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Original Article

Contracaecum sp. larvae (Nematoda: Anisakidae) parasitizing *Astyanax lacustris* (Lütken, 1875) (Characiformes, Characidae) and *Geophagus brasiliensis* (Quoy & Gaimard, 1824) (Cichliformes, Cichlidae) in the Pardo River, São Paulo State, southeast Brazil

Larvas de *Contracaecum* sp. Railliet & Henry, 1912 (Nematoda: Anisakidae) parasitando *Astyanax lacustris* (Lütken, 1875) (Characiformes, Characidae) e *Geophagus brasiliensis* (Quoy & Gaimard, 1824) (Cichliformes, Cichlidae) no Rio Pardo, Estado de São Paulo, Região Sudeste do Brasil

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Abstract

Astyanax lacustris (n = 26) and *Geophagus brasiliensis* (n = 28) were collected from two sampling sites along the Pardo River, in Botucatu, São Paulo, Brazil, and examined for parasites. Nematodes were found encysted in the intestinal serosa, heart, and fatty tissue. Morphological analysis identified the helminths as L3 larvae of *Contracaecum*, which was further confirmed by molecular analyses using cytochrome c oxidase subunit 2 (COX II) and large subunit ribosomal RNA (28S) genes. Phylogenetic inferences placed these larvae within a well-supported clade of Neotropical and Panamanian *Contracaecum*, containing sequences of *Contracaecum jorgei* (Argentina and Colombia) and other sequences identified as *Contracaecum* sp. and *Contracaecum multipapillatum* (Mexico, Costa Rica and Guatemala). Molecular distance analyses suggest that this Neotropical/Panamanian lineage of *Contracaecum* represents a single species, highlighting the fact that *C. multipapillatum* is currently non-monophyletic, with divergent lineages distributed across the Nearctic, Palearctic, Oriental, and Neotropical regions. Haplotype analysis supports this hypothesis with evidence of recent speciation events or ongoing gene flow within these Neotropical/Panamanian *Contracaecum* lineages. This new record of *Contracaecum* sp. larvae infecting fish in the Pardo River, Brazil, provides valuable insight into the phylogeny of this genus, which is suspected to have zoonotic potential, though no documented human infections have been reported in the Americas.

Keywords: biodiversity, molecular phylogeny, haplotype, helminth, neotropical.

Resumo

Astyanax lacustris (n = 26) e *Geophagus brasiliensis* (n = 28) foram coletados de dois locais de amostragem ao longo do Rio Pardo, em Botucatu, São Paulo, Brasil, e examinados para parasitas. Nematoides foram encontrados encistados na serosa intestinal, coração e tecido adiposo. A análise morfológica identificou os helmintos como larvas L3 de *Contracaecum*, o que foi posteriormente confirmado por análises moleculares usando genes da subunidade 2 do citocromo c oxidase (COX II) e do DNA ribossômico da subunidade grande (28S). Inferências filogenéticas colocaram essas larvas dentro de um clado bem suportado de *Contracaecum* neotropical e panamenho, contendo sequências de *Contracaecum jorgei* (Argentina e Colômbia) e outras sequências identificadas como *Contracaecum* sp. e *Contracaecum multipapillatum* (México, Costa Rica e Guatemala). Análises de distância molecular sugerem que esta linhagem Neotropical/Panamenha de *Contracaecum* representa uma única espécie, destacando o fato de que *C. multipapillatum* é atualmente não monofilético, com linhagens divergentes distribuídas pelas regiões Neártica, Paleártica, Oriental e Neotropical. A análise de haplótipos apoia esta hipótese e sugere ainda que eventos recentes de especiação ou fluxo gênico em andamento podem estar ocorrendo dentro destas linhagens Neotropicais/Panamenhas de *Contracaecum*. Este novo registro de larvas de *Contracaecum* sp. infectando peixes no Rio Pardo, Brasil, permite que interpretações importantes sejam feitas sobre a filogenia deste gênero, que é suspeito de ter potencial zoonótico, embora nenhuma infecção humana tenha sido relatada nas Américas.

