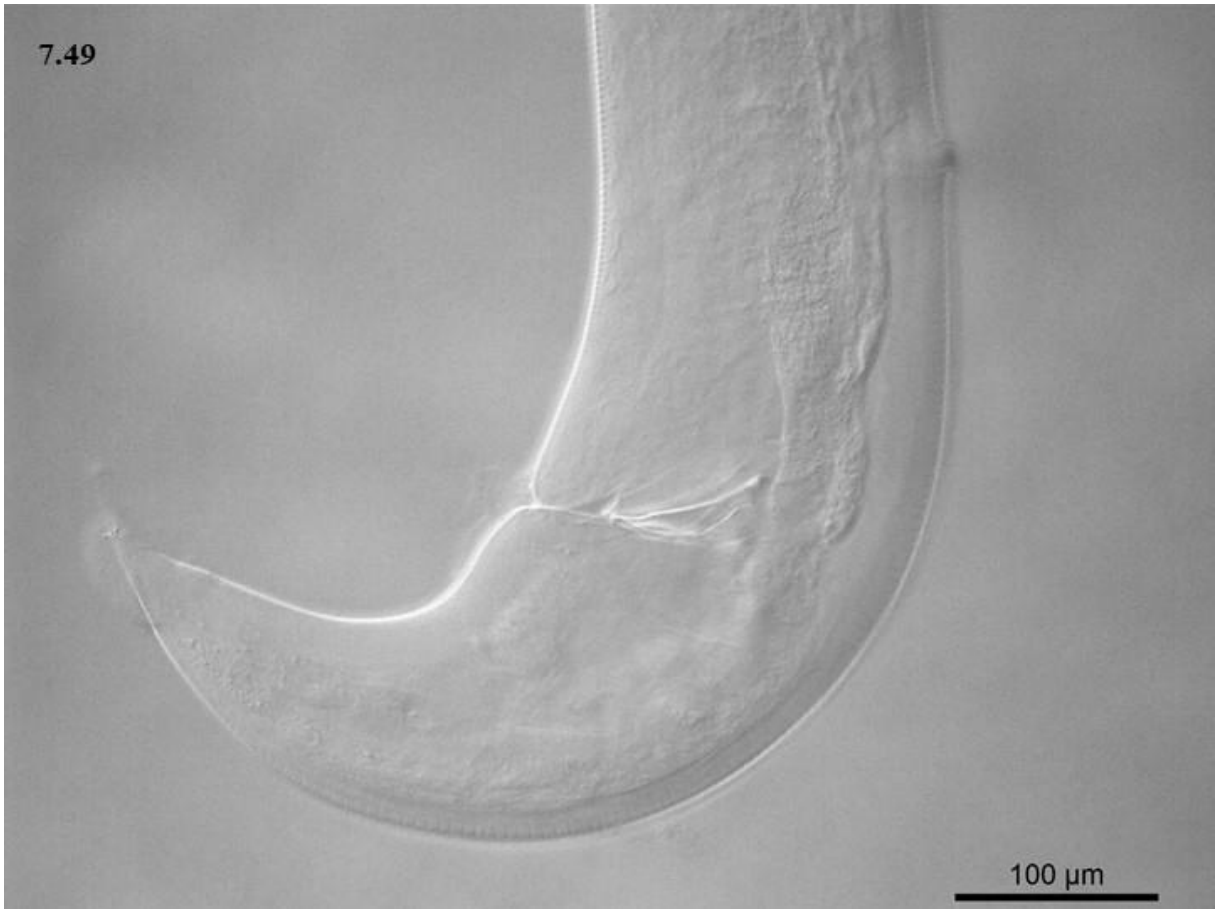
Continuação da ***Figura 7****.* 7.25 Larva de Nematoda; 7.26 Larva de *Physaloptera* sp.*;* 7.27 Larva de *Strongyluris;* 7.28 *Ochoterenella digicauda;* 7.29 *Ochoterenella* sp.*;* 7.30 *Oswaldocruzia* sp.*;* 7.31 *Oxyascaris* sp.*;* 7.32 *Parapharyngodon silvoi*.



Continuação da ***Figura 7****.* 7.33 *Parapharyngodon* n. sp.*;* 7.34 *Parapharyngodon* sp.1*;* 7.35 *Parapharyngodon* sp.2*;* 7.36 *Physalopteroides venancioi;* 7.37 *Raillietnema* sp.*;* 7.38 *Raillietnema spectans;* 7.39 *Rhabdias breviensis;* 7. 40 *Rhabdias cf. stenocephala*.



Continuação da ***Figura 7***. 7.41 *Rhabdias* n. sp.1*;* 7. 42 *Rhabdias* n. sp.2; 7.43 *Rhabdias* n. sp.3; 7.44 *Rhabdias pseudosphaerocephala;* 7.45 *Rhabdias* n. sp.4*;* 7.46 *Rhabdias* n. sp.5; 7.47 *Rhabdochona fuscovarius;* 7.48 *Schrankiana* sp.1.



Continuação da ***Figura 7***. 7.49 *Schrankiana* sp.2.

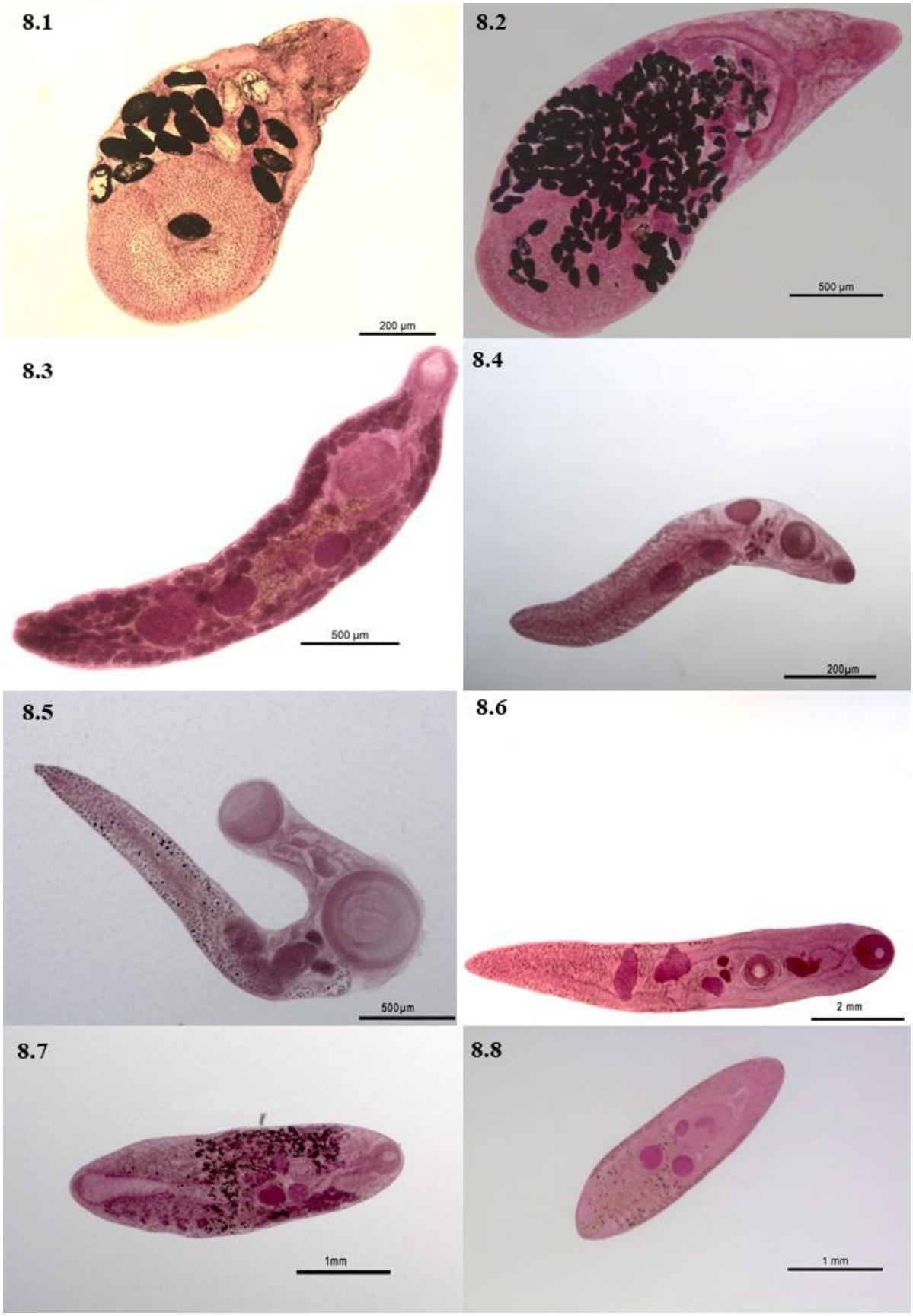
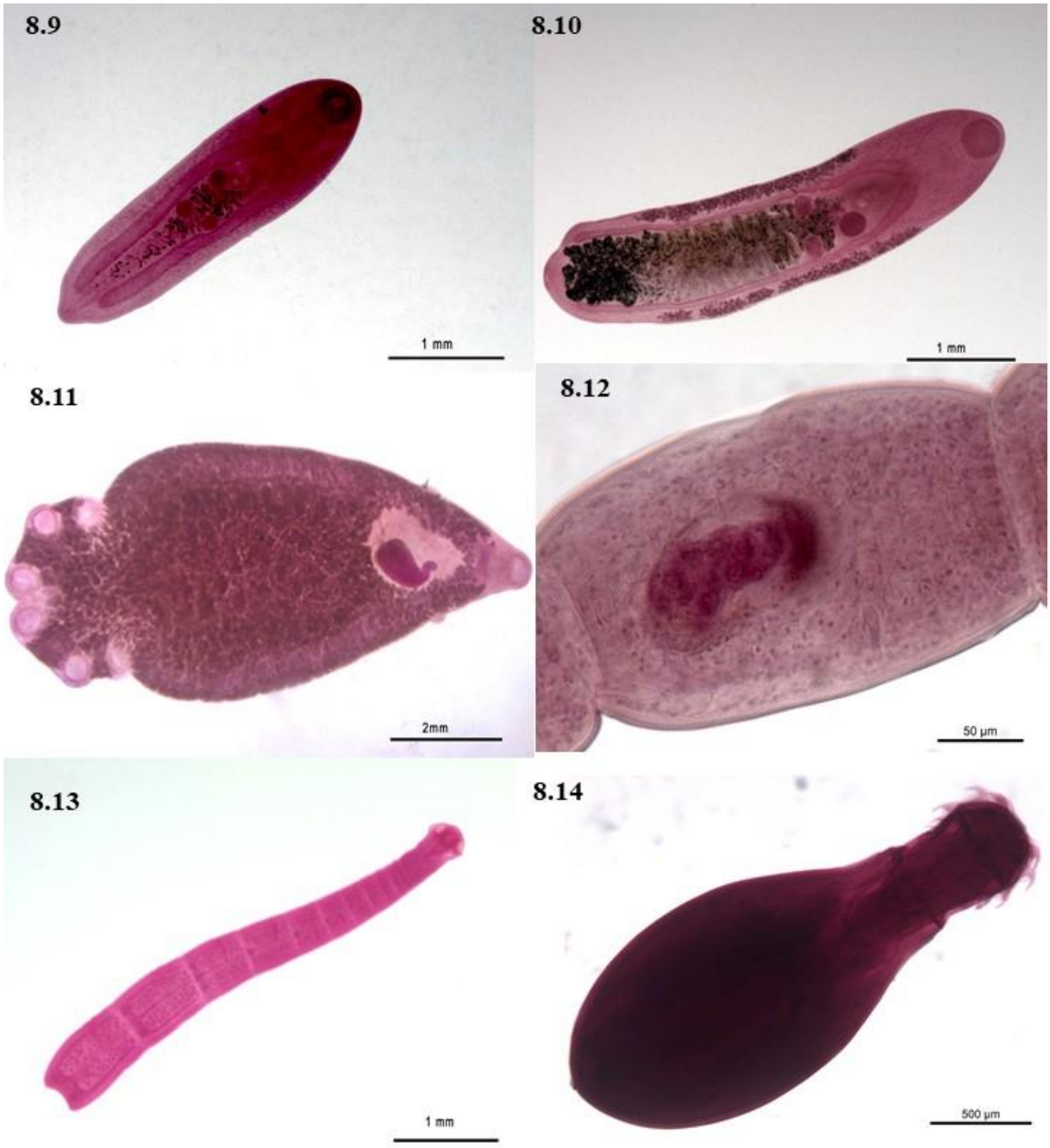


Figura 8. Espécies de platelmintos registrados em anuros brasileiros. 8.1 *Catadiscus propinquus;* 8.2 *Catadiscus marinholutzi;* 8.3 *Creptotrema* n. sp.; 8.4 *Gorgoderina cedroi;* 8.5 *Gorgoderina diaster;* 8.6 *Gorgoderina parvicava;* 8.7 *Mesocoelium monas;* 8.8 *Rauschiella* sp.1.



Continuação da ***Figura 8***. 8.9 *Rauschiella* sp.2; 8.10 *Rauschiella* sp.3*;* 8.11 *Polystoma* sp.; 8.12 *Cylindrotaenia americana.;* 8.13 *Proteocephalus* sp.; 8.14 Cistacanto de Oligacanthorhynchidae.

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Capítulo 1

Integrative taxonomy in the genus *Rhabdias* Stiles et Hassall, 1905 from anurans in Brazil, with the description of three new species and phylogenetic analyses[1]

Integrative taxonomy in the genus *Rhabdias* Stiles et Hassall, 1905 from anuran in Brazil, description of two new species and phylogenetic analyses

Abstract

About 20 valid species of the genus *Rhabdias* are known in the Neotropical region. The present study aimed to describe two new species of *Rhabdias* parasitizing the lungs of *Leptodactylus macrosternum* and *Leptodactylus podicipinus* from Brazil. Distinctive characteristics between these species are numerous and based on body size, size of the buccal capsule, shape and size of the oesophagus, and position of the vulva. Molecular data based on ribosomal genes 28S and ITS region and mitochondrial COI of the two species are presented. Molecular analysis and comparison of the partial mitochondrial COI sequence of *Rhabdias matogrossensis* n. sp. and *Rhabdias guaianensis* n. sp. revealed a genetic divergence between these new species and the sequences of *Rhabdias* spp. previously deposited in GenBank. In the phylogenetic analysis, *R. matogrossensis* n. sp. was grouped with *R. breviensis* species complex, and *R. guaianensis* n. sp. was grouped as a sister group of *R.* cf. *stenochepala.* This study contributes to improving the diversity of known species of *Rhabdias* described in Brazilian anurans.

Keywords: Anura. Nematoda. Taxonomy. Morphology. Molecular identification

1. Introduction

Rhabdias Stiles and Hassal, 1905 includes 90 species of nematodes that are parasitic in the lungs of amphibians and some reptiles and species of this genus are distributed in almost every continent [1]. About 20 valid species are described in the Neotropical region and until now only *Rhabdias breviensis* Nascimento, Gonçalves, Melo, Giese, Furtado and Santos, 2013, *Rhabdias galactonoti* Kuzmin, Melo, Filho and Santos, 2016, *Rhabdias glaurungi* Willkens, Rebêlo, Santos, Furtado, Vilela, Tkach, Kuzmin and Melo, 2019, *Rhabdias nicaraguensis* Bursey, Goldberg and Vitt, 2007, *Rhabdias pocoto* Morais, Melo and Müller, 2020, *Rhabdias pseudosphaerocephala* Kuzmin, Tkach and Brooks, 2007, and *Rhabdias waiapi* Tavares–Costa and Melo, 2022 have been described using a combination of morphology and molecular data [2,3,4,5,6].

During a survey of anuran parasites in the Brazilian Midwest (states of Goiás, Mato Grosso, and Mato Grosso do Sul) and Southeast (state of São Paulo) regions, nematodes were found with characteristics that corresponded to the diagnosis of *Rhabdias* [3,7], however, due to particular morphological characteristics, these could not be assigned to any known species of the genus. Thus, the present study describes two new species of *Rhabdias* parasitizing the lungs of anuran using both morphological and molecular phylogenetic analyses, based on the partial ribosomal (28S) gene, complete ITS region (ITS1-5.8S-ITS2) and partial mitochondrial (COI) gene.

[1] Manuscrito submetido para a revista Parasitology International.

2. Material and Methods

2.1. Sampling and morphological analysis

During a helminthological survey performed in 2018-2020, six anuran hosts infected with rhabdiasid nematodes were collected in five different localities (Fig. 1), as follows: one *Leptodactylus macrosternum* Miranda-Ribeiro, 1926 from the bank of the Pindaíba River next to the Japonês Inn, municipality of Araguaiana (14°35'45.79"S, 51°42'59.20"W) and two from the Boa esperança Farm, municipality of Cocalinho, both in the Mato Grosso state (14°46'32.37"S, 51°32'50.60"W), two *Leptodactylus podicipinus* (Cope, 1862) from the Carandá Farm, municipality of Araraquara, São Paulo state (21°52'30.28"S, 48°13'50.78"W) and one from the Sertaneja Retiro farm, municipality of Cocalinho, Mato Grosso state (14°25'52.42"S, 51°36'9.60"W).

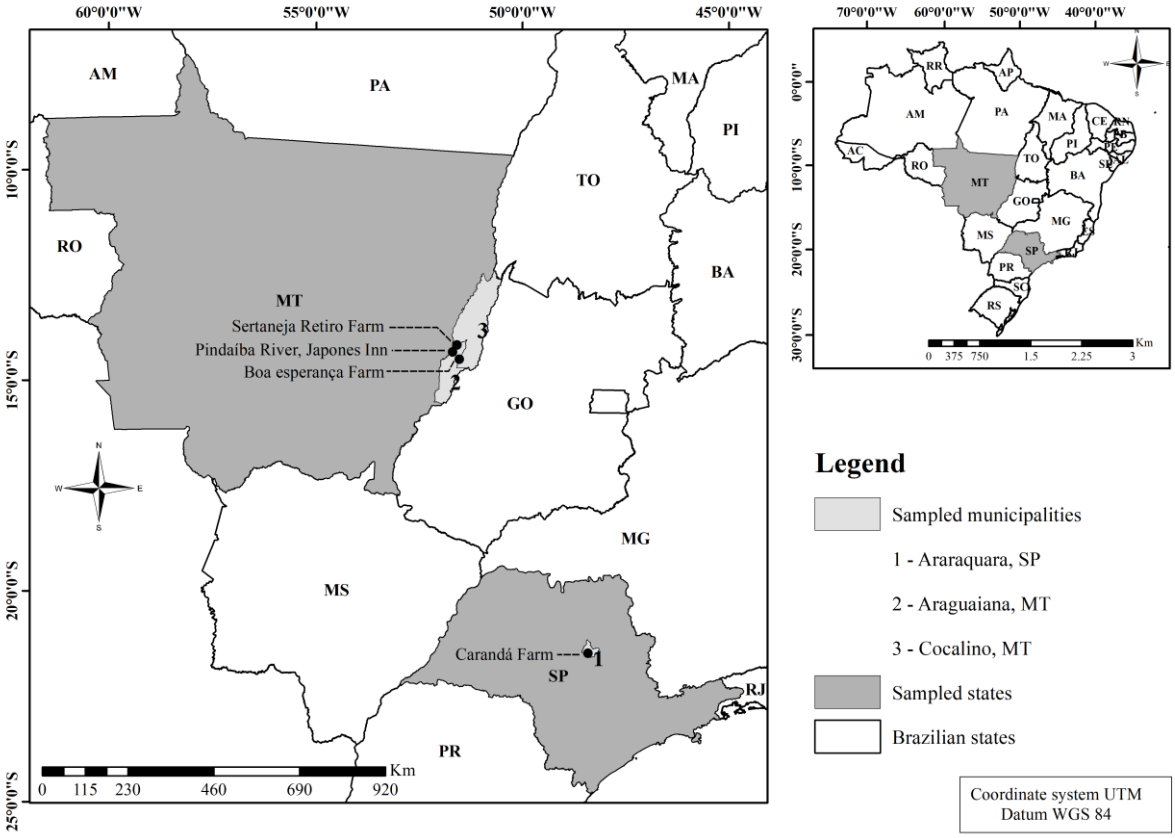
All applicable international, national, and/or institutional guidelines for the ethical handling of animals were followed (ICMBio SISBIO #60640-1; CEUA-UNESP 1061; SISGEN). Hosts have been deposited in the Herpetological Collection of the Universidade Federal do Ceará (CHUFC), municipality of Fortaleza, Ceará State, Brazil (16069, 16107, 16066, 16219-16221).