Palavras-chave: biodiversidade, filogenia molecular, haplótipo, helmintos, neotropical.

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1. Introduction

Fish are remarkable vertebrates that host a diverse array of metazoan parasites, ranging from cnidarians to arachnids (Woo et al., 2011; Thatcher, 2006). Compared to other vertebrate groups, fish exhibit the highest diversity of helminth parasites (Carlson et al., 2020), thriving in marine, brackish, and freshwater environments. Among these helminths, nematodes are particularly important as they often act as endoparasites of several fish species and play crucial roles in the parasitic dynamics of aquatic ecosystems (Anderson, 2000).

Both adult and larval stages of nematodes can affect their hosts in various ways, by appropriating the fish nutrients, attacking host tissues, organs, fluids, or blood, and causing direct physical harm that triggers inflammatory responses (Menezes et al., 2006; Molnár et al., 2006; Abdelmonem et al., 2010; Santoro et al., 2013; Horbowy et al., 2016; Sayyaf-Dezfuli et al., 2016; Austin et al., 2022). They can also cause hematological alterations (Kundu et al., 2016), atrophy of the liver and striated musculature (Margolis, 1970; De Buron and Roumillat, 2010), impair host fecundity (Oliva et al., 1992; Hesp et al., 2002), and significantly increase the mortality rate of infected hosts, especially in severe infections (Molnár et al., 2006; Horbowy et al., 2016).

In addition to the injuries caused to hosts, certain helminths in fish pose significant health risks to humans, particularly the larvae of nematodes from the family Anisakidae, known to cause anisakidosis, a zoonotic threat. Although considered rare by some medical practitioners (Khan and Williams, 2016), its incidence has been increasing, perhaps due to globalization in culinary habits of consuming raw fish (Shamsi and Barton, 2023). However, the failure to perform differential diagnoses, especially in individuals consuming raw or undercooked fishes, has resulted in numerous misdiagnoses due to symptoms resembling those of other conditions, such as cancer and Crohn's disease (Ishikura et al., 1993; Shamsi and Barton, 2023).

Anisakidosis is primarily caused by larval anisakids from the genera *Anisakis* Dujardin, 1845, *Contracaecum* Railliet & Henry, 1912, *Pseudoterranova* Mozgovoi, 1951, and *Phocanema* Mozgovoi, 1951, which parasitize fish and other hosts (Ishikura et al., 1993; Falla-Zuñiga et al., 2021; Shamsi and Barton, 2023). *Anisakis simplex* (Rudolphi, 1809) is the most frequently identified species in human infections (Ishikura et al., 1993; Shamsi and Barton, 2023), but taxonomic challenges complicate species identification, as larval morphology is difficult to assess (Shamsi and Barton, 2023). Moreover, molecular phylogenetic studies have led to reclassifications, including the reassignment of *Phocanema decipiens* (Krabbe, 1878) and *Skrjabinisakis physeteris* (Baylis, 1923), previously classified under *Pseudoterranova* and *Anisakis*, respectively (Ishikura et al., 1993; Safonova et al., 2021; Bao et al., 2023; Shamsi and Barton, 2023). Additionally, *Hysterothylacium* species, once grouped within Anisakidae, are now placed in Raphidascarididae (Pereira and Luque, 2017; Li et al., 2018; Shamsi and Barton, 2023).

In South America, anisakidosis remains an underrecognized risk. Despite this, immunological responses to *Anisakis* have been reported, including cases involving pregnant women (Figueiredo Junior et al., 2013, 2015; Eiras et al., 2018; Falla-Zuñiga et al., 2021). Yet, endoscopic observations have also linked larvae resembling *Anisakis* to gastrointestinal lesions (Rosa-da-Cruz et al., 2010). While marine fish are the primary source of infection, freshwater species can also carry anisakids, and the rising consumption of freshwater fish in Brazil (FAO, 2024) may elevate the risk.

Brazilian freshwater fish harbor diverse anisakid larvae, with *Contracaecum* being the most prevalent (Luque et al., 2011). However, both taxonomic and phylogenetic understanding of these larvae remain limited. Some studies have classified them based on morphometric traits, identifying two morphotypes: *Contracaecum* sp. Type 1, characterized by an elongated ventricular appendix, and *Contracaecum* sp. Type 2, which is larger but has a proportionally shorter appendix (Moravec, 1998). Despite these efforts, no direct associations with adult specimens have been established. The advent of DNA sequencing has made such connections more feasible, providing a more robust approach to resolving taxonomic uncertainties (see Sardella et al., 2020). Nevertheless, there are still few *Contracaecum* sequences from Brazil available in GenBank (Borges et al., 2014; Jacobus et al., 2016; Sayers et al., 2022; Santana et al., 2023), with none originating from freshwater lineages. Therefore, expanding research in this area is critical for developing a comprehensive framework to support public health policies.

Astyanax lacustris (Lütken, 1875) (Characidae) and *Geophagus brasiliensis* (Quoy and Gaimard, 1824) (Cichlidae) are two common and native freshwater fish species distributed in rivers from Brazil (Froese and Pauly, 2005), with established commercial productions (Castellani and Barrella, 2005) and frequently consumed by the population. *Contracaecum* larvae have been documented infecting *A. lacustris* (Negrelli et al., 2018) and *G. brasiliensis* (Bellay et al., 2012) in Brazil. During a parasitological survey of fish from the Pardo River in the Paranapanema River Basin, São Paulo, numerous *Contracaecum* sp. larvae were discovered infecting the internal organs of both fish species. Considering the potential public health significance of this finding, our objective was to provide a description of these larvae based on morphology, molecular analysis and phylogenetic inferences.

2. Material and Methods

2.1. Fish and parasite collection

We analyzed 26 specimens of *A. lacustris*: standard length = 6.57 (3.30–10.30) cm; weight = 10.51 (0.94–33.80) g and 28 specimens of *G. brasiliensis*: standard length = 7.44 (4.20–10.40) cm; weight = 16.57 (3.95–45.58) g caught using different fishing methods (*e.g.* cast nets, drag nets, fishing sieves, gills nets, rod fishing) from June, 15 to October, 01, 2020. Two localities (Figure 1) in the Pardo River, municipality of Botucatu, São Paulo State, were selected for the host sampling: an area near the river source of a

Figure 1. Collection sites of the fish hosts, in the Pardo River, Paranapanema River Basin, Botucatu, São Paulo, Brazil. (A) Locality 1 (22° 59' 59.76" S, 48° 22' 37.38" W); (B) Locality 2 (22° 59' 34.00" S, 48° 26' 52.13" W).

tributary of Pardo River, in the Boa Vista Farm (Locality 1: 22° 59' 59.76" S, 48° 22' 37.38" W) (Figure 1A), and a stream tributary of the main channel of the Rio Pardo, in the Paineiras Farm (Locality 2: 22° 59' 34.00" S, 48° 26' 52.13" W) (Figure 1B). Fish sampling was authorized by the Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio through the System of Authorization and Information on Biodiversity (SISBIO # 60640-1) and all procedures followed the recommendations of the Ethical Commission for Animal Experimentation from the São Paulo State University (Unesp), Institute of Biosciences, Botucatu, Brazil (CEUA # 9415260520).

The fishes were killed and refrigerated, then transported to the laboratory for necropsy. In the laboratory, the internal organs were removed, placed separately in Petri dishes, and analyzed under a stereomicroscope to survey parasites. The parasites found in the organs were fixed in a hot AFA solution and preserved in 70% ethanol for morphological analysis. For molecular biology, fresh samples were transferred to absolute ethanol.