Anurans were killed with 2% lidocaine hydrochloride or sodium thiopental [8] and the internal organs were removed, dissected, and surveyed for *Rhabdias* spp. under a stereomicroscope (Bel Photonics STM Pro, Brazil). The nematodes found in the lungs were rinsed in saline solution and fixed in pre-heated 70% ethanol for morphological analysis or 96% ethanol (Merck, Darmstadt, Germany) for molecular analysis. For the morphological analysis, the specimens were cleared with lactophenol on temporary slides and analyzed under a microscope with a computerized image analysis system (Qwin Lite 3.1, Leica Microsystems, Wetzlar, Germany).

Some specimens had their apical morphology studied using scanning electron microscopy (SEM). For this, specimens were post-fixed in 1% osmium tetroxide, dehydrated in a graded alcohol series, and dried at the critical point of CO_2. The specimens were then mounted on an aluminum stub using conductive double-sided tape, coated with gold-palladium, and examined using a Vega3 electron microscope (TESCAN, Brno, Czech Republic) at the Laboratory of Histology and Embryology of the Federal Rural University of Amazonia (Universidade Federal Rural da Amazônia, UFRA).

The measurements are expressed in micrometres unless otherwise indicated. The range represents the measurements of the paratypes while for the holotype the values are presented in parentheses. However, the range for paratype is not possible for *Rhabdias guaianensis* n. sp., since it is described based on only two specimens.

Fig. 1: Map showing the distribution of samples of amphibians throughout the Brazilian southeast (1) and midwest of Brazil (2-3). (1) Carandá Farm, municipality of Araraquara, São Paulo (SP) state (21°52'30.28"S, 48°13'50.78"W); (2) Pindaíba River, Japones Inn, municipality of Araguaiana (14°35'45.79"S, 51°42'59.20"W); (3) Boa esperança Farm, municipality of Cocalinho (14°46'32.37"S, 51°32'50.60"W) and Sertaneja Retiro Farm, municipality of Cocalinho, Mato Grosso state (14°25'52.42"S, 51°36'9.60"W).



2.2. Molecular analysis

For PCR, the medial part of the nematode was cut, transferred to absolute ethanol (Merck), and stored in a freezer at -20ºC until de DNA extraction. The genomic DNA was extracted using DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol, with a final volume of 30 µl. DNA fragments of 28S (partial) containing D2/D3 divergent domains, internal transcribed spacer (ITS: complete), and COI (partial) genes were amplified with the primers and cycling conditions according to Müller et al. [9].

The reactions were made with a final volume of 25 µl, being 3 µl of DNA extract, 0.5 µl of each primer, 21 µl of ultrapure water (Sigma-Aldrich, United Kingdom), and Master Mix MyFiTM Mix Bioline®. 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 then sequenced using PCR primers on a 3,500 Genetic Analyzer capillary sequencer (Applied Biosystems) and after 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). The alignment (concatenated genes ITS + 28S) included the sequence of *Pneumonema tiliquae* Johnston, 1916 as an outgroup and sequences of all available species of *Rhabdias* from the Neotropical region. This alignment was used for the phylogeny of the amplified newly sequences that were performed using the default parameters of the algorithm Muscle [10] implemented in Geneious 7.1.3 [11]. The Akaike information criterion in the JModelTest software [12] was used to determine the best-fit model for nucleotide substitution.

Phylogenetic analysis using the Maximum Likelihood method, RAxML [13] was used with bootstrap support values of 1,000 repetitions. The Bayesian Inference was performed with MrBayes 3.2 [14], the Markov chain Monte Carlo (MCMC) run with 50,000,000 generations saving one tree every 1,000 generations, and burn-in of 25% with only probabilities greater than 90% were considered well supported. Both ML and BI phylogenetic analyses were performed on CIPRES [15].

The Bayesian analysis was run using the following nucleotide substitution model settings for the 28S dataset: lset nst = 1, rates = gamma, ncat = 4, shape = estimate, inferrates = yes and basefreq = empirical, which correspond to the K2+G model. The Bayesian analysis of the ITS dataset was performed as follows: lset nst = 2, rates = gamma, ncat = 4, shape = estimate, inferrates = yes and basefreq = empirical, corresponding to the T92+G model. Bayesian analysis of the COI dataset was performed as follows lset nst = 1, rates = gamma, ncat = 4, shape = estimate, inferrates = yes and basefreq = empirical, which correspond to the GTR+G.

The FigTree v.1.3.1 software was used for both trees (ML and BI) editions and visualization. The genetic divergence using the mitochondrial COI gene was calculated using the Kimura 2-parameter model with 1,000 bootstrap replicates in MEGA 7 software [16].

3. Results

The present study describes two new species of *Rhabdias* in Brazilian anurans.

3.1. *Morphological description*

Order Rhabditida Chitwood, 1933

Family Rhabdiasidae Railliet, 1915

***Rhabdias matogrossensis* n. sp. Alcantara, Müller, Morais, and Silva** (Figs 2-4; Table 1)

Based on the holotype and 4 paratypes, all gravid hermaphrodites. The morphological measurements of the specimens are presented as the values of the holotype followed by the mean of the paratypes and range in parentheses (reported in micrometers, except where indicated) in accordance with the standardization pro posed by Willkens et al. [4].

Body 3.35; 3.56 (2.98–3.852) mm long, curved dorsally, gradually tapering from mid-region to anterior end and expanding gradually from mid-region to posterior part of the body, with a gradual narrowing of the caudal end (Fig. 2).

Anterior end rounded, posterior end extremely pointed. Body cuticle typically not swollen or only slightly swollen, especially at anterior end, thin and smooth (Fig. 2A-C). Lateral pores and ducts present. Body width at

vulva 263.9; 267.1 (231.2–301.4), width at oesophagus-intestinal junction 154.3; 172.3 (144.5–191.8). Oral opening surrounded by six lips, four smaller lips close to edge of oral opening, two lateral lips somewhat larger, each lip has a papilla on its inner edge and two amphidial openings located at base of lateral lips (Fig.3).

Vestibulum cylindrical, cuticularized, narrow lumen. Buccal capsule small, well developed, almost rounded in enface view, cup-shaped in lateral view (Fig. 4). Maximum diameter of buccal capsule 15; 16 (15–17), total depth 15; 13 (11–16). Oesophagus club-shaped, 470.5; 475.9 (437.5–507.9) long, representing 14.02%; 13.5% (11.7–15.9%) of body length, without anterior dilation, oesophagus width 36.1; 37 (32.9–40.3) in anterior part, 35.7; 39.6 (36.5–40.7) at middle part, and 39.4; 46.4 (43.6–49.4) in posterior part. Bulb width 59.5; 61.1 (52.9–66.6). Nerve-ring surrounding oesophagus approximately the previous dilation, 142.5; 166 (142.5–207.9) from anterior end of body (Fig. 2C). Excretory pore not observed. Intestine thick-walled, contents of intestine brown throughout length.

Genital system typical of Rhabdiasidae, amphidelphic with anterior and posterior ovaries. Vulva post-equatorial, small, vulvar lips distinct, not salient, aperture short and transverse, and weakly cuticularized, representing 61.7%; 63.5% of body length (Fig. 2D). Distance from anterior end to vulva 2.07; 2.37 mm, Uteri joined, sac-like, containing numerous eggs (>100 in total); eggs near vulva with fully developed larvae (Fig. 2E). Eggs 84.9–113.8 × 44.9–61.9 (N = 14, measured in uteri close to vulva of holotype). Both genital tubes U-bent in proximal parts of seminal receptacles. Genital system occupying 59.2% of body. Distance from anterior end of body to anterior U-bend 998.3; 1082.6 (1070.1–1098.3), distance from posterior U-bend to tail end 527.9; 431.7 (316.7–538.6). Tail comparatively short, conical (Fig. 2B), 210; 198.7 (165.9–229.7) long. Phasmid located 167.2; 173.4 (168.9–177.9) from tail.

Taxonomic summary

Type host: Leptodactylus macrosternum Miranda-Ribeiro, 1926 (Leptodactylidae).

Type locality: Boa Esperança Farm, municipality of Cocalinho, Mato Grosso state, Brazil, (14°46'32.37"S, 51°32'50.60"W).

Other locality: municipality of Araguaiana, Mato Grosso state, Brazil (14°35'45.79"S, 51°42'59.20"W).

Site of infection: Lungs.

Type material: Holotype; Paratypes (Specimens will be deposited after acceptance of the article).

Numbers of specimens/hosts: 18 nematodes were found in three frogs. Host 1: 5 nematodes (Japones Inn, municipality of Araguaiana, Mato Grosso state); host 2 and 3, 10 and 3 specimens, respectively (both from Boa Esperança Farm, municipality of Cocalinho, Mato Grosso state).

GenBank Accession number: (sequences will be submitted to GenBank after manuscript acceptance).

ZooBank registration: The species will be deposited after acceptance of the article

Sisgen: (Species will be registered after manuscript acceptance)

Etymology: The epithet refers to the type locality of the new species, Mato Grosso state.

Fig. 2. Line drawings of *Rhabdias matogrossensis* n. sp. (A) Entire body, lateral view; (B) posterior end, lateral view, (C) anterior end; lateral view; (D) Vulva, lateral view (E) eggs.

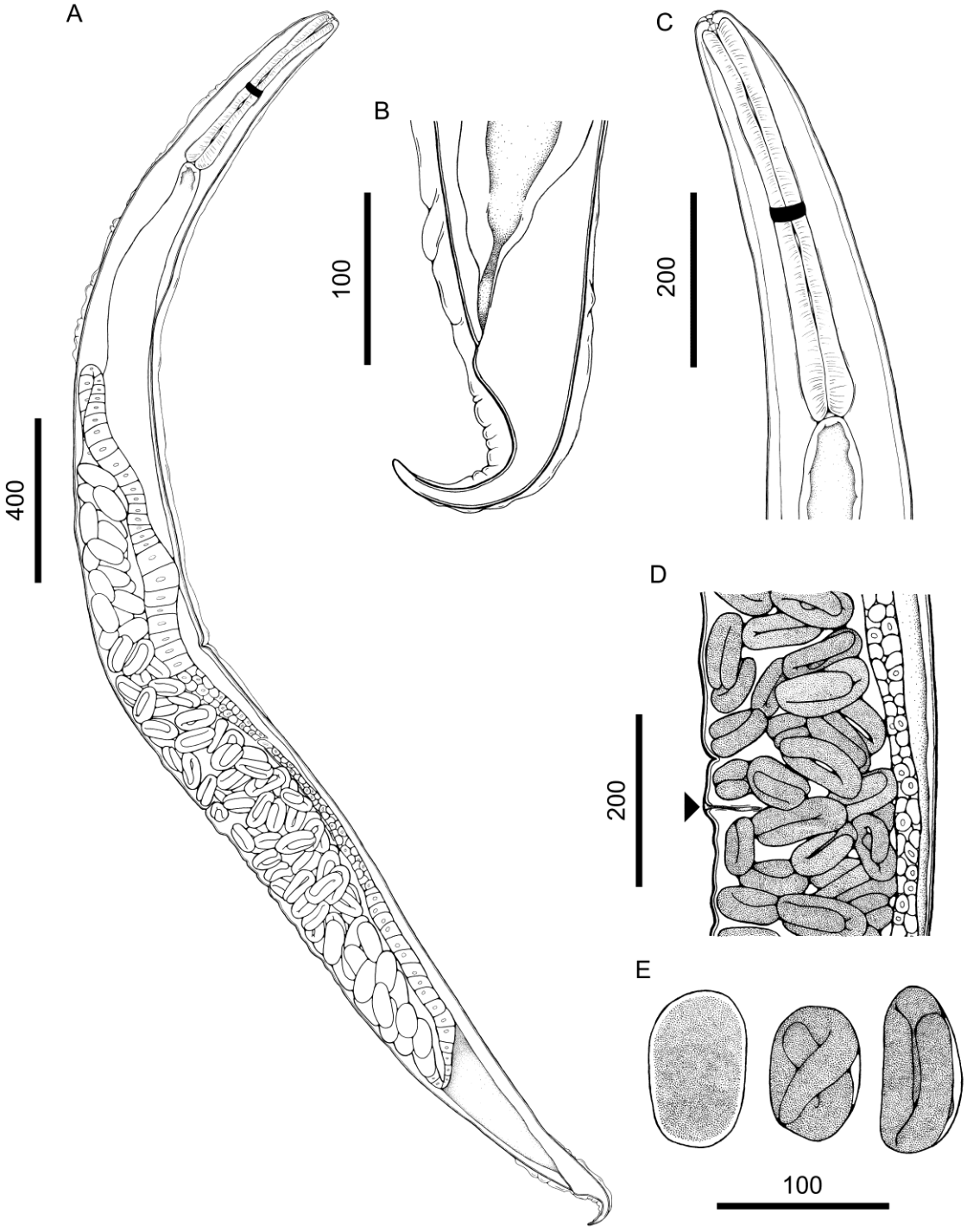


Fig. 3. Scanning electron micrographs apical view of the *Rhabdias matogrossensis* n. sp. Apical view (arrowheads, cephalic papillae; asterisks, amphids; lines, LL - lateral lips; S L-submedian lips).

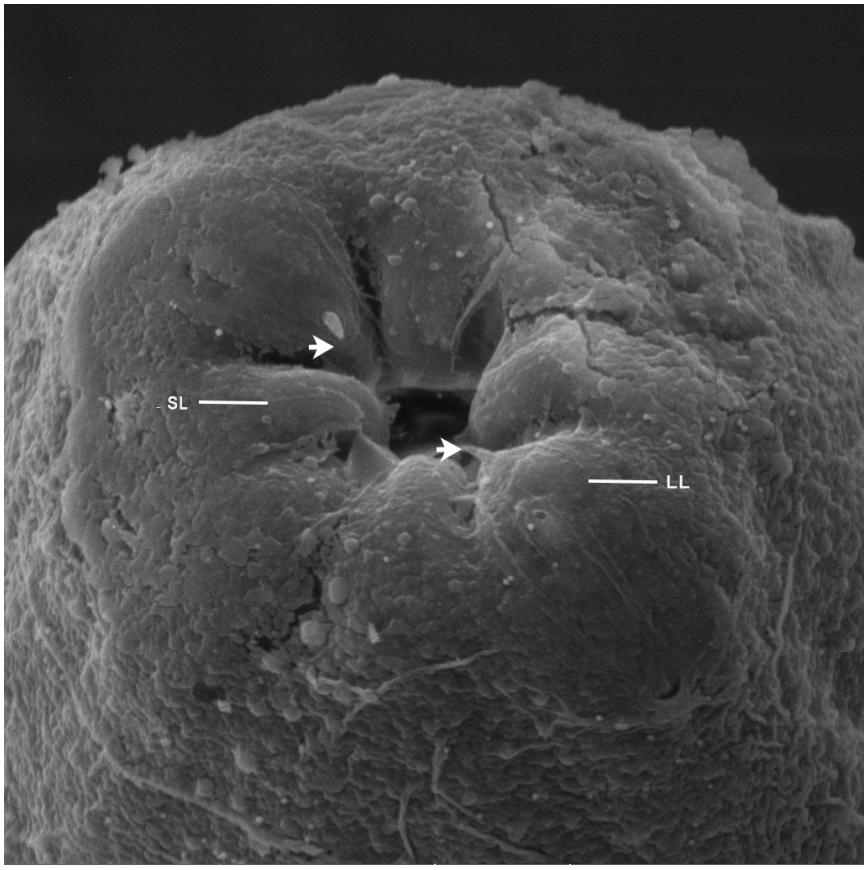
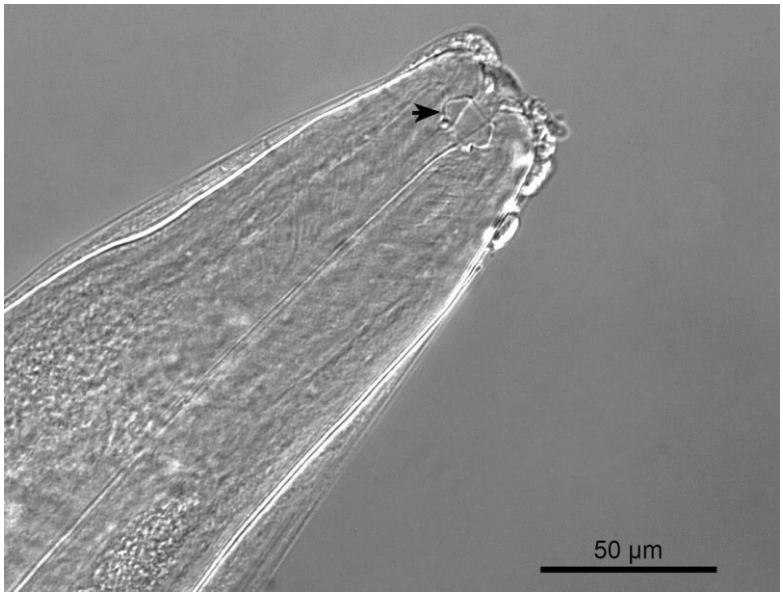


Fig. 4. Anterior end of *Rhabdias matogrossensis* n. sp. Lateral view (arrowheads, bucal capsule).



Remarks

Rhabdias matogrossensis n. sp. is assigned to the genus *Rhabdias* based on molecular data and the following morphological characteristics: inflated body cuticle, small buccal capsule, amphidelphic reproductive system with short transverse vagina and joined uteri, embryonated eggs, and due to its parasitism in the lungs in the amphibian hosts [3, 7].

Rhabdias matogrossensis n. sp. differs from the other species reported for the Neotropical region by the combination of a unique set of morphological traits, size and proportion of the buccal capsule, shape of cuticular inflation, esophagus size, and lip position and size.

Therefore, considering the morphological characters in *R. matogrossensis* n. sp., the new host, and molecular data, we herein propose a new species and will compare the new taxon with only those species reported in the Neotropical region which are morphologically similar to *R. matogrossensis* n. sp. In addition to these species, we will compare the new species with *R. hermaphrodita* Kloss, 1971, because it is a Neotropical species for which there is no information about the arrangement of the oral structure in their original descriptions [17,18]. On the other hand, as proposed by Willkens et al. [4], *Rhabdias mucronata* Schuurmans-Stekhoven, 1952 and *Rhabdias truncata* Schuurmans-Stekhoven, 1952 will not be included in the comparison because data on the hermaphrodite forms of these species are not available, as the descriptions these species were performed based on juvenile forms.

Based on the arrangement of the oral structure, *R. matogrossensis* n. sp. is similar to the group of species with six lips, four submedial lips, and two lateral lips: *Rhabdias androgyna* Kloss, 1971; *R. breviensis* Nascimento, Gonçalves, Melo, Giese, Furtado, and Santos, 2013; *Rhabdias fuelleborni* Travassos, 1926; *R. galactonoti* Kuzmin, Melo, da Silva Filho, and Santos, 2016; *R. glaurungi* Willkens, Rebêlo, Santos, Furtado, Vilela, Tkach, Kuzmin, and Melo, 2020; *Rhabdias manantlanensis* Martínez-Salazar, 2008; *R. pocoto* Morais, Melo, and Müller, 2020; *Rhabdias stenocephala* Kuzmin, Melo, da Silva Filho, and Santos, 2016; *Rhabdias tobagoensis* Moravec and Kaiser, 1995, and *R. waiapi* Tavares-Costa and Melo, 2022 (Table 1).

Rhabdias androgyna is parasitic in bufonids, hylids, and odontophrynids (Table 1), accordingly Kloss [17] and Kuzmin et al. [18]. *Rhabdias androgyna* has shoulder-like circular dilatation with an extended cuticular swelling disposed of in two layers, while the new species does not have such characteristics. Besides, *R. matogrossensis* n. sp. is much smaller than *R. androgyna* (2.98–3.85 vs 9.35-13.39), has a smaller buccal capsule (15–17 in *R. matogrossensis* n. sp.2 vs 19–27 in *R. androgyna*), smaller oesophagus (437.5–507.9 in *R. matogrossensis* n. sp. vs 577-618 in *R. androgyna*).

Rhabdias breviensis parasitic in anurans of Leptodactylidae, Bufonidae, Hylidae, and Odontophrynidae, has a particular, curved body shape, but the curved body shape is probably due to the method used for fixation. The new species resembles *R. breviensis* in body length, the post-equatorial position of the vulva, and the shape of the buccal capsule. Moreover, the total depth of the buccal capsule in *R. breviensis* is just slightly smaller than in *R. matogrossensis* n. sp. (4–9 x vs 11–16) and the oesophagus in *R. breviensis* is smaller than *R. matogrossensis* n. sp. (238–410 vs 437.5–507.9). In addition, the location of the nerve ring in relation to anterior end is smaller than *R. matogrossensis* n. sp (41–84 vs 142.5–207.9).

Rhabdias fuelleborni was described in *Rhinella marina* (=*Rhinella icterica*) (Bufonidae) and is considered a common parasite from bufonids in South America. *Rhabdias matogrossensis* n. sp. resembles *R. fuelleborni* in the size of the oesophagus. In contrast to the new species, the depth of the buccal capsule in *R. fuelleborni* is 9–9

(vs 11–16 wide in the new species) and *R. fuelleborni* has a tail larger than *R. matogrossensis* n. sp. (370-420 vs 165.9–229.7), despite specimens of *R. fuelleborni* being larger than those of *R. matogrossensis* n. sp. (10–12 vs 2.98–3.85 mm long).

Rhabdias matogrossensis n. sp. is similar to *R. galactonoti* parasitic in dendrobatids, in the transitional areas between the Cerrado and the Amazon Forest, Brazil in size of the buccal capsule. However, the shape of the buccal capsule is differing, in the new species is cup-shaped and in the *R. galactonoti* is doliiform. The position of the vulva in *R. galactonoti* is pre-equatorial, while in the new species the position of the vulva is post-equatorial. The body is larger in *R. galactonoti* (5.60–6.04 vs 2.98–3.85 in the new species).

Rhabdias matogrossensis n. sp. is similar to *R. glaurungi* parasitic in hylids from the Brazilian Amazon in the post-equatorial position of the vulva and buccal capsule. The new species differs from *R. glaurungi* in the body size (2.980–3.85 vs 3.54–6.73 in *R. glaurungi*), and the oesophagus in *R.matogrossensis* n. sp. is significantly longer than in *R. glaurungi* (437.5–507.9 vs 292–332). The distance from the anterior extremity to the nerve ring is larger in the new species (142.5–207.9 vs 108–140 in *R. glaurungi*).

The description of *R. hermaphrodita* is superficial and incomplete, the buccal capsule or the structure of the apical region was not presented or measured in the original [5]. The new species is smaller than *R. hermaphrodita* (2.98–3.85 vs 12.9). Furthermore, the position of the vulva in the new species is post-equatorial and equatorial in *R. hermaphrodita*.

Rhabdias matogrossensis n. sp. resembles *R. manantlanensis* parasite of craugastorid from Mexico in shape and size depth of buccal capsule (Table 1). In contrast to the new species, the vulva in *R. manantlanensis* is usually equatorial (slightly pre-equatorial), the buccal capsule is 19-27 wide (vs 15–17 wide in the new species) and *R. matogrossensis* n. sp. is smaller than *R. manantlanensis* (2.98–3.85 vs 6.48-9.64).

Rhabdias matogrossensis n. sp. clearly differs from *R. pocoto* parasitic in leptodactylid from Caatinga, Brazil by the position of the vulva and body size. Besides, *R. pocoto* has two external pores situated at the edge of the oral region, each connected to amorphous material, and a glandular-like internal structure, which is not observed in the new species.

Rhabdias matogrossensis n. sp. is similar to *R. stenocephala* parasitic in leptodactylid from Pará, Brazil in position vulva (Table 1). In contrast to the new species in the shape of the buccal capsule (ring-shape vs cup-shape in the new species). *Rhabdias stenocephala* is larger than *R. matogrossensis* n. sp. (6.9–8.1 vs 2.98–3.85) and the new species has a tail small than *R. stenocephala* (165.9–229.7 vs 266–339 in the *R. stenocephala*). The anterior end of *Rhabdias stenocephala* has a narrow anterior part separated from the remainder of the body by a distinct constriction, which was not observed in *R. matogrossensis* n. sp.

In comparison to *Rhabdias matogrossensis* n. sp., *R. tobagoensis* parasitic in craugastorid from Tobago Island has a larger body size (7.34-7.56mm vs 2.98–3.85 in the new species), and a larger buccal capsule (18-21 vs 15–17 in the *Rhabdias matogrossensis* n. sp.). In addition, the position vulva in the new species is post-equatorial while in the *R tobagoensis* is equatorial (slightly pre or postequatorial).

Rhabdias matogrossensis n. sp. is similar to *R. waiapi* parasitic in craugastorid from Amapá State, Brazil in the position of the vulva (Table 1) and lip shape. Nonetheless, *R. waiapi* is larger than *R. matogrossensis* n. sp.2 (3.36–5.38 mm vs 2.98–3.85 in the new species) and also has a larger tail (266–339 in *R. waiapi* vs 165.9–229.7 in the *R. matogrossensis* n. sp.) (Table 1).

***Rhabdias guaianensis* n. sp. Alcantara, Müller and Silva (Fig 5-7; Table 1)**

Based on 2 specimens, gravid hermaphrodites. The morphological measurements of the specimens are presented as the values of the holotype followed by the paratype (reported in micrometers, except where indicated). Small worms, anterior end somewhat truncated, and posterior end tapering (Fig. 5A). Body 2.90; 3.56 mm long, 208.6; 200.4 wide at vulva level, and 121.6; 109.3 wide at oesophagus-intestine junction.

Anterior end rounded, posterior end extremely pointed. Body cuticle thin, inflation of cuticle more prominent at anterior and posterior body end (Fig. 5A-C). Oral opening round. Four submedian lips transformed into low, flattened, and close to oral opening and two lateral pseudolabia, all lips and papillae arranged in circle surrounding oral opening present on apical surface. Two amphidial openings located at base of lateral lips. (Fig. 6).

Buccal capsule well developed, almost rounded in enface view, and cup-shaped in lateral view (Fig. 7). Maximum diameter of buccal capsule 13; 14, total depth 14; 11. Oesophagus club-shaped, 266.9; 274.6 long, with slight dilation in middle of anterior half, representing 9.2%; 7.7% of body length. Width of oesophagus anterior end 23.2; 20.9, 28.5; 22.5 at middle part, 27.7; 29.5 in posterior part. Bulb width 47.7; 42.4. Nerve ring 113.9; 112.7 from anterior end (Fig .5B). Excretory pore not observed. Intestine filled with brown content. Rectum funnel-shaped, wide anteriorly, tapering posteriorly, prominently cuticularised.

Genital system amphidelphic. Vulva post-equatorial, 1.87; 2.24 mm from anterior end, representing 64.7%; 63% of body length. Vulva lips not salient (Fig. 5D). Vagina short, transverse, cuticularised. Uteri joined, thin-walled, containing numerous eggs (>100 in total); eggs near vulva with fully-developed embryos (Fig. 5E). Eggs 80.5–88.9 × 41.4–50.2 (N = 11, measured in uteri of holotype). Both genital tubes U-bent in proximal parts of seminal receptacles. Genital system occupying 78.1% of body. Distance from anterior end of body to anterior U-bend 512.2; 435.5, distance from posterior U-bend to tail end 404.8. Testis zones not observed. Tail narrow, gradually tapering (Fig. 5C). Slight elevation of body wall present on ventral side posterior to anus. Tail length 160.4; 186.9. Phasmids located at 120.8; 122 from tail end.

Taxonomic summary

Type host: Leptodactylus podicipinus (Cope, 1862) (Leptodactylidae).

Type locality: Carandá Farm, municipality of Araraquara, São Paulo state, Brazil (21°52'30.28"S, 48°13'50.78"W).

Other locality: Sertaneja Retiro Farm, municipality of Cocalinho, Mato Grosso state, Brazil (14°25'52.42"S, 51°36'9.60"W).

Site of infection: Lungs.

Type material: Holotype; Paratypes (Specimens will be deposited after acceptance of the article).

Numbers of specimens/hosts: Seven nematodes were found in three frogs. Host 1: 3 nematodes (Sertaneja Retiro Farm, municipality of Cocalinho, Mato Grosso state); host 2 and 3, 3 specimens each host (both from Carandá Farm, municipality of Araraquara, São Paulo state).

GenBank Accession number: (sequences will be submitted to GenBank after manuscript acceptance).

ZooBank registration: The species will be deposited after acceptance of the article

Sisgen: (Species will be registered after manuscript acceptance)

Etymology: The epithet refers to the Guaianás Indians, who originally inhabited the Araraquara region where the new species was found.

Fig 5. Line drawings *of* Rhabdias guaianensis n. sp. A) Entire body, lateral view; (B) anterior end; lateral view, (C) posterior end, lateral view; (D) Vulva, lateral view (E) eggs.

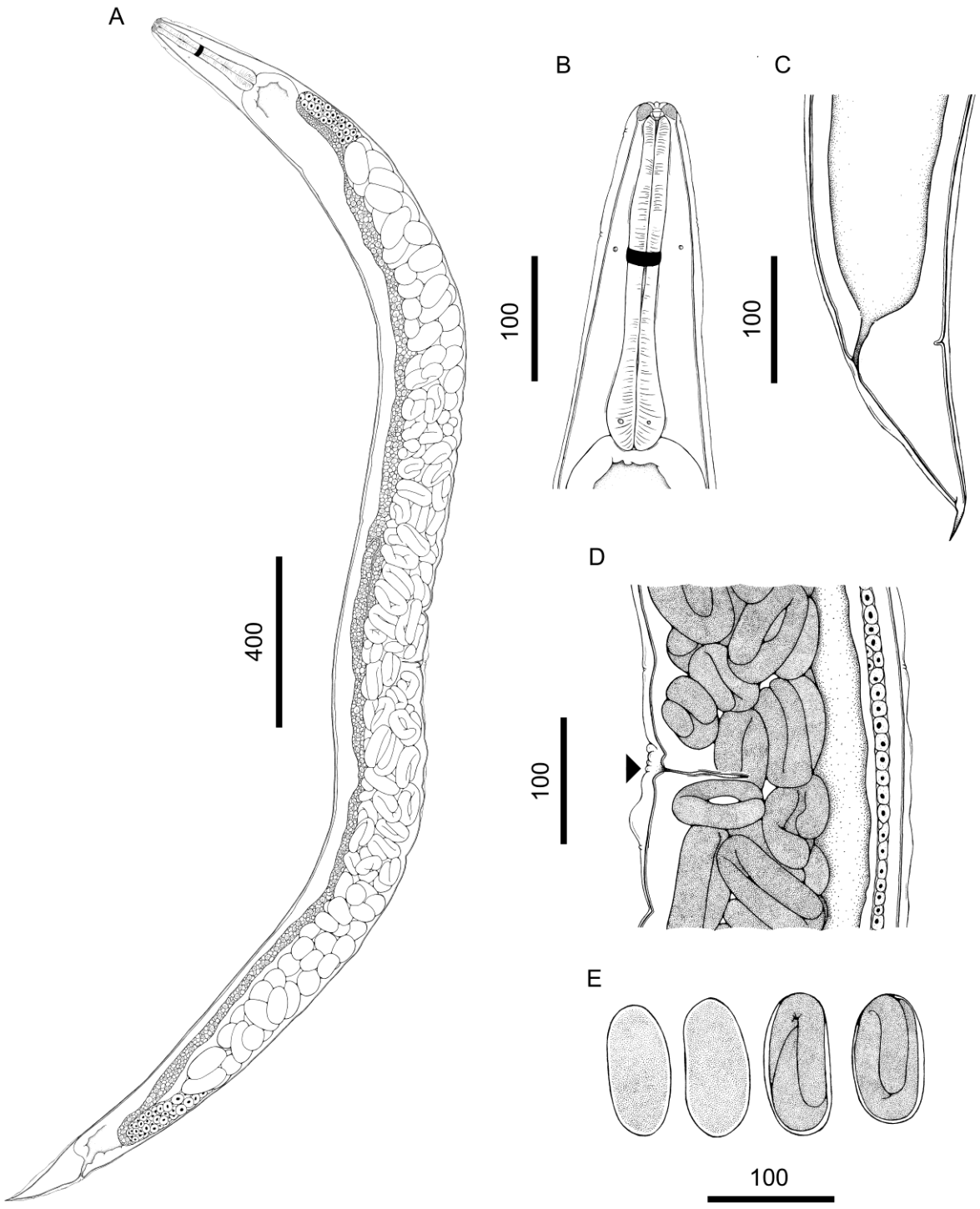


Fig. 6. Scanning electron micrographs apical view of the *Rhabdias guaianensi* n. sp. Apical view (arrowheads, cephalic papillae; asterisks, amphid; lines, LL - lateral lips; SL-submedian lips).

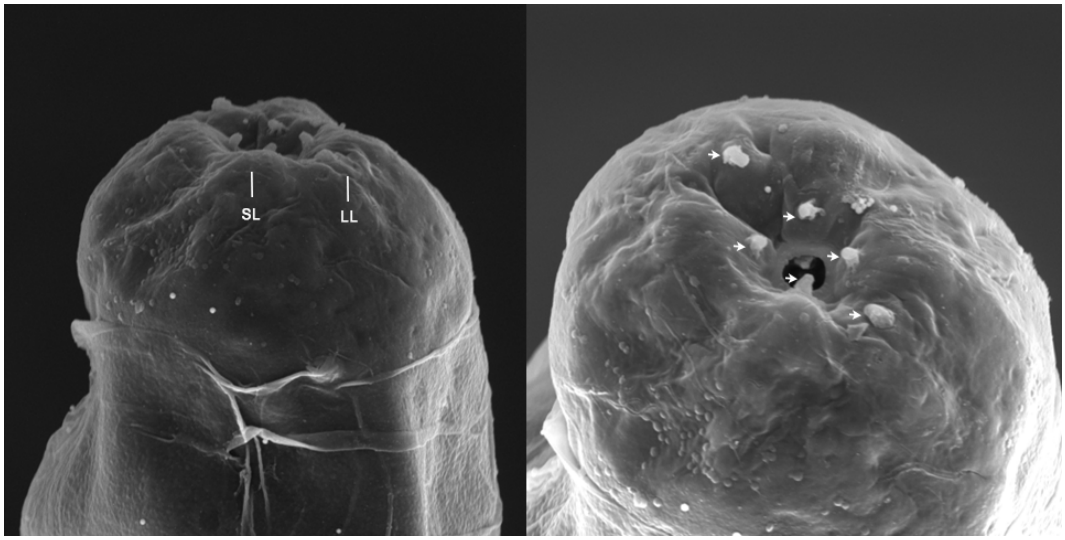
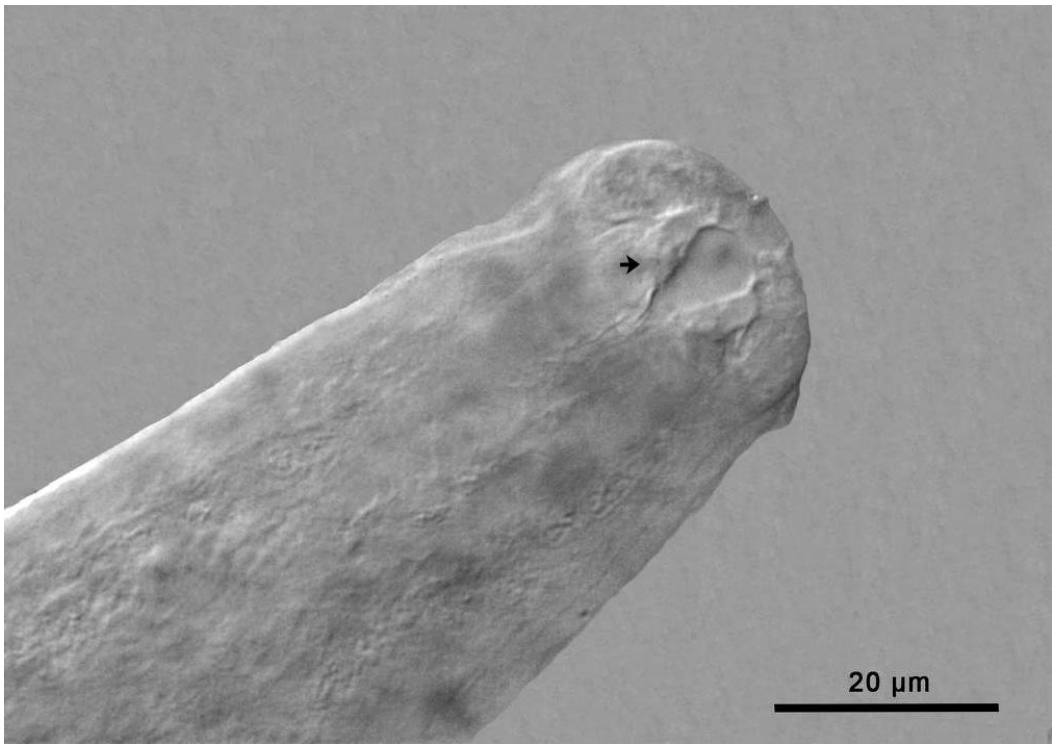


Fig. 7. Anterior end of *Rhabdias guaianensi* n. sp. Lateral view (arrowheads, bucal capsule).



Remarks

The new species was assigned to the genus *Rhabdias* based on molecular data and the following morphological traits: inflated body cuticle, the small buccal capsule with thick walls, the morphology of the reproductive system, and its parasitism in the lungs of amphibian hosts [3, 7].

Rhabdias guaianensis n. sp. differs from the other species reported for the Neotropical region by the combination of a unique set of morphological traits: body size, size and proportion of the buccal capsule, the position of the vulva, esophagus size, and tail size.

Therefore, considering the morphological characters in *R. guaianensis* n. sp., the new host, and molecular data, we herein propose a new species and will compare the new taxon with only those species reported in the Neotropical region which are morphologically similar to *R. guaianensis* n. sp. In addition to these species, we will compare the new taxon with *R. hermaphrodita*, because it is a Neotropical species for which there is no information about the arrangement of the oral structure in their original descriptions [17, 18].

Based on the arrangement of the oral structure, *R. guaianensis* n. sp. is similar to the group of species that present four submedian lips and two lateral pseudolabia, as follows: *Rhabdias kuzmini* Martinez-Salazar and Leon-Regagnon, *Rhabdias manantlanensis* Martinez-Salazar, 2008, *R. pseudosphaerocephala*, and *R. savagei* Bursey and Goldberg, 2005 (Table 1).

Rhabdias hermaphrodita parasite of *Rhinella crucifer* (Wied-Neuwied, 1821) (Bufonidae) is larger than *R. guaianensis* n. sp. (12.9 mm vs 2.9 mm–3.56 mm in the new species) and possesses larger oesophagus (268-663 vs 266.9–274.6 in *R. guaianensis* n. sp.) and tail (226–524 vs 160.4–186.9 in *R. guaianensis* n. sp.). The position of the vulva in *R. hermaphrodita* is equatorial, while in the *R. guaianensis* n. sp. is post-equatorial.