2.2. Statistical analysis on parasitic intrapopulation

Quantitative descriptors of parasite populations (prevalence, mean abundance, and mean intensity of infection) were calculated following Bush et al. (1997). For *G. brasiliensis*, the abundance and intensity of infection with *Contracaecum* larvae found in localities 1 and 2 were compared using the Mann-Whitney test. Prevalence was compared using Fisher's exact tests, Chi-square tests with Yates' correction, and Chi-square tests with Monte Carlo simulation. All statistics were performed in R version 4.3.1 (R CoreTeam, 2021), running on RStudio version 2023.06.0+421 (RStudio Team, 2024). The significance level was 5%.

2.3. Morphological analysis

For the morphological analysis, nematodes were cleared with lactophenol and analyzed using a system for image analysis (Leica Application Suite software - LAS V3) coupled in a light microscope equipped with differential interference contrast (DIC). The taxonomical identification was according to Moravec (1998) and Luque et al. (2011). Some specimens were analyzed using scanning electron microscopy (SEM). The nematodes were dehydrated in a graded ethanol series (96%, 70%, 50%, and 30%), dried, and mounted on a strip of carbon conductive tape. Samples were sputter coated with gold and observed at 10 Kv in a Hitachi Stereoscan Model SU1510 (Hitachi Ltd, Tokyo, Japan).

Voucher specimens were deposited in the Coleção Helmintológica do Instituto de Biociências de Botucatu (CHIBB) under collection numbers CHIBB 10636–10644.

2.4. Molecular analysis and phylogenetic inferences

The samples for DNA extraction were prepared by cutting the nematodes into three sections: (I) anterior or cephalic – including buccal parts, nerve-ring, and esophagus; (II) middle-carrying portions of the digestive tract; and (III) posterior – with tail, anal opening, and papillae. Anterior and posterior sections were selected for morphological identification, while the middle sections were stored in absolute ethanol for molecular purposes. The genomic DNA was extracted from the samples using DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol, with a final volume of 30 µl. In order to generate proper molecular and phylogenetic analyses, the cytochrome c oxidase subunit 2 (COX II) and large subunit ribosomal RNA (28S) genes were selected for this paper due to the richness of sequences available, but also to include a highly conserved nuclear ribosomal gene widely used for resolving deep phylogenetic relationships (28S rDNA) and a gene (COX II) that evolves more rapidly than nuclear markers, making it particularly useful for resolving recent divergences and distinguishing closely related species.. Polymerase chain reactions (PCR) were executed using an Eppendorf Mastercycler® thermocycler. The primers and cycling conditions for the amplification of the 28S rDNA (partial) gene were according to García-Varela and Nadler (2005): primer forward 502F (5'-CAAGTACCGTGAGGGAAAGTTGC) and primer reverse 536R (5'-CAGCTATCCTGAGGGAAAC), cycling parameters including denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 30 seconds, annealing at 55 °C for 15 seconds, and extension at 72 °C for 1.5 min, followed by a post-amplification incubation at 72 °C for 10 min. The primers and cycling conditions for amplification of the cytochrome oxidase subunit II (COX II) gene were according to Marigo et al. (2015): primer forward COXF (5'-TTGRTTTCATAAYTTTAATTGTAG) and primer reverse 210R (5'-CACCAA CTCTTAAAATTATC) set to denaturate at 95 °C for 3 min, followed by 30 cycles of 95 °C for 45 seconds, annealing at 47.5°C for 30 seconds, and extension at 72 °C for 45 seconds, followed by a post-amplification incubation at 72 °C for 5 min. The reactions were made with a final volume of 25 µl: 4 µl of DNA, 1 µl of each primer, 6.5 µl of ultrapure water (Sigma- Aldrich, United Kingdom), and 12.5 µl Master Mix MyFiTM Mix Bioline®.

The 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 3500 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 the software Geneious® 7.1.3 (Kearse et al., 2012). Newly generated sequences were compared with other sequences (Table S1) through the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) to build molecular matrices for both genes. Specific sequence searches targeting certain clades or regions that did not appear in BLAST were conducted using the Nucleotide Advanced Search Builder (Sayers et al., 2022).

To infer phylogeny, two distinct algorithms were employed, both executed on the CIPRES Science Gateway (Miller et al., 2010). Bayesian Inference (BI) was performed using MrBayes on XSEDE (Ronquist et al., 2012), while Maximum Likelihood (ML) was conducted via RAxML HPC-2 on ACCESS (Stamatakis, 2015). For the COX II matrix, *Phocanema* species were selected as the outgroup, whereas the 28S matrix included *Ascaris lumbricoides* Linnaeus, 1758 along with other anisakids from genera *Anisakis*, *Pseudoterranova*, *Skrjabinisakis*, and *Phocanema*. The ML tree branches were supported by bootstrapping with 1000 repetitions. For BI, two independent Markov Chain Monte Carlo (MCMC) analyses were run, each consisting of two chains with 10,000,000 generations, sampling every 100 generations, and the initial 25% of samples were discarded as burn-in. The evolutionary model used was 'lset nst = mixed' and 'rates = invgamma'. Phylogenetic trees were visualized and edited using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape version 1.3 (Bah, 2011). To evaluate molecular divergence among species, a distance analysis was conducted in Mega 11 (Tamura et al., 2021), utilizing the p-distance substitution method, variance estimation based on 1000 bootstrap replicates, Gamma Distributed evolution rate, and gaps treated as Pairwise deletion. The distance matrices for both genes are available as Supplementary Material (Tables S2 and S3). The resulting trees of the phylogenetic searches were constructed based on the topology of the Bayesian Inference and complemented by the bootstrap values of Maximum Likelihood. In both trees, a legend was added to indicate the country of origin, the zoogeographical region (Holt et al., 2013), and the host's species and environment for each of the sequences (excluding the outgroups).

We also conducted a haplotype network analysis focusing on the COX II gene of the phylogenetically and molecularly close *Contracaecum* spp. These analyses were performed using the 'pegas' package (Paradis et al., 2023) implemented in R version 4.3.1 (R Core Team, 2021), running on RStudio version 2023.06.0+421 (RStudio Team, 2024). Additionally, haplotype network analyses were carried out using the Population Analysis with Reticulate Trees (PopART) software (Leigh et al., 2015), where we also prepared a georeferenced haplotype analysis. For these analyses, the files were prepared in DNA Sequence Polymorphism (DnaSP) software (Rozas et al., 2017).

The specimens were registered in SISGEN (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado) according to Brazilian laws under the registration number (A1100BF). Additionally, the *Contracaecum* sp. sequences obtained in this study were deposited in GenBank under accession numbers PQ115179–PQ115183 and PQ108980–PQ108984 (Table S1).

3. Results

Specimens of *A. lacustris* were exclusively collected at locality 1, and out of the 26 specimens sampled, only five were found to be infected with *Contracaecum* sp. larvae (Table 1). Individuals of *G. brasiliensis* were collected from both localities, with a higher number of *Contracaecum* sp. larvae found in locality 1. However, the prevalence ($p > 0.05$), the mean abundance ($U = 585.5$; $p = 0.840$) and infection intensity ($U = 10{,}000$; $p = 0.857$) were similar across both areas (Table 1).

3.1. Taxonomic summary

Phylum Nematoda Rudolphi, 1808
Class Chromadorea Inglis, 1983
Order Rhabditida Chitwood, 1933
Family Anisakidae Railliet & Henry, 1912
Genus *Contracaecum* Railliet & Henry, 1912
Contracaecum sp.

Hosts: *Astyanax lacustris* (Lütken, 1875) (Characidae) and *Geophagus brasiliensis* (Quoy and Gaimard, 1824) (Cichlidae).

Localities: River source of a tributary of the Pardo River, in the Boa Vista Farm (Locality 1 - 22° 59' 59.76" S, 48° 22' 37.38" W); a stream tributary of the main channel of the Rio Pardo, in the Paineiras Farm (Locality 2 - 22° 59' 34.00" S, 48° 26' 52.13" W), municipality of Botucatu, São Paulo State, Brazil.