Rhabdias kuzmini from *Incilius occidentalis* (Camerano, 1879) (Bufonidae) described by Martinez-Salazar and Leon-Regagnon (2007) differs from *R. guaianensis* n. sp. by the larger body size (about 14.2–19.2 mm vs 2.90–3.56), larger buccal capsule (35–47 vs 13–14), and larger oesophagus (833–1,008 vs 266.9–274.6). In addition, *R. kuzmini* presents two lateral pseudolabia instead of the lateral lips, a truncated shape of the anterior end, and the cuticular swelling more prominent mostly in the anterior and posterior parts of the body [19].

Rhabdias guaianensis n. sp. differs from *R. manantlanensis* parasite of *Craugastor occidentalis* (Taylor, 1941) (Craugastoridae) by a smaller buccal capsule (13–14 vs 19–27 in *R. manantlanensis*) and smaller body size (2.9 mm –3.56 mm vs 6.48- 9.64 in *R. manantlanensis*). Additionally, *R. manantlanensis* has a mostly pre-equatorial vulva and larger oesophagus (387-515 vs 266.9–274.6 in *R. guaianensis* n. sp.).

Rhabdias pseudosphaerocephala from *Rhinella marina* (Linnaeus, 1758) is larger than *R. guaianensis* n. sp. (6.17–9.60 vs 2.9–3.56 mm in the new species) and possesses a larger oesophagus (290–380 vs 266.9–274.6 in *R. guaianensis* n. sp.) and larger tail (310–410 vs 160.4–186.9 in *R. guaianensis* n. sp.). The position of the vulva in *R. pseudosphaerocephala* is almost equatorial, while in the *R. guaianensis* n. sp. is post-equatorial.

Rhabdias savagei parasite of *Lithobates forreri* (Boulenger, 1883) differs from *R. guaianensis* n. sp. by the larger body size (4.2–5.3 mm vs 2.9–3.56 mm), larger buccal capsule (18–24 vs. 13–14) and larger oesophagus (366–415 vs 266.9–274.6).

3.2. Molecular analyses and phylogenetic study

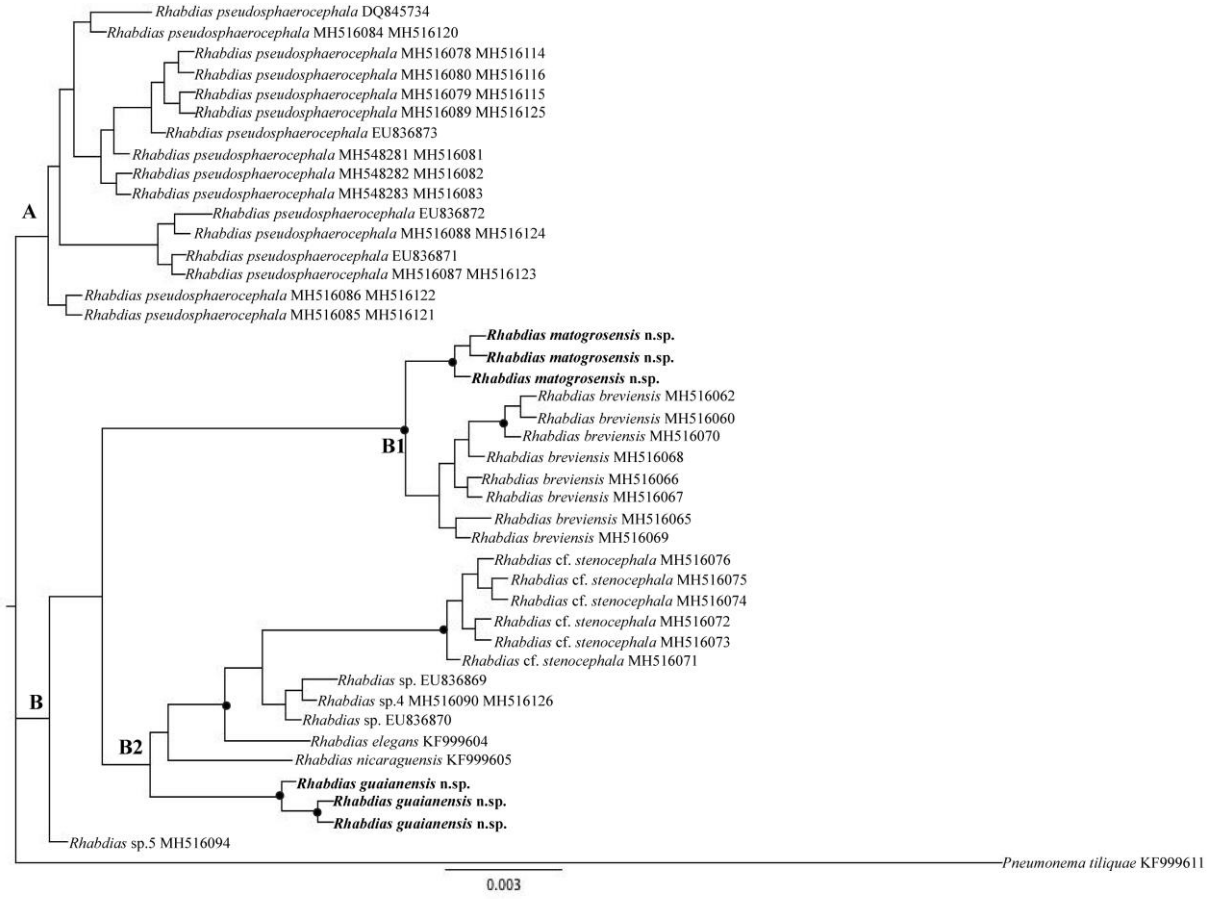
We obtained the partial COI mitochondrial gene from four sequences from *R. matogrossensis* n. sp., 406, 408, 411, and 412 bp), and three sequences from *R. guaianensis* n. sp. (383, 415, and 426 bp) (Table 2). The

alignment of our sequences with those available in GenBank from the Neotropical region generated a matrix of 327 base pairs. Pairwise genetic divergence comparison, considering *Rhabdias* spp. and *Serpentirhabdias viperidicus* (KX350054) as the outgroup, revealed *R. matogrossensis* n. sp. as the closest species from *R. breviensis* complex, with a genetic difference of 1.55%–1.86%. *R. guaianensis* n. sp. is the closest species *Rhabdias* sp.4 from Müller et al. [9] which is demonstrated to be *Rhabdias fuelleborni* [20], with a genetic difference of 7.35%. Relating pairwise distance data from the tree, the two new species here described have shown an interspecific divergence ranging from 0.3% to 20.6% (see Table 2).

We obtained sequences from *R. matogrossensis* n. sp. three from the partial 28S ribosomal gene (588, 593, and 581 bp), three sequences from ITS region (725, 722, and 716 bp), and sequences from *R. guaianensis* n. sp. from the partial 28S ribosomal gene (589, 576, and 585 bp) and three sequences from ITS region (713, 711, and 691 bp). Alignment I comprised the partial 28S gene dataset of *Rhabdias* spp. (424bp) from the Neotropical region and *Pneumonema tiliquae* available in GenBank together with the six newly generated sequences. Alignment II comprised the ribosomal ITS dataset of *Rhabdias* spp. (560 bp) from the Neotropical region and *Pneumonema tiliquae* GenBank together with the six newly generated sequences,

The final concatenated alignment comprised the ITS + 28S datasets and yielded a total of 984 positions with 26 sequences. The topology produced two main clades (labeled A and B; Fig. 8) with Neotropical *Rhabdias* spp. Clade A with low support supported (0.08PP) and comprised *R. pseudosphaerocephala* complex. Clade B was low-supported (0.62PP). This clade was subdivided into two other clades (labeled B1 and B2) (Fig. 8). *Rhabdias matogrossensis* n. sp. clade B1, appeared as a sister to *R. breviensis*, in a clade well-supported (1PP) (Fig. 8). Clade B2, comprising the *R.* cf. *stenocephala* complex, was related to *Rhabdias* spp. (EU836869 and EU836870 from Brazil), *Rhabdias* sp.4 (from Brazil and considered *R. fuelleborni* according to Müller et al. [20], *R. nicaraguensis*, and *R. elegans. Rhabdias guaianensis* n. sp. grouped as a sister species with clade B2 with low support (0.52PP).

Fig. 8. Bayesian phylogenetic tree of Rhabdias spp. from Neotropical region with concatenated internal transcribed spacer (ITS) and 28S ribosomal genes. Black circles in the nodes indicate posterior probabilities (PP) above 90%.



4. Discussion

Rhabdias matogrossensis n. sp. and *Rhabdias guaianensis* n. sp. are the 21rd and 22th nominal species of *Rhabdias* known in the Neotropical Region. Selected diagnostic features of 20 valid Neotropical species and that of the present parasites are presented in Table 1. Based on the set of morphological and molecular data, we describe two new species of *Rhabdias* for the Neotropical region, increased to 22 species in the Neotropical region and 12 species in Brazil.

According to recent molecular and morphological studies [4, 5, 6, 9, 21], *Rhabdias* spp. from the Neotropical region can be divided into three groups concerning the standard lip arrangement: (1) four submedian lips and two lateral pseudolabia (*R. manantlanensis*, *R. savagei*, *R. kuzmini*, and *R. pseudosphaerocephala*), (2) six equal lips (*R. androgyna*, *R. fuelleborni*, *R. breviensis*, *R. galactonoti*, *R. glaurungi*, *R. pocoto*, *R. stenocephala*, *R. tobagoensis*, and *R. waiapi*), and (3) absence of lips *(R. alabialis*, *R. elegans*, and *R. paraensis*) [4, 5, 6, 9, 21, 22]. In their original descriptions of *Rhabdias mucronata*, *R. truncata*, and *R. hermaphrodita*, there is no information about the arrangement of the oral structure [4].

Rhabdias matogrossensis n. sp., coincide in the number of lips of the second group of species (six lips). However, as well as *Rhabdias waiapi*, it has submedian smaller lips and two lateral larger ones. *Rhabdias*

guaianensis n. sp. coincide in the number of lips of the first group of species (four submedian lips and two lateral pseudolabia). One of the most useful taxonomic characteristics for species classification has been the configuration of the circumoral structures of these nematodes [21]. However, Müller et al. [9] reported that despite anterior end structures of Rhabdiasidae, the main morphological characteristics used in taxonomical descriptions, there is no evidence that these structures reflect phylogeny/relationships. According to Moravec and Sey [23], the host specificity and geographical distribution of *Rhabdias* spp. are auxiliary data for comparisons with its congeners. Therefore, our results are similar to recently published works [6, 9, 21], corroborating the hypothesis that species of *Rhabdias* spp. belonging to the same clade may have different patterns of apical structure, while phylogenetically distant species may have the same apical pattern.

In the phylogenetic analysis (28S+ITS), *R. matogrossensis* n. sp. was positioned as a sister of the *R. breviensis* species complex, that could be found in hosts from four different families (Bufonidae, Odontophrynidae, Leptodactylidae, and Hylidae), demonstrating that host switching and ecological fitting were evolutionary more important than association with particular host taxa [21, 24]. *Rhabdias guaianensis* n. sp. was positioned as a sister of the *R.* cf. *stenocephala* and other *Rhabdias* spp., however, with low support. According to our results, the terminal nodes of *R. matogrossensis* n. sp. and *R. guaianensis* n. sp. were strongly supported (1 and 0.96, respectively). Nonetheless, *R. pseudosphaerocephala*, *R. breviensis*, *R.* cf. *stenocephala, Rhabdias* sp., *Rhabdias* sp.5, *Rhabdias* sp.4., *R. elegans*, and *R. nicaraguensis* were not (Fig. 8). According to Tkach et al. [21], this might be due to problems with the molecular ribosomal markers (ITS and 28S), which might not be appropriate for analyzing the relationships between the main lineages of Rhabdiasidae.

The hermaphroditic females of *Rhabdias* spp. have few morphological characteristics that can be used for species comparison. For this reason, the two new species described here show subtle differences among them and also among the species already described in the Neotropical region. However, the molecular characterization of these species corroborates with the morphological data here presented and allows the erection of the three new rhabdiasid species. These data show how important it is to use an integrative taxonomy to determine the diversity of *Rhabdias* species from anurans in this biodiversity hotspot. Although in the present study the diversity of known species of *Rhabdias* described in Brazilian frogs has increased significantly, there are still several other species to be described in this area. Future studies should include screening for more species in Brazilian anurans in different families.

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Declaration of Competing Interest

None.

Ethical approval All applicable institutional, national, and international guidelines for the care and use of animals were followed. North-West University ethics approval no CEUA-UNESP 106.

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Capítulo 2

Phylogenetic position of *Gorgoderina parvicava* Travassos, 1922 (Digenea: Gorgoderidae), a parasite of *Leptodactylus labyrinthicus* (Spix, 1824) (Anura: Leptodactylidae) in Brazil

Abstract

During a parasite survey in Brazilian amphibians from São Paulo state, Brazil, *Gorgoderina parvicava* Travassos, 1922 was found in the urinary bladder (11 adult worms) and (five juvenile worms) in the kidneys of the pepper-frog *Leptodactylus labyrinthicus* (Spix, 1824). Parasites were examined by microscopy and 28S rDNA and COI gene were sequenced and analyzed for the molecular study. The phylogenetic reconstructions resulted in identical topologies with highly supported values in the nodes in most clades using Maximum likelihood and Bayesian inference methods and positioned G. parvicava in the subclade formed by species of subfamily Gorgoderinae parasitizing the urinary bladder of amphibians. Molecular phylogenetic data showed that this species is related to other species of *Gorgoderina*. In addition, new molecular data and the analyses of genetic distances provide extra comparative data, which can be applied in further investigations on the taxonomic status and the diversity among *Gorgoderina* spp. and host-parasite relationships.

Keywords: Amphibian, Helminths, Trematoda, Phylogeny.

Resumo

Posição filogenética de *Gorgoderina parvicava* Travassos, 1922 (Digenea: Gorgoderidae), parasita de *Leptodactylus labyrinthicus* (Spix, 1824) (Anura: Leptodactylidae) no Brasil

Durante o levantamento de parasitas de anfíbios brasileiros do estado de São Paulo, Brasil, *Gorgoderina parvicava* Travassos, 1922 foi encontrado na bexiga urinária (11 vermes adultos) e nos rins (cinco vermes juvenis) da rã-pimenta *Leptodactylus labyrinthicus* (Spix,

1824). Os parasitas foram examinados por microscopia e os genes 28S rDNA e COI foram sequenciados e analisados para o estudo molecular. As reconstruções filogenéticas resultaram em topologias idênticas com valores suportados nos nós na maioria dos clados, usando métodos de máxima verossimilhança e inferência bayesiana e posicionaram *G. parvicava* no subclado formado por espécies da subfamília Gorgoderinae parasitando a bexiga urinária de anfíbios. Dados filogenéticos moleculares mostraram que esta espécie está relacionada a outras espécies de *Gorgoderina*. Além disso, novos dados moleculares e as análises de distâncias genéticas fornecem dados comparativos extras, que podem ser aplicados em futuras investigações sobre o status taxonômico e a diversidade entre *Gorgoderina* spp. e relações parasita-hospedeiro.

Palavras-chaves: Anfíbio**,** Helmintos, Trematoda, Filogenia.

1. Introduction

The Pepper-frog *Leptodactylus labyrinthicus* (Spix, 1824) has a wide distribution through Brazil, Paraguay, and Bolivia (Heyer, 2005; Santos and Haddad, 2006; Heyer et al. 2021; Frost, 2021). In Brazil, the species occurs mainly near wetlands and has been recorded in open habitats throughout the Cerrado, Caatinga, and central Amazonia (Heyer, 1979; Larson and De Sá, 1998; Carvalho et al., 2013).

Some digenean species were previously reported infecting *L. labyrinthicus* from South America, as follows: *Gorgoderina parvicava* Travassos, 1922, *Choledocystus elegans* (Travassos, 1926) Ruiz, 1949, *Neohaematoloechus neivai* (Travassos and Artigas, 1927) Odening, 1960, *Rauschiella linguatula* (Rudolphi, 1819) Razo-Mendivil, Leon-Regagnon and Pérez-Ponce de Leon, 2006, and *Rauschiella palmipedis* (Lutz, 1928) (Freitas, 1960; Dobbin, 1957; Campião et al., 2014; Kohn and Fernandes, 2014).

Gorgoderina Looss, 1902 comprises 57 described species, widely distributed around the world that are parasites of the urinary bladder of several anuran and caudata species (Mata-López et al., 2005; Kohn and Fernandes, 2014; Bursey et al., 2014; Velázquez-Urrieta and Pérez-Ponce de León, 2021). In Brazil, seven *Gorgoderina* species have been reported. Despite the large amount of information available on *Gorgoderina* spp., there are no molecular studies for these species in Brazil.

Studies have demonstrated that molecular analysis is an important tool for the identification and phylogenetic analyses of trematodes (Gomes et al., 2013; Müller et al., 2021; Queiroz et al., 2021; Franceschini et al., 2021). Here, we provide the morphological and the first molecular assessment of *G. parvicava* from *L. labyrinthicus* in Brazil using the mitochondrial gene cytochrome c oxidase I (COI) and the large subunit of the 28S ribosomal gene.

Materials and methods

One adult male specimen of *L. labyrinthicus*, snout-vent length (SLV) 145 mm and weight (W) 245 g, was collected on January 14, 2020, at the Carandá Farm (21°52'30.28"S, 48°13'50.78"W), municipality of Araraquara, São Paulo state, Brazil. During necropsy, digeneans were found in the urinary bladder and kidneys.

The parasites found were fixed in alcohol-formalin-acetic acid solution under the light pressure of a coverslip for 10 min and transferred to 70% alcohol for further processing. Three fresh specimens were added to 96% ethanol for molecular analyses. Digeneans were stained with carmine, cleared with eugenol, and analyzed in a computerized system for image analysis (V3 Leica Application Suite, Leica Microsystems, Wetzlar, Germany) in a microscope with differential interference contrast. Voucher specimens were deposited in the Helminthological Collection of the Instituto de Biociências, Universidade Estadual Paulista

(UNESP), municipality of Botucatu, São Paulo State, Brazil, under #CHIBB 9200. The frog host was deposited at the Herpetological Collection of the Universidade Federal do Ceará, municipality of Fortaleza, Ceará State, Brazil, under #CHUFC- A10088.

The morphological analysis was based on five whole-mounted adult specimens. Measurements are presented as range and are expressed in micrometers, except in the indicated cases.

Genomic DNA was extracted from a portion of three specimens using a DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol, with a final volume of 30 µl. DNA fragments were amplified using a partial 28S rDNA gene containing D1/D3 divergent domains and cytochrome c oxidase subunit 1 (COI). The primers and cycling conditions used to amplify and sequence the partial 28S ribosomal DNA (rDNA) (D1–D3) followed Mendoza-Palmero et al. (2015) and for the mtDNA, COI was amplified using the primers (JB3 and JB4) and polymerase chain reaction (PCR) conditions described by Morgan and Blair (1998). The PCR reactions were performed using 3 µl of DNA extract, 1 µl of each primer, 7.5 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 amplicons (3 µl) were visualized on GelRed (Biotium, Fremont, CA, USA) and purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Sequencing reactions were performed directly on the purified PCR products, with the same PCR primes, using a BigDye v3.1 Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) and sequences were run on an ABI 3500 DNA genetic analyzer (Applied Biosystems).

Contiguous sequences from each molecular marker were assembled and edited in Sequencher v. 5.2.4 (Gene Codes, Ann Arbor, MI). Sequences of partial 28S rDNA and COI were aligned with sequences of *Gorgoderina* spp. and *Phyllodistomum* spp. downloaded from

GenBank for comparative purposes. Sequences of *Glypthelmins tuxtlasensis* Razo-Mendivil, León-Règagnon and Pérez-Ponce de León 2004 was used as an outgroup of COI analysis and *Degeneria halosaur* (Bell 1887) Campbell 1977 was used as an outgroup of 28S rDNA.

Two datasets of DNA sequences were constructed and analyzed. Alignment I comprised the partial 28S gene dataset of *Gorgoderina* spp. and representative species of gorgoderids. This molecular marker was used because it is the molecular marker with the largest number of sequenced species, thus allowing us to test the position of our species in the family. The alignment II comprised the mitochondrial COI gene dataset of *Gorgoderina* spp.. Although there are not many *Gorgoderina* sequences, this marker provides information on intragenotypic differences.

Newly obtained sequences were aligned using the MUSCLE (Edgar, 2004) implemented in Geneious version 11.1.4 (Kearse et al., 2012). To evaluate the occurrence of substitution saturation, the Iss index was estimated in DAMBE 6 (Xia, 2013). The best-fit model for nucleotide substitution was determined by the Akaike information criterion in jModelTest program (Posada, 2008). Phylogenetic analyses were performed using Bayesian inference and maximum likelihood (ML) using MrBayes and RaxML analysis was carried out using the computational resource CIPRES (Miller et al., 2010). The COI sequences were assessed using the default parameters of the Muscle algorithm (Edgar, 2004) implemented in Geneious 7.1.3 (Kearse et al., 2012). Stop codons and indels were also checked in Geneious 7.1.3 (Kearse et al., 2012), translation frame 1, invertebrate mitochondrial. The substitution saturation index was estimated in DAMBE 5 (Xia, 2013) to calculate its occurrence.

The Bayesian Bayesian inference for both analyses was run with the nucleotide substitution model GTR+I+G. To search with the Markov chain Monte Carlo method, chains were run with 10,000,000 generations, saving one tree every 1,000 generations. On the burn-in, the first 25% of generations were discarded, and the consensus trees were estimated using

the remaining trees. Bayesian posterior probabilities (BPP) cutoff was considered > 90%. The branch support for ML was determined by performing 1,000 bootstrap replicates. The Bootstrap cutoff was considered > 75%. The trees were visualized in FigTree v1.3.1 (Rambaut, 2009). Genetic divergence was calculated using the Kimura 2-parameter model in MEGA7.0.20 software (Kimura, 1980; Tamura et al., 2013).

Results

Sixteen digeneans were found, 11 adults in the urinary bladder and 5 juveniles in the kidneys of *L. labyrinthicus*. Based on the observed morphological features and morphometric analyses, the digenean was identified as *G. parvicava* (Figures 1-2; Table 1).

Figure 1. *Gorgoderina parvicava* Travassos, 1922 (Gorgoderidae) parasite of *Leptodactylus labyrinthicus* (Spix, 1824) (Leptodactylidae) from Carandá Farm, municipality of Araraquara, São Paulo state, Brazil. Ventral view.

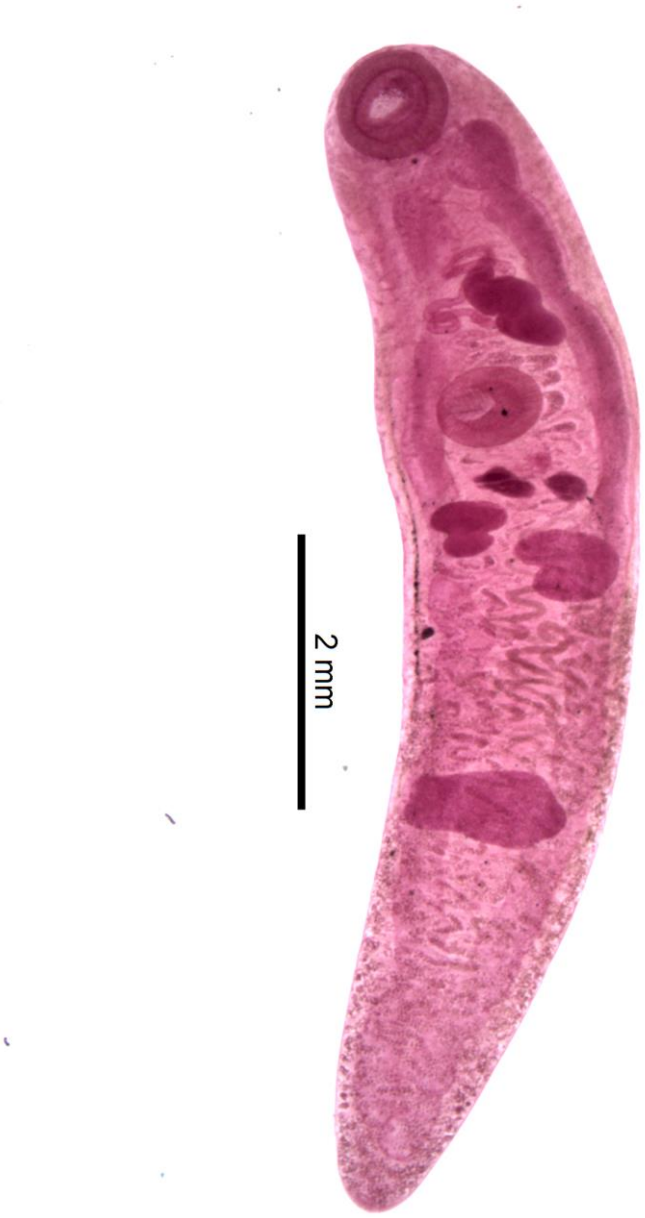
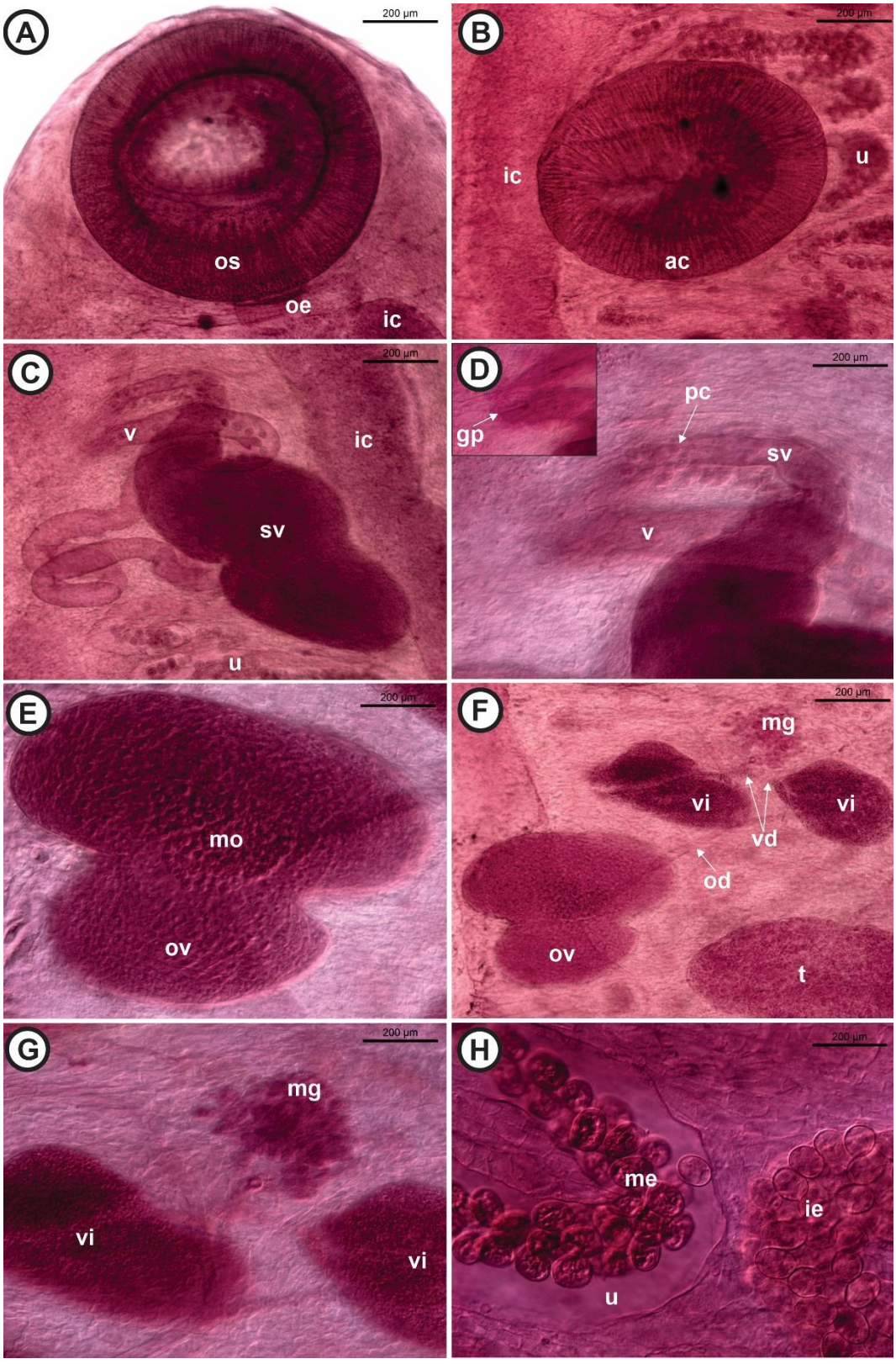


Figure 2. *Gorgoderina parvicava* Travassos, 1922 (Gorgoderidae) parasite of *Leptodactylus labyrinthicus* (Spix, 1824) (Leptodactylidae) from Carandá Farm, municipality of Araraquara, São Paulo state, Brazil. A) Detail of the oral sucker, oesophagus, and intestinal caeca; B) Acetabulum; C) Detail of the region of seminal vesicle and vagina; D) Detail of the terminal portion of the seminal vesicle and vagina, highlighting the genital (left above corner); E) Ovary; F) Region of the ovary highlighting the Mehlis' gland and vitelline follicles; G) Detail of the Mehlis' gland; H) Eggs in the uterus, highlighting part of the descending loop of the uterus with immature eggs and an ascending part with mature eggs. Legend: ac – acetabulum, gp – genital pore, ic – intestinal caeca, ig – immature eggs, me – mature eggs, mg – Mehlis' gland, mo – mature oocytes, od – ovary duct, oe – oesophagus, os – oral sucker, ov – ovary, prostate cells, sv – seminal vesicle, t – testis, u – uterus, v – vagina, vd – vitelline ducts, vi – vitelline follicles.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The *G. parvicava* specimens found in *L. labyrinthicus* presented the following morphology: Elongated body with tapered ends. Smooth tegument, without spines. Oral sucker subterminal. Acetabulum cylindrical to ovoid, slightly smaller than oral sucker. Pharynx absent. Esophagus short and frequently sinuous. Intestinal caeca robust extending until almost the body end. Excretory vesical I-shaped. Excretory pore terminal. Genital pore located after cecal bifurcation, at the middle distance between oral sucker and acetabulum, and slightly dislocated to the right side. Seminal vesicle placed just anteriorly to acetabulum, sinuous, enlarged at basal portion, and thin at distal portion, where prostate composed of few and large cells are distributed. Testes mainly intercaecal, with irregular contours. Anterior testis dislocated to left side, just behind the ovary. Posterior testis postequatorial, transversally to the body. Ovary smaller than testes, placed between anterior testis and vitelline follicles, with irregular contours. Vitelline follicles composed of two follicles, compacts, and with regular contour. Mehlis gland located in middle position, anterior e near vitelline follicles. Ovary duct and short vitelline ducts pass to the Mehlis' gland. Canal de Laurer not oberverd. Uterus distributed from Mehlis' gland to posterior end of body, numerous loops, inter and extracaecal. Descending loops filled with immature eggs and ascending loops filled with mature eggs (Figures 1-2).

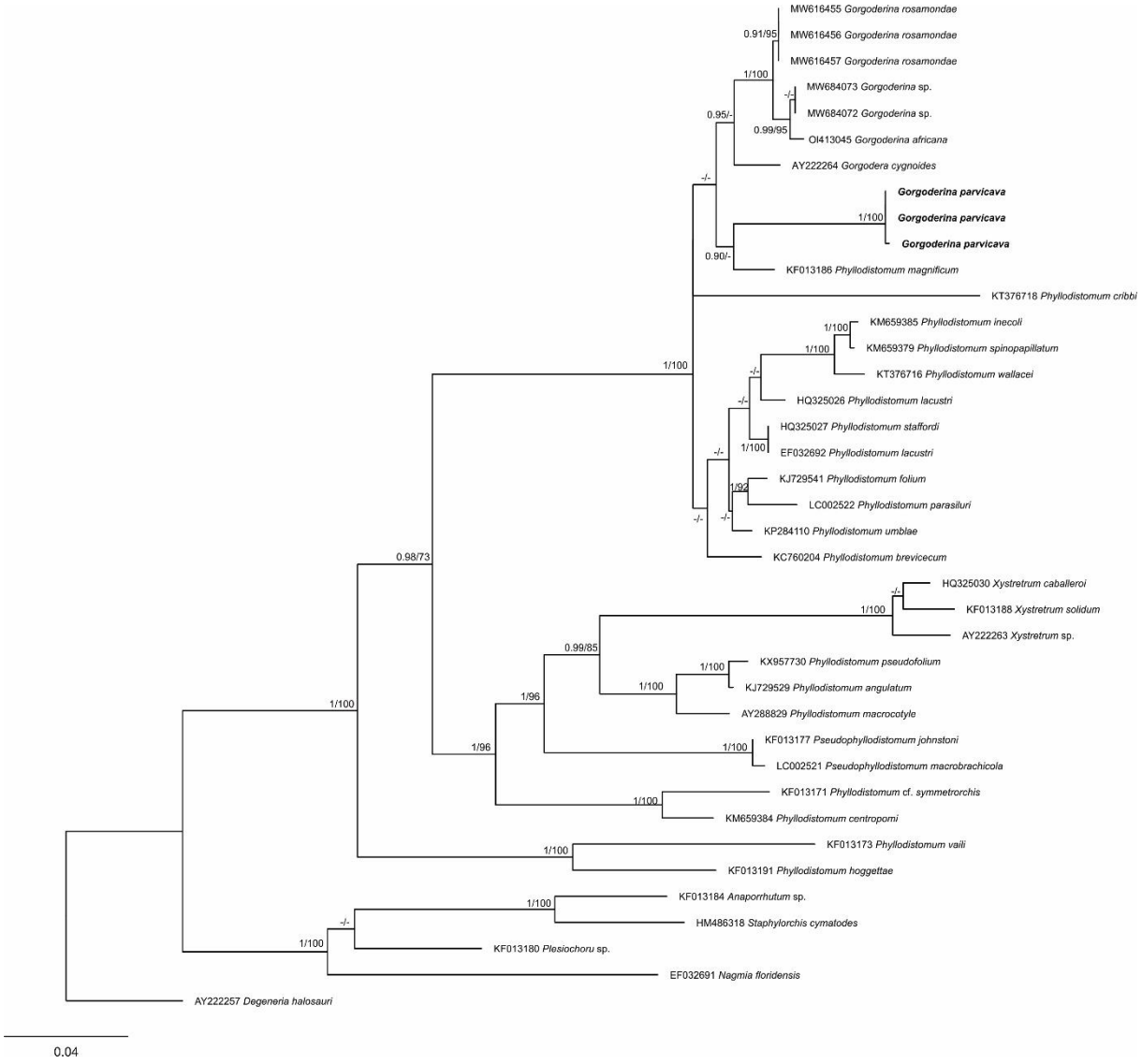
The genomic DNA was extracted from three adult worms and five new sequences were obtained; three partial sequences of the 28S rDNA gene (1,527 bp, 1,558 bp, and 1,537 bp) and two of the COI mtDNA (446 bp and 405 bp). DNA sequences were generated and two alignments were built. The first one included 28S rDNA sequences of *G. parvicava* and other gorgoderid trematodes to test their phylogenetic position within the family (Figure 3). The 28S rDNA final alignments included 36 sequences of Gorgoderoidean digeneans available in the Genbank. Sequences from all individuals were trimmed to match the shortest consensus sequence. The final 28S rDNA alignment consisted of 745 bp long. Genetic

divergence among specimens of *G. parvicava* was 0.1% (Supplementary Table 1), indicating conspecificity. The genetic distance of sequences of *G. parvicava* varied 9.4% from *Gorgoderina africana* Meskal, 1970 parasite of *Hyperolius viridiflavus* (Duméril and Bibron, 1841) (Hyperoliidae), 7% from *Gorgoderina rosamondae* Velázquez-Urrieta and Pérez-Ponce de León 2021 parasite of *Lithobates berlandieri* (Baird, 1859) (Ranidae) and 8.9%-9.1% from *Gorgoderina* sp. (MW684072-73) parasite of *Lithobates vaillanti* (Brocchi, 1877) (Supplementary Table 1).

The 28S Bayesian inference (BI) and maximum likelihood (ML) method resulted in identical topologies with highly supported values in the nodes in most clades (Figure 3). The 28S tree shows that only *Pseudophyllodistomum* Cribb, 1987and *Xystretrum* Linton, 1910 form natural groups. The phylogenetic analysis yielded two main clades labeled as A and B; clade A grouped two species of *Phyllodistomum* parasite of fishes. The main clade B is divided into two subclades B1 and B2 (Figure 3). The subclade B1 high PP (posterior probability) and bootstrap support for the clade comprising species of *Phyllodistomum* Braun, 1899, *Pseudophyllodistomum*, and *Xystretrum* species. Subclade B2 presented well-supported nodes and was subdivided into clades B2.1 and B2.2. The B2.1 presented low support and is formed by *Phyllodistomum* species and the subclade B2.2 presented low support and is formed by *Gorgoderina* and *Gorgodera* species (Figure 3), plus two species of *Phyllodistomum*. *Gorgoderina parvicava* appears in the subclade formed by species of subfamily Gorgoderinae parasitizing the urinary bladder of amphibians. The genus is not monophyletic since *Gorgodera* is nested within the genus.

Figure 3. Maximum Likelihood topology based on partial 28S ribosomal DNA sequences of gorgoderid trematodes. GenBank accession numbers are indicated next to species names. Numbers above nodes represent supported nodes by posterior probabilities for Bayesian

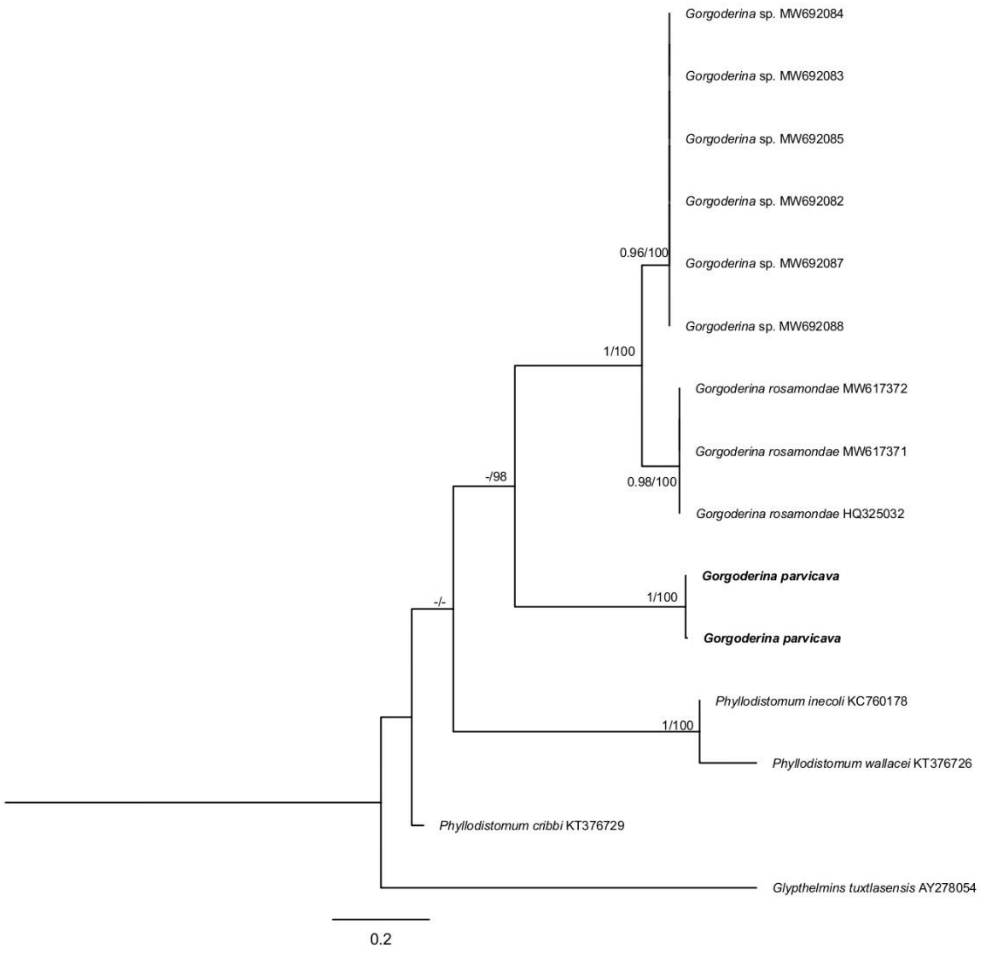
Bayesian inference and bootstrap for maximum likelihood analyses respectively (posterior probabilities > 0.90 and bootstrap scores > 70). Branch length scale bar indicates the number of substitutions per site.