3.2. Morphological data

The nematode larvae in this study were found encysted, were white in color, and presented a transversely striated cuticle (Figures 2A–C). The cephalic extremity was rounded with three underdeveloped lips and a small ventral tooth (Figures 2A, 2C). There were four cephalic papillae surrounding the oral aperture (Figure 2A). The excretory pore was located just below the cephalic tooth (Figures 2A, 2C). The esophagus was narrow, with a small and round ventricle and a short ventricular appendix (Figures 2D–F). It also had a long intestinal caecum (Figures 2D). The tail was conical with a pointed end (Figure 2B). No morphological or morphometric difference could be observed between the specimens of all *Contracaecum* sp. larvae here examined (Tables 2, S2 and S3).

3.3. Molecular and phylogeographic findings

Five 28S rDNA sequences ranging from 814 to 855 base pairs (bp) and five COX II sequences spanning 524 to 576 bp

Table 1. Total of fish collected, number of infected hosts, number of nematode parasites, and ecological data.

	Locality	Total	Infected	Parasites	P (%)	MA ± SE (Min-Max; CI)	MII ± SE (Min-Max; CI)	Site of infection
Astyanax lacustris (n = 26)	1	26	5	22	19.2	0.85 ± 0.47 (0-11; 0.96)	4.40 ± 1.78 (1-11; 4.94)	Intestine and fatty tissue
Geophagus brasiliensis (n = 28)	1	23	6	8	26.1	0.35 ± 0.14 (0-2; 0.28)	1.33 ± 0.21 (1-2; 0.54)	Heart and gastrointestinal
	2	5	2	3	40	0.60 ± 0.40 (0-2; 1.11)	1.50 ± 0.50 (1-2; 6.35)	Heart and gastrointestinal

P (%) = Prevalence; MA = Mean abundance; MII = Mean intensity of infection; SE = standard error; Min = minimum value; Max = maximum value; CI = 95% Confidence Interval.