The two newly generated COI sequences have shown 0.3% of genetic divergence among them (Supplementary Table 2). Both phylogenetic analyses (ML and BI) revealed similar phylogenetic patterns; *G. parvicava* clustered with *Gorgoderina* spp. from Mexican frogs (Figure 4). The COI pairwise distance between *G. parvicava* and *G. rosamondae* has shown a genetic divergence of 25% and 26% between *G. parvicava* and *Gorgoderina* sp..

Regarding the *G. rosamondae* and *Gorgoderina* sp., the divergence reported was 9% (Supplementary Table 2).

Figure 4. Maximum Likelihood topology based on COI sequences of *Gorgoderina*, showing the phylogenetic position of the adults of *Gorgoderina parvicava* from Carandá Farm, municipality of Araraquara, São Paulo State, Brazil. Numbers above nodes represent supported nodes by posterior probabilities for Bayesian Bayesian inference and bootstrap for maximum likelihood analyses respectively (posterior probabilities > 0.90 and bootstrap scores > 70). Branch length scale bar indicates the number of substitutions per site.



Discussion

Gorgoderina spp. are urinary-bladder parasites of amphibians. They have body elongated and hindbody slender and elongated; oral sucker subterminal; ventral sucker prominent in anterior third of body; pharynx absent; oesophagus short; caeca simple, terminate near posterior extremity; two testes, intercaecal, postovarian, slightly diagonal; cirrus-sac absent; seminal vesicle in forebody; genital pore median or submedian, immediately postbifurcal; ovary in middle third of body, submedian; vitelline masses compact, smooth or lobed, paired, pre-ovarian; excretory vesicle slender, I-shaped; excretory pore terminal; and eggs embryonated. (Bray et al. 2008). These characteristics were clearly diagnosed in our specimens found in *L. labyrinthicus*.

Gorgoderina parvicava here reported had its morphology compared to other studies with this digenean species (Travassos, 1922; Fernandes, 1958; Suriano, 1965) (Table 1) and its identification was confirmed, except for Suriano (1965), who included a young specimen in its analysis, incorrectly expanding the morphological variation known for the species.

The geographical distribution of this parasite in the Neotropics is wide, and many anurans host were previously reported parasitized with *G. parvicava*, as follows: *Leptodactylus chaquensis* Cei, 1950, *L. labyrinthicus*, *L. latrans* (Steffen, 1815) (= *L. ocellatus*), *L. pentadactylus* (Laurenti, 1768), *R. crucifer* (Wied, 1821), *Rhinella diptycha* (Cope, 1862), *R. icterica* (Spix, 1824), *R. marina* (Linnaeus, 1758), and *Pseudis paradoxa* (Linnaeus, 1758) from Brazil; *L. chaquensis*, *L. latrans*, and *Rhinella fernandezae* (Gallardo, 1957) from Argentina; *Atelopus bomolochos* Peters, 1973, *Leptodactylus rhodonotus* (Günther, 1869), *Rhinella limensis* (Werner, 1901), *Telmatobius culeus* (Garman, 1876), *Telmatobius jelskii* (Peters, 1873), *Telmatobius macrostomus* (Peters, 1873), *Telmatobius peruvianus* Wiegmann, 1834, and *Telmatobius* sp. from Peru; *Rhinella diptycha* from Paraguay; *L. latrans* from Uruguay; and *Pseudis paradoxa* and *Lithobates palmipes* (Spix, 1824) from Venezuela (Campião et al., 2014; Kohn and Fernandes, 2014; Toledo et al.,

2017). Infection with *G. parvicava* in *L. labyrinthicus* has also been reported (Travassos, 1922; Fernandes, 1958; Suriano, 1965). Besides the wide distribution and the number of hosts associated with this digenean, few works provided morphometric data of the species and none of them carried out molecular approaches for *G. parvicava*.

Velázquez-Urrieta and Ponce de León (2021) divided *Gorgoderina* into two main groups according to ventral/oral sucker ratio: (1) the ventral sucker is less than twice the size of the oral sucker; and (2) the ventral sucker is more than twice the size of the oral sucker. Considering this criterium, *G. parvicava* should be included in group 1 while the other *Gorgoderina* spp. used in the 28S phylogeny belong to group 2. This morphological distinction could explain the position of these species in two clades and the supposed paraphilia. Besides the morphology of *G. parvicava* being well known, this is the first sequenced species of group 1 and also in South America. The description of new sequences of *Gorgoderina* spp. from South America can change this scenario. Future studies will be necessary to prove the paraphyly of *Gorgoderina*.

The characteristics commonly used for species delimitation of *Gorgoderina* such as the body length, caeca length, location and shape of the ovary, testes, and vitelline follicles are variable among individuals of the same species (Bravo-Hollis, 1948). For the species delimitation, a set of tools would be needed: analysis of scanning electron microscopy, life cycle, and molecular data (Krull, 1935; Rankin, 1939; Mata-López and León-Règagnon, 2005; Bolek et al., 2009; Mata-López et al., 2005). Nonetheless, molecular data for congeneric species is very limited and few species were studied for molecular biology: *Gorgoderina Africana* (18S and 28S genes) from Central Africa, *Gorgoderina rosamondae* (28S, ITS, and COI) and *Gorgoderina* sp. (28S, ITS and COI) from Mexico, *Gorgoderina attenuata* (Stafford, 1902) Stafford, 1905 (ITS), and *Gorgoderina simplex* (Looss, 1899) (ITS) from USA.

According to the present results in the 28S phylogenetic analyses, some nodes were strongly supported. However, the clade with the *Gorgoderina* spp. sequences were poorly supported (Figure 3). This may result from limitations of the molecular ribosomal marker (28S), which may not be suitable for the analysis of interrelationships of major lineages of Gorgoderidae. Sequences of the 28S rRNA gene were used to test the phylogenetic position of *G. parvicava* within the family Gorgoderidae, confirming the paraphyletic nature of the *Phyllodistomum* spp. (Velázquez-Urrieta and Pérez-Ponce de León, 2021, Cutmore et al., 2013), and suggesting the paraphyly of the genus *Gorgoderina.*

The high genetic divergence between *G. parvicava* and the other *Gorgoderina* spp. and the position of the new sequence in the tree suggest a further revision of the genus. Although not supported by ML, Bayesian inference is supported in the clade of the sequence with *Phyllodistomum magnificum* Cribb 1987 (Figure 3).

The phylogenetic analysis using COI mtDNA has shown the three species (*Gorgoderina rosamondae*, *Gorgoderina* sp., and *G. parvicava)* comprising different subclades and *G. parvicava* appears as a sister group to *G. rosamondae* and *Gorgoderina* sp. That result is expected because both *G. rosamondae* and *Gorgoderina* sp. are from the same locality (Los Tuxtlas, Mexico).

Here we provide new 28s and mtDNA COI sequences of morphologically identified *G. parvicava* along with phylogenetic analyses This study gives preliminary information that should be accessed in future studies to help unravel phylogenetic relationships in Gorgoderidae and the genus *Gorgoderina* in the Neotropical region, contributing to the knowledge of digeneans worldwide.

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Capítulo 3

Morphology, genetic characterization, and phylogeny of *Creptotrema* n. sp. (Trematoda: Allocreadiidae) from the Schmidt's Spinythumb frog *Crossodactylus schmidti* (Amphibia: Anura) [2]

Abstract

A new allocreadiid trematode, *Creptotrema* **n. sp.**, is described from the Brazilian anuran *Crossodactylus schmidti*. The new species can be distinguished from other congeners by the presence of a shell-shaped structure in the ventral sucker. In addition, it differs from other congeners by the combination of the following characters: body size, ventral sucker size, cirrus size, and small eggs. DNA sequences were obtained from *Creptotrema* **n. sp.** including 28S and COI. Genetic divergences among the new species and *Creptotrema* spp. varied from 2.1 to 4.1% for 28S, and 15.1 to 16.8% for COI. Phylogenetic analysis (28S and COI) placed the newly generated DNA sequences of *Creptotrema* **n. sp.** grouped together with all the other sequences of *Creptotrema* in a monophyletic clade (except for *Creptotrema funduli*). Among the *Creptotrema* clade, *Creptotrema* **n. sp.** (the only species that parasitizes an anuran represented in the analyses) appeared as the most basal species. Our results enable us to describe a new species using morphological and molecular data, which will contribute substantially to future studies about the relationships among *Creptotrema* and Allocreadiids in general.

Keywords: Anura, Digeneans, Neotropical region, Phylogeny.

Introduction

Digenea of the genus *Creptotrema* Travassos, Artigas & Pereira, 1928 are worldwide distributed parasites of freshwater teleosts and anurans (Franceschini et al., 2021; Liquin et al. 2022). To date, the genus includes 20 valid species (Franceschini et al., 2021; Liquin et al. 2022), which 16 of those occur in the South America, i.e., the type-species *Creptotrema creptotrema* Travassos, Artigas & Pereira, 1928, *Creptotrema conconae* Franceschini, Aguiar, Zago, Yamada, Ebert & Silva**,** 2021, *Creptotrema diagonale* (Curran, Tkach & Overstreet, 2011), *Creptotrema foliaceum* (Curran, Tkach & Overstreet, 2011), *Creptotrema guacurarii* Montes, Barneche, Croci, Balcazar, Almirón, Martorelli & Pérez-Ponce de León, 2021, *Creptotrema lamothei* Curran, 2008**,** *Creptotrema lynchi* Brooks, 1976, *Creptotrema macrorchis* (Szidat, 1954), *Creptotrema megacetabularis* Franceschini, Aguiar, Zago, Yamada, Ebert & Silva, 2021, *Creptotrema ocloya* comb. nov. Liquin, Gilardoni, Cremonte, Saravia, Cristóbal & Davies, 2022, *Creptotrema pati* Lunaschi, 1985, *Creptotrema paraense* Vicente, Santos & Souza, 1978, *Creptotrema platense* (Szidat, 1954), *Creptotrema schubarti* Franceschini, Aguiar, Zago, Yamada, Ebert & Silva**,** 2021, *Creptotrema stenopteri* (Mañé-Garzón & Gascón, 1973), and *Creptotrema sucumbiosa* Curran, 2008**.**

The diversity of amphibians in Brazil is extraordinarily high (1,206 species), with the vast majority of anurans (1,162 species; 2 invasive exotics) followed by caecilians (39 species) and salamanders (five species in a single family and genus) (Frost 2022; Segalla et al. 2021). Predicting the complete diversity of trematodes is a challenging task since fewer than 8% of those possible host species have been examined for parasites (Campião et al., 2014). Therefore, our current knowledge of the composition of the helminth fauna of amphibians in Brazil

[2] Manuscrito a ser submetido para a revista Systematic Parasitology

is far to be complete. This highlights the need of further parasitological surveys to increase our understanding of parasite species richness and host-parasite relationships, as well as the use of distinct tools for morphological analyses, such as light microscopy and SEM, and molecular investigations, as the employment of different markers for phylogenetic analyses.

During a helminthological survey regarding Brazilian anurans, some *Creptotrema* trematodes were collected from the Schmidt's Spinythumb frog *Crossodactylus schmidti* Gallardo, 1961 (Anura: Hylodidae) in Paraná state. Trematodes were identified using morphological and molecular analyses (28S rDNA gene and COI mtDNA gene). Their examination using integrative approaches revealed that these specimens represented a new species of *Creptotrema*.

Materials and methods

Ethics

All applicable institutional, national, and international guidelines for the ethical handling of animals and collection of zoological material were followed (SISBio #61940) including recommendations from the Ethics Committee for Animal Experimentation (CEUA-UNESP #1061). According to Brazilian laws, species registration for scientific research purposes was carried out at SisGen (X).

Host sampling and parasitological procedures

We collected 27 specimens of *C. schmidti* from Parque Nacional do Iguaçu, municipality of Céu Azul, (25°9’4.036"S, 53°50’28.777"W), Parque Estadual Rio Guarani, municipalilty of Três Barras, Paraná state, (25°26´42.871"S, 53°9´37.879"W), and Parque Estadual do Turvo, municipalities of Derrubadas (27°8'31.68"S, 53°52'39.35"W) and Frederico Westphalen (27°21'43.36"S, 53°24'38.32"O), Rio Grande do Sul state, and municipality of São Miguel do Oeste (26°45'36.10"S, 53°31'30.47"O), Santa Catarina state. Frogs were killed with 2% lidocaine hydrochloride and the internal organs were removed, dissected, and analyzed under a stereomicroscope.

Trematodes were collected from the intestines of the anurans and two fresh specimens were transferred directly to 96% ethanol for molecular diagnosis while the other specimens were fixed in alcohol-formalin-acetic acid solution under light pressure of a coverslip for 10 min and transferred to 70% alcohol for further processing. In the laboratory, digeneans were stained with carmine, cleared with eugenol, and analyzed in a computerized system for image analysis (V3 Leica Application Suite, Leica Microsystems, Wetzlar, Germany) in a microscope with differential interference contrast. Morphological descriptions followed recommendations of Travassos et al. (1928), Kohn (1984), and observations by Scholz et al. (2004) and Razo-Mendivil et al. (2014). All measurements are presented in micrometers (μm) and are expressed as means, followed by the range in parentheses. Illustrations of the structures were produced with aid of a camera lucida mounted on a Leica DMLS microscope with phase-contrast optics.

Holotype and paratypes of the new species of *Creptotrema* will be deposited in the Helminthological Collection of the Oswaldo Cruz Institute (CHIOC), Rio de Janeiro State, Brazil. Other vouchers will be deposited in the Helminthological Collection of the Department of Biostatistics, Plant Biology, Parasitology and Zoology, Institute of Biosciences, São Paulo State University, UNESP (CHIBB), Botucatu municipality, São

Paulo State, Brazil. The frog host was deposited he Museu de Zoologia "Prof. Adão José Cardoso," Universidade Estadual de Campinas (Unicamp), Campinas, São Paulo, Brazil.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from two *Creptotrema* isolates using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol. Fragments of the 28S rDNA gene and the COI mtDNA gene were amplified using the primers and cycling conditions described in Franceschini et al. (2021)**.** Conventional polymerase chain reaction (PCR) amplifications were performed on a final volume of 25 µl containing 12,5 µl of 2× MyFiTM Mix (Bioline, Taunton, MA, USA), 3.0 µl of extracted DNA, 7,5 µl of pure water and 1.0 µl of each PCR primer. PCR products (2.0 µl) were run on an agarose gel (1%) using GelRed™ fluorescent nucleic acid dye and loading buffer to confirm amplicon size and yield. PCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen), following the manufacturer's instructions. Automated sequencing was performed directly on purified PCR products using a BigDye v.3.1 Terminator Cycle Sequencing Ready Reaction kit on an ABI 3500 DNA genetic sequencer (Applied Biosystems). Forward and reverse sequences were assembled and edited using Sequencher v. 5.2.4 (Gene Codes, Ann Arbor, MI, USA).

Phylogenetic analyses

To perform the phylogenetic analyses, two independent datasets were created: the first contained the newly generated 28S rDNA sequences, published sequences of Allocreadiidae retrieved from GenBank and sequences of species from the genera *Prosthenhystera* Travassos, 1922 (Callodistomidae), *Dicrocoelium* Dujardin, 1845 (Dicrocoeliidae), *Degeneria* Campbell, 1977 (Gorgoderidae) and *Phyllodistomum* Braun, 1899 (Gorgoderidae), which were used as outgroup. The second contained the newly generated COI mtDNA sequences, published sequences of Allocreadiidae retrieved from GenBank and sequences of *Phyllodistomum parasiluri* Yamaguti, 1934 (Gorgoderidae), *Dicrocoelium dendriticum* (Rudolphi, 1819) and *Dicrocoelium chinensis* (Sudarikov and Ryjikov, 1951) Tang and Tang, 1978 (Dicrocoeliidae) as outgroup.

The alignments of the two datasets were performed separately using MUSCLE algorithm implemented on Geneious 7.1.3 (Kearse et al., 2012) with default settings. The presence of stop codons and indels for COI mtDNA alignment was verified by amino acid translation using the trematode mitochondrial code table on Geneious 7.1.3 (Kearse et al., 2012).

The best-fitting model of nucleotide substitution for the aligned datasets was selected in the JModelTest software (Posada, 2008) using the Akaike information criterion, as GTR + I + G for the 28S rDNA dataset and HKY + G for the COI mtDNA dataset.

Phylogenetic trees were obtained using Bayesian Inference (BI) and Maximum Likelihood (ML). The BI was performed with MrBayes 3.2 (Ronquist et al., 2012) performed at the online platform CIPRES. The Markov chain Monte Carlo (MCMC) was run with 10,000,000 generations saving one tree every 100 generations, with a burn-in set to 25% of the trees. Only nodes with posterior probabilities (pp) greater than 90% were considered well supported. Phylogenetic analysis using Maximum Likelihood was run in RAxML (Guindon & Gascuel, 2003) and performed at the online platform CIPRES with 1,000 bootstrap replicates. Only nodes with bootstrap values greater than 70% were considered well supported. The ML and BI trees were visualized in FigTree v. 1.3.1 software (Rambaut, 2009) and edited in CorelDRAW X6.

Pairwise genetic distances within and among sequences were calculated using the Kimura-2-parameter (K2P) model and a bootstrap procedure with 1000 replicates in the MEGA7 program (Kimura,1980; Kumar et al., 2016).

Results

Morphological descriptions

Allocreadiidae Looss, 1902

***Creptotrema* Travassos, Artigas & Pereira, 1928**

Type species: *Creptotrema creptotrema* Travassos, Artigas & Pereira, 1928

***Creptotrema* n. sp. Alcantara and Silva (Fig 1-3)**

Taxonomic summary

Type host. ***Crossodactylus schmidti* Gallardo, 1961.**

Type locality Parque Nacional do Iguaçu, municipality of Céu Azul, Paraná state, Brazil (25°9’4.036"S, 53°50’28.777"W)

Other locality. Parque Estadual Rio Guarani, municipality of Três Barras, Paraná state (25°26´42.871"S, 53°9´37.879"W) and municipality of São Miguel do Oeste Santa Catarina state, Brazil (26°45'36.10"S, 53°31'30.47"W).

Site of infection. Large intestine.

Level of infection: Nine specimens were found in the intestine of seven host specimens.

Description

(Based on seven adult specimens). Body elongated, 2.24 (1.95–2.86) mm long, 559.1 (436.2–688.3) wide, short pre-pharyngeal. Oral sucker subterminal, subspherical to funnel-shaped, 208.5 (203.9–241.1) long, 213.9 (181.6–214.8) wide, with a single muscular lobe on either side of oral sucker (“auricles”), with a broad base, stretching from ventrolateral to dorsolateral side and presenting free dorsal ends. Pharynx muscular, subspherical 89.4 (89.1–117.5) long, 99.3 (99.3–127.7) wide. Esophagus 88.2 (88.2–161.8) long. Intestinal ceca blind, extending to posterior end of body. Ventral sucker pre-equatorial, 357.6 (355.1–406) long, 347.5 (274.5–393.6) wide. Ventral sucker opening thin, elongated, with two bivalve shell-like musculatures. Genital pore aperture close to intestinal bifurcation, anterior to ventral sucker. Cirrus-sac well-developed, 768.2 (576.6–768.2) long, 58.9 (54.5–69.4) wide sinuous, passing posterior to ventral sucker, reaching ovary posteriorly, with seminal vesicle, and unarmed and eversible cirrus. Pars prostatica observed. Two testes rounded, in tandem, right testis 231.3 (151–290.8) long, 204.3 (171.7–247.8) wide, and left testis 221.7 (209.5–275.6) long, 210.9 (184.7–301.2) wide juxtaposed next to each other, intercecal. Ovary posterolateral to ventral sucker, pretesticular, obliquely oval to irregular in shape, with entire margin, 179.9 (142.9–234.2) long, 213.3 (142.3–213.3) wide. Vitelline follicles marginal, large, extra- and intra-cecal, not overlapping sexual structures, extending from esophagus level to posterior end of body, completely separated into two lateral fields but confluent in post-testicular region. Mehlis’ gland close to ovary. Laurer’s canal not observed. Uterus pretesticular, intra-cecal. Eggs operculate,

(52.7–63.2) long, (35.3–39.7) wide; few eggs in adult specimens in the early development phase than in completely developed adults (usually > 10). Excretory pore terminal; excretory vesicle I-shaped, reaching anterior testis.

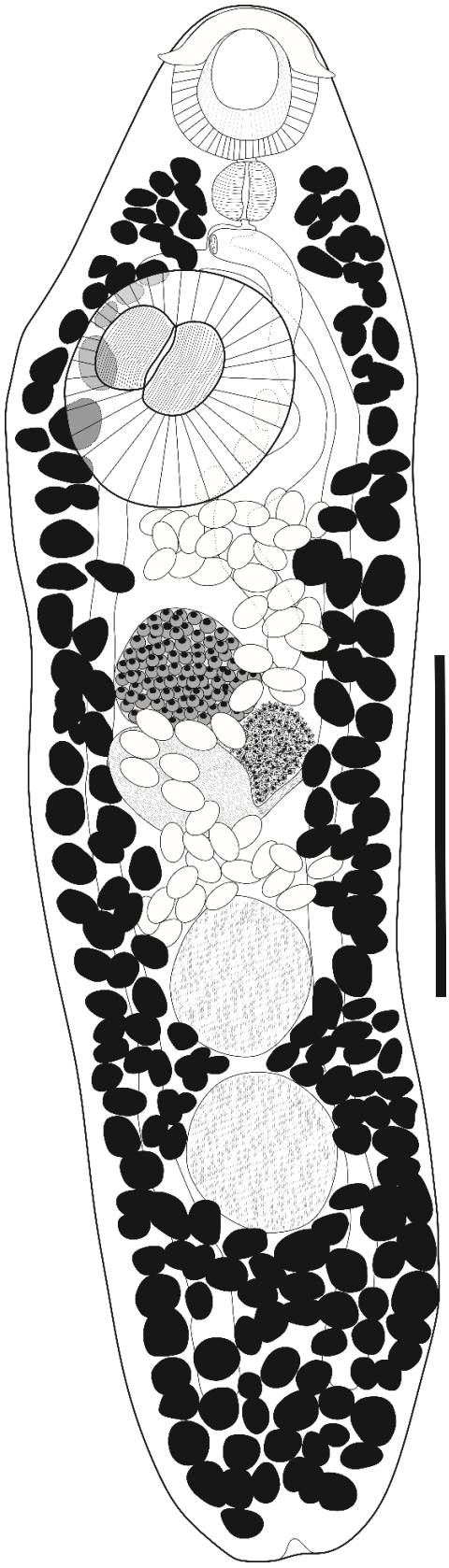

Fig. 1. *Creptotrema* **n. sp.** (Holotype) from *Crossodactylus schmidti* Gallardo, 1961. Scale: 500 μm.

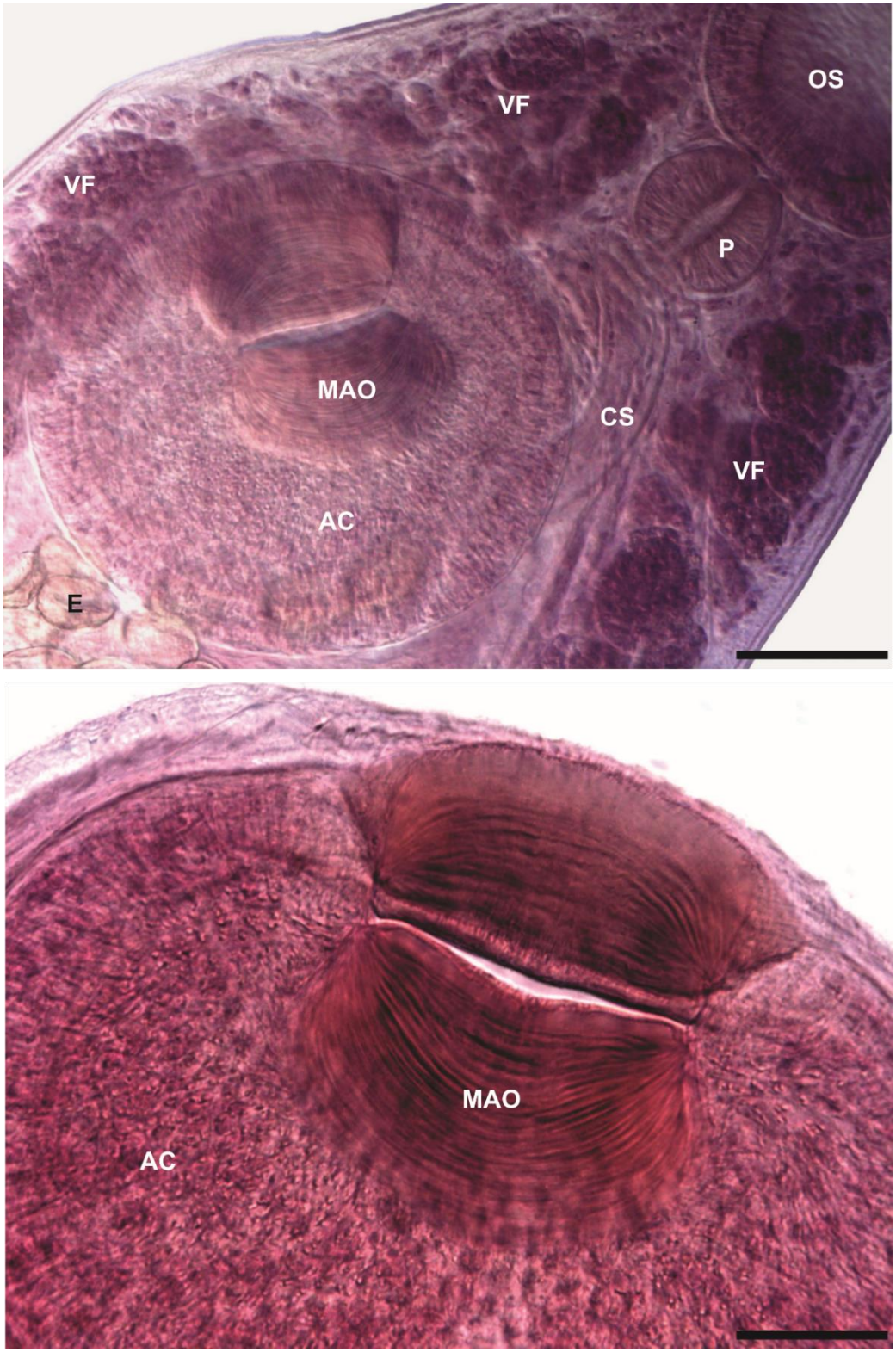

Fig. 2. Anterior region of *Creptotrema* **n. sp.,** highlighting the musculature associated with acetabulum opening. A) general view. B) Detail of the musculature of acetabulum opening. AC, acetabulum; CS, cirrus sac; MAO,

musculature associated with the acetabulum opening; OS, oral sucker; P, Pharynx; VF, vitelline follicles. Scale bar: 100 μm (A) and 50 μm (B).

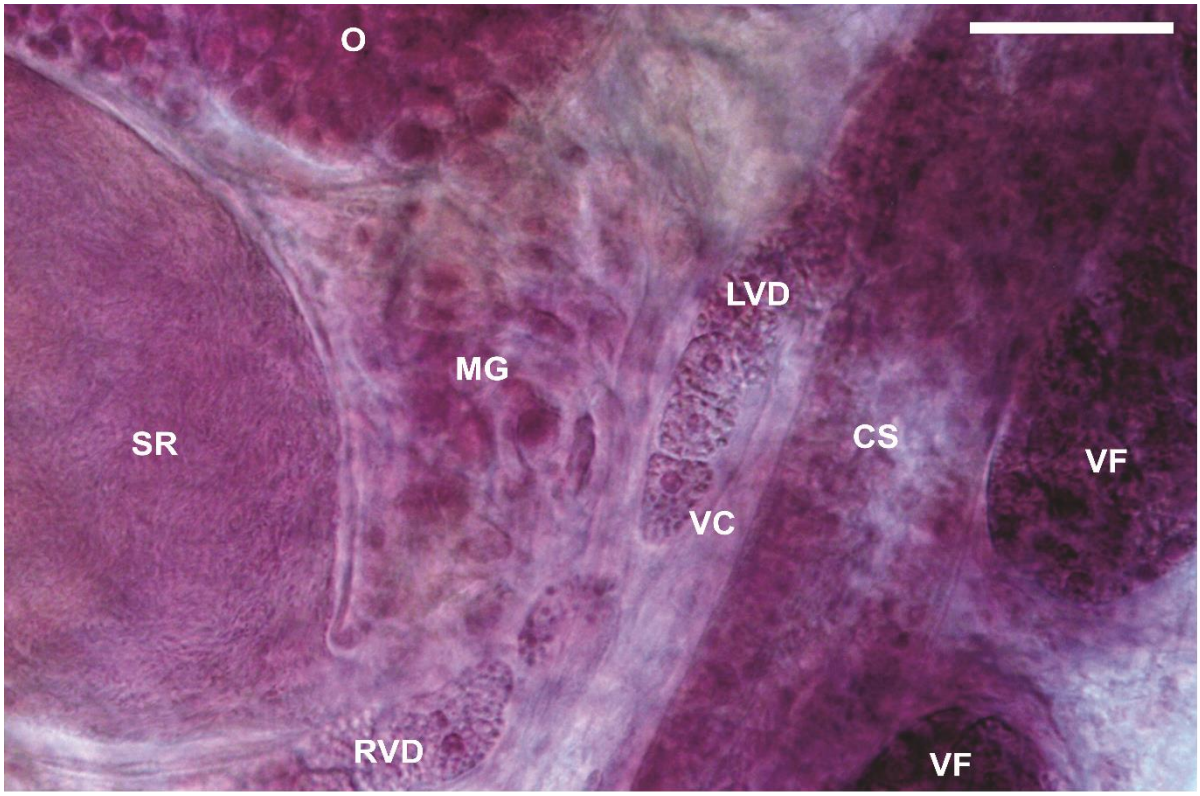

Fig. 3. Detail of the Mehlis' gland region of *Creptotrema* **n. sp.** CS, cirrus sac; LVD, left vitelline duct; MG, Mehlis' gland; O, ovary; RVD, right vitelline duct; SR, seminal receptacle; VC, vitelline cells. Note the nucleus and the cluster of shell protein globules; VF, vitelline follicles. Scale bar: 50 μm.

Molecular and Phylogenetic results

We successfully obtained two partial sequences of the 28S rDNA (GenBank accession numbers XXXXX and XXXXX – sequences will be deposited in GenBank after the acceptance of the manuscript) and one partial sequence of the COI mtDNA (GenBank accession number XXXXX - sequence will be deposited in GenBank after the acceptance of the manuscript) of *Creptotrema* **n. sp.**

The two newly generated 28S rDNA sequences of *Creptotrema* **n. sp.** were 1,254 and 1,253 bp in length and the final alignment was 1,036 bp long. The ML and BI analyses of the partial 28S rDNA alignment produced phylograms with consistent topologies and most clades with supported values (Fig. 4). Both analyses recovered the Allocreadiidae as a monophyletic group (pp = 1; bootstrap = 84). The newly generated sequences of *Creptotrema* **n. sp.** grouped together with all the other sequences of *Creptotrema* in a monophyletic clade, except for *Creptotrema funduli* Mueller, 1934 (pp = 1; bootstrap = 97), with *Wallinia* spp. recovered as its sister group. Among the *Creptotrema* clade, *Creptotrema* **n. sp.** (the only species that parasitizes an anuran represented in the analyses) appeared as the most basal species.

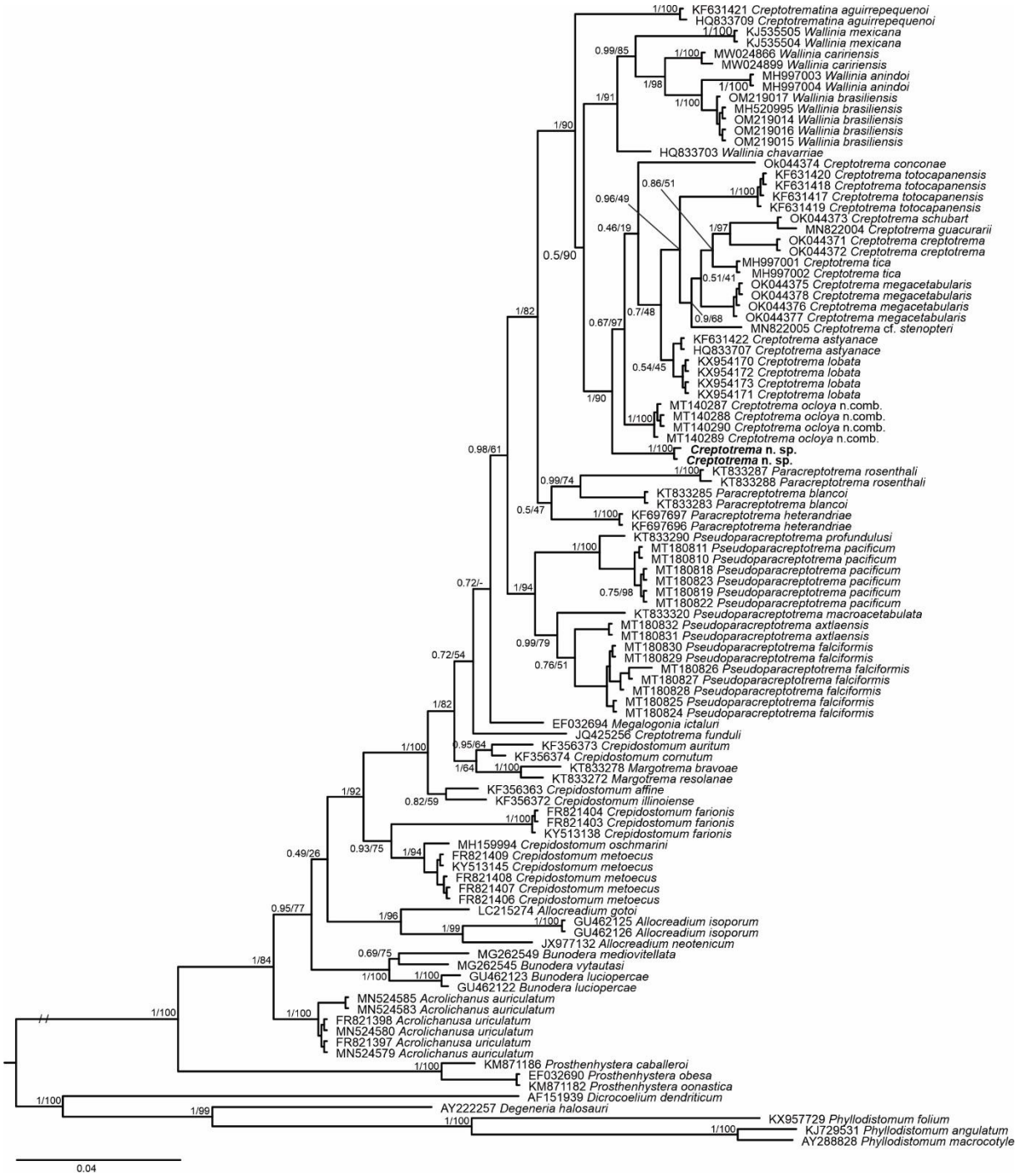

Fig. 4. Bayesian Inference phylogram based on 28S rDNA sequences of Allocreadiidae, showing the phylogenetic position of the new species, *Creptotrema* **n. sp.** from the Schmidt's Spinythumb frog *Crossodactylus schmidti* Gallardo, 1961**.** Numbers above nodes represent posterior probabilities values for Bayesian Inference analyses followed by bootstrap values for Maximum Likelihood analyses (posterior probabilities > 0.90 and bootstrap values> 70 were considered well supported). Branch length scale bar indicates the number of substitutions per site. The new sequences are highlighted in bold.

For the partial 28S rDNA, the interspecific genetic divergences found among the sequences of the new species and *Creptotrema* spp. (except *C. funduli*) varied from 2.1 (*C. astyanacea* (Scholz, Aguirre-Macedo & Choudhury, 2004) to 4.1% (*C. guacurarii* (Montes, Barneche, Croci, Balcazar, Almirón, Martorelli & Pérez-Ponce de León, 2021)) and among *Creptotrema* **n. sp.** and Wallinia spp. (its sister clade), varied from 3.6 and 5.7%. Intraspecific genetic divergence was observed between the two *Creptotrema.* **n. sp.** sequences (0.1%) (Supplementary Table S1).

The newly generated COI mtDNA sequence of *Creptotrema* **n. sp.** was 446 bp in length and the final alignment was 351 bp long. The ML and BI analyses of the partial COI mtDNA alignment recovered identical phylograms with most clades well supported (Fig. 5). Both analyses also recovered the sequences of Allocreadiidae as a monophyletic group (i.e., *Creptotrema* spp., *Margotrema* spp., *Wallinia chavarriae* Choudhury, Daverdin & Brooks, 2002, and *Allocreadium lobatum* Wallin, 1909) (pp = 1; bootstrap = 95). The newly generated sequences of *Creptotrema* **n. sp.** grouped together with all the other sequences of *Creptotrema* in a monophyletic clade (pp = 0.99; bootstrap = 100), with *Wallinia* spp. also recovered as its sister group. As in the partial 28S rDNA analyses, the new species was recovered as the most basal species among the *Creptotrema* clade.

For the COI mtDNA gene, the interspecific genetic divergences among the new species and *Creptotrema* spp. varied from 15.1% (*C. megacetabularis*) to 16.8% (*C. conconae*) (see Supplementary Table S2).

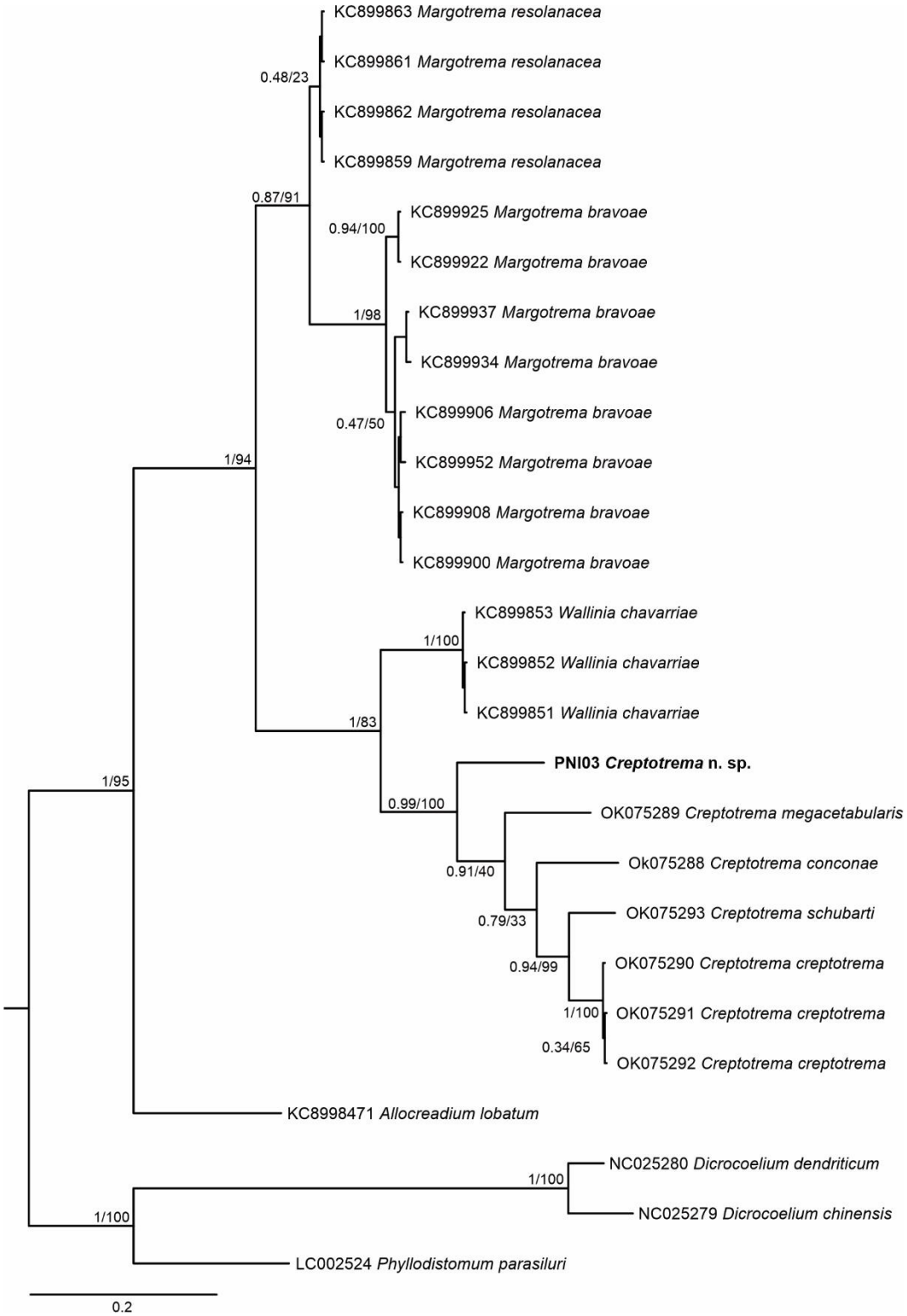

Fig. 5. Bayesian Inference phylogram based on COI mtDNA sequences of Allocreadiidae, showing the phylogenetic position of the new species, *Creptotrema* **n. sp.** from the Schmidt's Spinythumb frog *Crossodactylus schmidti* Gallardo, 1961. Numbers above nodes represent posterior probabilities values for Bayesian Inference analyses followed by bootstrap values for Maximum Likelihood analyses (posterior probabilities > 0.90 and bootstrap values> 70 were considered well supported). Branch length scale bar indicates the number of substitutions per site. The new sequences are highlighted in bold.

Discussion

The morphological analyses and the phylogenetic positioning of our isolates provided in this study allowed us to formally describe a new species of *Creptotrema*. The main morphological characteristic of the new species is the presence of a subterminal oral sucker with a single muscular lobe on either side of the oral sucker, Ventral sucker round, pre-equatorial, larger than oral sucker, vitelline follicles lateral, exclusively extracecal or extra and intracecal, with large or small follicles extending from level of the oral sucker to posterior end of the body.

Creptotrema **n. sp.** parasite of anuran can be distinguished from other congeners by the presence of a shell-shaped musculature in the opening of ventral sucker. In addition, it differs from other congeners by the combination of the following characters: body size, ventral sucker size, cirrus size, and small eggs (Table 1). *Creptotrema* **n. sp.** is the second species of the genus known from anuran and the 17th species known from South America.

The positioning of *Creptotrema* **n. sp.** within the *Creptotrema* clade by both phylogenetic analyses of the 28S rDNA and COI mtDNA genes corroborate its correct assignation. The lowest interspecific genetic divergences were found between the partial 28S rDNA and COI mtDNA sequences of *Creptotrema* **n. sp.** and its congeners (2.1% *C. astyanace* and 15.1% *C. megacetabularis*, respectively), associated to the set of morphological variations shown between the new species and those from *Creptotrema* spp. were considered strong evidence in favor of the erection of the new species. In addition, the *C. astyanace* and *C. megacetabularis* are parasites of fishes while *Creptotrema* **n. sp.** is a parasite of frogs.

Recently, a revised diagnosis of *Creptotrema* was proposed by Franceschini et al. (2021). By using molecular evidence, those authors identified the genus *Auriculostoma* as a synonym of *Creptotrema*, reallocating all described species of the former *Auriculostoma* in their new correct assignation as members of *Creptotrema*. Therefore, considering the new diagnosis of *Creptotrema* proposed by Franceschini et al. (2021), *Auriculostoma ocloya* (Liquin et al. 2022), which was described after the new revision of the genus, should be considered as *Creptotrema ocloya* comb. n.

According to our 28S rDNA phylogenetic inference (see Results section and Fig 4), *C. funduli* was the only sequence of the genus that was not positioned within the *Creptotrema* clade, suggesting that this species might not belong to *Creptotrema* and should be reevaluated, corroborating the results already demonstrated by Franceschini et al. (2021), which proposed the species as *species inquirenda*.

Species identification based only on morphological characteristics is considered biologically unsatisfactory (De Queiroz, 2005; Baum & Donoghue, 1995). Currently, the use of genetic data is essential for the systematic taxonomy of parasites, especially for the description of new species, as it enables the resolution of taxonomic conundrums within some groups through molecular phylogenies, the description of gene flow patterns between species and populations, and, mainly, to understand the diversity in groups of complex parasites (Tkach et al., 2014; Müller et al., 2018, Poulin et al., 2019).

The current knowledge of the helminth fauna richness in Brazilian amphibians is still incomplete and further parasitological surveys are needed to increase our understanding of parasite diversity and host-parasite relationships, implying the use of new tools for morphological analyses and molecular investigations. Our results enable us to describe a new species using morphological and molecular data, which will contribute substantially to future studies about the relationships among *Creptotrema* and Allocreadiids in general.

Table 1. Summary of morphometrics for the most useful features distinguishing species of *Creptotrema*. The morphological measurements were reported in micrometers unless otherwise indicated. Measurements are sourced from original descriptions plus selected redescriptions.

Species	Host type	Country	Body length (mm)	Body widt	Oral sucker	Pharynx	Esophagus	Ventral sucker	Ovary	Testis	References
***Creptotrema* n. sp.**	***Crossodactylus schmidti***	**Brazil**	**1.95–2.86**	**436.2–688.3**	**203.9–241.1 x 181.6–214.8**	**89.1–117.5 x 99.3–127.7**	**88.2–161.8**	**355.1–406 x 274.5–393.6**	**142.9–234.2 x 142.3–213.3**	**151–290 x 171–301**	**Presenty study**
Creptotrema astyanace	*Astyanax fasciatus*	Nicaragua	1.90–2.90	400–488	202–234 x 256–304	93–115 x 90–118	70–141	250–285 x 250–330	160–230 x 144–202	166–349 x 157–218	Scholz et al. 2004
Creptotrema conconae	*Imparfinis mirini*	Brazil	0.99–1.86	126–421	126–188 x 127–209	(31–50 x 57–61	128–156	116–169 x 150–179	100–175 x 87–163	93–179 X 100–176	Franceschini et al. 2021
Creptotrema creptotrema	*Megaleporinus obtusidens*	Brazil	1.12–1.41	513–663	141–206 x 175–194	67–82 x 68–93	87–216	232–278 x 223–305	151–181 x 119–182	125–184 x 89–143	Franceschini et al. 2021
Creptotrema diagonale	*Stethaprion cf. erythrops*	Peru	1.21–1.6	352–474	70–190 x 170–208	45–57 l x 63–68		213–221 x 213–227	156–161 x 102–145	193–304 x 110–159	Curran et al. 2011
Creptotrema foliaceum	*Bryconops cf. caudomaculatus*	Peru	1.99	450	279 x 374	95 x 100	111	207 x 202	145 x 117	106 - 167 x 134 - 145	Curran et al 2011
Creptotrema guacurarii	*Characidium heirmostigmata*	Argentina	1.08–1.44	259–456	93–137 x 106–16)	41–60 x 50–69	41–76	122–157 x 120–148	101–145 x 77–104	126–173 x 68–83	Montes et al. 2021
Creptotrema lamothei	*Ageneiosus brevifilis*	Paraguay	1.15-1.41	359-457	223-258 x 173-228	62-100 x 57-78		223-258 x 173-228	72-99 x 79-102	91-153 x 85-159	Curran 2008
Creptotrema lobata (Hernández-Mena, Lynggaard, Mendoza-Garfias & Pérez-Ponce de León, 2016)	*Brycon guatemalensis*	Mexico	2.54-3.01	502-639	153-234 x 243-245	114-139 x 82-112	71-141	238-319 x 233-324	164-217 x 92-165	267-396 x 167-274	Hernández-Mena et al. 2016
Creptotrema lynchi	*Rhinella marina*	Colombia	0.85-1.49	390-670	156-276 x 192-336	60-84 x 120		264-396 x 276-372	204-264 x 2160188	340-540 x 180-265	Brooks 1976
Creptotrema macrorchis	*Pachyurus bonariensis*	Argentina	1.5	500	180 x 200	70 x 50		150			Szidat, 1954
Creptotrema megacetabularis	*Auchenipterus osteomystax*	Brazil	0.93–1.55	332–625	101–151 x 115–190	37–56 x 45–68	52–54	190–270 x 160–247	116–185	105–213 x 78–134	Franceschini et al. 2021
Creptotrema ocloya n. comb.	*Heptapterus qenqo*	Argentina	0.85–1.52	216–393	117-176 x 113–193	49–74 x 56–86	49–73	122–193 x 113–208	95–154 x 95–184	110–174 x 98–149	Florencia Liquin et al 2022
Creptotrema paraense	*Pimelodus sp.*	Brazil	1.84	510	220 x 180	80 x 90		160 x 150	140 x 180	210 x 250	Vicente et al. 1978
Creptotrema pati	*Luciopimelodus pati*	Argentina	0.943-1.415	687-856	174-207 x 185-207	50-81 x 59-67		205-251 x 208-262	174-218 x 87-119	272-398 x 82-174	Lunaschi, 1985
Creptotrema platense	*Pimelodus maculatus*	Argentina	0.8	200	120	30 x 30		120			Szidat, 1954
Creptotrema schubarti	*Characidium schubarti*	Brazil	0.67–0.91	324–394	91–122 x 122–138	60 x 54	62–89	71–149 x 75–138	140 x 122	104–179 x 82–122	Franceschini et al. 2021
Creptotrema stenopteri	*Charax stenopterus*	Uruguay	0.75-1.32	216-308		60-96 x 60-88	40-80	148-190 x 140-240	52-120 x 32-52	60-120 x 72-150	Mañé-Garzón & Gascón, 1973
Creptotrema sucumbiosa	*Tetragonopterus argenteus*	Ecuador	1.75-1.88	472-584	212-296 x 234-279	89-100 x 67-83	89-100	250-340 x 284-307	156-170 x 122-140	307-390 x 139-195	Curran 2008
Creptotrema tica (Hernández Mena, Pinacho-Pinacho, García-	*Gymnotus maculosus*	Costa Rica	0.55–2.12	424–568	240–27 x 216–260	74–98 x 81–115		143–179 x 161–192	90–112 x 75–129	135–182 x 83–144	Hernández-Mena et al. 2018

Varela, MendozaGarfias & Pérez-Ponce de León, 2018)											
Creptotrema totonacapanense (Razo-Mendivil, Mendoza-Garfias, PérezPonce de Léon & Rubio-Godoy, 2014)	*Astyanax mexicanus*	Mexico	1.03–2.0	287–568	117–212 x 128–223	39–92 x 44–101	71–96	153–240 x 154–259	93–210 x 68–167	103–259 x 62–239	Razo-Mendivil et al. 2015

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Code availability Not applicable**.**

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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Consent to participate Not applicable.

Consent for publication Not applicable.

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Anexo 1

Anexo 1 – Artigo publicado no Journal of Helminthology

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)

Resumo

Cosmocerca spp. são parasitas nematóides comuns de anfíbios. Fornecemos aqui dados moleculares para duas espécies de *Cosmocerca* e descrevemos uma nova espécie, *Cosmocerca albopunctata* n. sp., usando microscopia de luz e dados moleculares (COI mtDNA). *Cosmocerca albopunctata* n. sp. pode ser facilmente distinguida de outras espécies congenéricas pela combinação de características como tamanho do corpo, comprimento dos espículos e gubernáculo, arranjos e número de papilas caudais (7+1:1+1:6). Os resultados filogenéticos baseados nas sequências parciais de mtDNA de COI agruparam a nova espécie em um clado monofilético junto com as demais sequências de *Cosmocerca* spp. Nossos resultados contribuem para o conhecimento da diversidade de espécies e dados genéticos de *Cosmocerca* spp. na região Neotropical.

Palavras-chave: Anura. Nematoda. Cosmocercidae. Morfologia. Identificação molecular.

Anexo 2

Anexo 2 – Artigo publicado na revista Zootaxa

Rhabdochona fuscovaria sp. n. (Nematoda: Rhabdochonidae) from the stomach of frog *Scinax fuscovarius* (Anura: Hylidae) in Brazil

Resumo

Uma nova espécie de nematoide, *Rhabdochona* (*Rhabdochona*) *fuscovaria* sp. n. (Rhabdochonidae), é descrita com base em espécimes coletados do estômago da snouted treefrog Scinax fuscovarius (Hylidae) na fazenda São Sebastião do Paraíso, município de Boa Esperança do Sul, sudeste do Brasil. Esta espécie é caracterizada por deirídeos pequenos, simples e em forma de estilete, prostom em forma de funil com 14 dentes, espículo esquerdo conspícua (585,7 µm), ponta distal ligeiramente alargada, moderadamente dilatada, espículo direito (132,9 µm), em forma de barco, sem farpa dorsal na ponta distal, 18 pares de papilas caudais (9 pares pré-cloacais e 9 pares pós-cloacais) e ovos não filamentosos. Esta é a quarta espécie descrita para a América do Sul e a 13ª para a Região Neotropical.

Palavras-chave: Anfíbio, Macroparasita, Região Neotropical, América do Sul

Anexo 3

Anexo 3 – Artigo publicado na revista Zootaxa

A new species of *Aplectana* (Nematoda: Cosmocercidae) in the Marsupial frog *Gastrotheca microdiscus* (Amphibia: Hemiphractidae) from Brazil

Abstract

Aplectana longa **n. sp.** (Ascaridida: Cosmocercidae) parasite de intestine delgado de *Gastrotheca microdiscus* (Amphibia: Hemiphractidae) é descrita e ilustrada aqui. A nova espécie é caracterizada pela combinação de um conjunto único de caracteres morfológicos: 1) Tamanho corporal grande em ambos os sexos; 2) Lateral asa ausente; 3) Gubernáculo presente e pequeno, e 4) Arranjo das papilas caudais (9+1:0:6). O padrão de distribuição das papilas caudais é semelhante *Aplectana chamaeleonsis*. No entanto, *Aplectana longa* **n. sp.** é facilmente diferenciada desta espécie pelo arranjo das papilas pré-cloacais. Esta é a 57ª espécie de *Aplectana* e a 16ª espécie registrada para o Brasil.

Palavras-chave: Nematoda, Parasita, Anura, América do Sul, região Neotropical

Anexo 4

Anexo 4 – Artigo publicado na revista Neotropical Helminthology

Intestinal helminths of *Hylodes heyeri* Haddad, Pombal & Bastos, 1996 (Anura: Hylodidae) in the Atlantic Forest, Brazil

Abstract

Cosmocercidae são parasitas comuns de anfíbios e répteis. Apesar de ser uma família bastante conhecida, falta conhecimento sobre a diversidade de seus hospedeiros. Neste trabalho, ampliamos o conhecimento da fauna parasitária de anuros da família Hylodidae e a distribuição geográfica de três espécies de nematóides cosmocercídeos (*Cosmocerca brasiliensis* Travassos, 1925, *Cosmocercoides sauria* Ávila, Strüssmann & Silva, 2010, e cosmocercídeo não identificado) encontrados em dois machos de *Hylodes heyeri* Haddad Pombal & Bastos, 1996. Nosso relatório inclui novos dados sobre a distribuição geográfica e a morfologia de cosmocercídeos parasitas e esclarece ainda mais as interações parasitárias de anfíbios neotropicais.

Palavras-chave: anfíbios - Cosmocercidae - endoparasitas - Nematoda - novo hospedeiro

Anexo 5

Anexo 5 – Artigo publicado na revista Journal of Parasitology

Helminths of *Dermatonotus muelleri* (Anura: Microhylidae) from Northeastern Brazil

Resumo

A helmintofauna associada ao Muller's térmite frog, *Dermatonotus muelleri*, da região sul do Estado do Ceará, Brasil, foi estudada. A riqueza de espécies foi de 6 táxons de helmintos, incluindo cistacantos de Acanthocephala e 5 espécies de nematóides: A*plectana membranosa, Parapharyngodon silvoi, Raillietnema spectans*, larvas de *Physaloptera* sp. e um nematóide não identificado. A prevalência geral foi de 88,6%, com intensidade média de infecção de 123,7 6 26,3. *Raillietnema spectans* apresentou a maior prevalência e foi o mais abundante (d = 0,670). O tamanho do corpo do hospedeiro não influenciou a intensidade da infecção nem a riqueza de espécies de helmintos. Este estudo aumenta o conhecimento sobre a diversidade de helmintofauna associada a *Dermatonotus muelleri* de nordeste do Brasil, ampliando o registro de hospedeiros e a distribuição geográfica dessas espécies de helmintos.

Considerações finais

CONSIDERAÇÕES FINAIS

Os resultados obtidos durante os estudos realizados para o desenvolvimento desta tese trazem importante contribuição para o conhecimento da riqueza de helmintos em anuros brasileiros. Através da caracterização morfológica e molecular dos parasitas realizada até o presente momento foi possível identificar 64 *taxa* de helmintos, dos quais 15 são novas espécies, sendo 14 espécies de Nematoda e 1 espécie de Trematoda. Ainda é possível que esse número aumente, pois algumas espécies ainda carecem de analises morfológicas e moleculares que serão realizadas posteriormente.

O presente estudo amplia o conhecimento taxonômico da helmintofauna associada a anuros brasileiros, através de análises de caracteres morfológicos e moleculares, promovendo descrições de espécies de helmintos e um inventário estruturado das espécies que fazem parte da anurofauna brasileira. A análise filogenética realizada até o presente momento, com as espécies helmintos das famílias Cosmocercoidae, Rhabdisiadae e Gorgoderidae que parasitam anuros brasileiros, as quias terão suas sequências parciais disponíveis no GenBank, nos possibilitaram fazer inferências sobre as relações filogenéticas dessas espécies.

A grande variedade de helmintos encontrada nas espécies de anuros alvos deste estudo evidenciam a necessidade de se intensificar o estudo da diversidade e de análises filogenéticas destes parasitos.