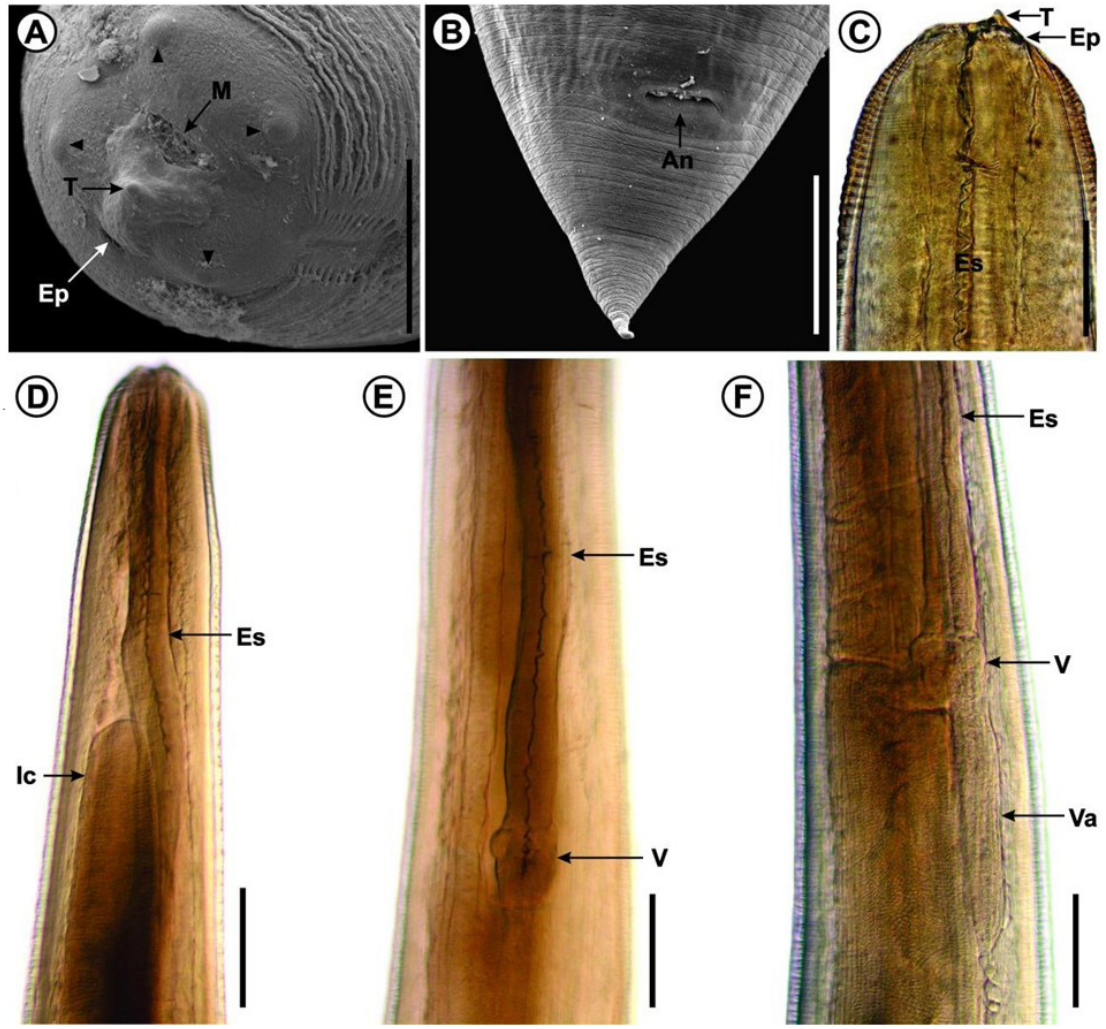

Figure 2. Morphology of the *Contracaecum* larvae found in *Astyanax lacustris* and *Geophagus brasiliensis*: (A and B) Scanning electron micrograph of *Contracaecum larvae* (scale-bar = 50 µm). (A) Apical view of the worm's mouth. Arrows indicate the four papillae on the lips; (B) posterior end of the worm, showing anus and conical tail. (C, D, E and F) Light microscopy pictures of *Contracaecum* larvae; (C) Anterior portion of the worm, ventral tooth and excretory pore visible; (D) Anterior portion of the worm, showing esophagus and intestinal caecum (E) Middle portion of the worm, showing the end of the esophagus and the ventriculus connected to it; (F) Middle portion of the worm, showing ventriculus and ventricular appendix. Abbreviations: M = mouth opening, T = ventral tooth, Ep = excretory pore, Es = esophagus, Ic = intestinal caecum, V = ventriculus, Va = ventricular appendix, An = anus.

were successfully obtained. The 28S rDNA gene matrix included 43 taxa and 763 characters, while the COX II gene matrix comprised 42 taxa and 494 characters. For BI using the 28S gene, the most likely DNA substitution model selected was the GTR submodel 123343. In the BI using COX II, the highest probability was assigned to the GTR submodel 123323. A total of 200,002 trees were read in 2 files for each analysis, from which 150,002 trees were sampled. For ML analysis, the best evolutionary model selected was the General Time Reversible (GTR) model with gamma rates.

In both phylogenetic analyses, inferred using 28S (Figure 3) or COX II (Figure 4), the five sequences of *Contracaecum* sp. obtained in this study, consistently formed a well-supported clade (posterior probability = 1; bootstrap value = 100) composed by *Contracaecum* species from Panamanian and Neotropical freshwater environments. In the 28S tree (Figure 3), our sequences grouped with *Contracaecum* sp. from Mexico forming the clade C. In the COX II tree (Figure 4), our sequences clustered with *Contracaecum multipapilatum* (Rudolphi, 1819) from Colombia, Costa Rica, Guatemala, and Mexico, and with *Contracaecum jorgei* Sardella, Mancini, Salinas, Simões & Luque, 2020 from Argentina and Colombia. Internal phylogenetic relationships in both trees were inconclusive due to low support for their subclades. In the 28S tree (Figure 3), there were only two highly supported subclades, one formed by sequences of *Contracaecum* sp. from Brazil (PQ108983 and PQ108981, posterior probability = 0.92) and the other, by sequences of *Contracaecum* sp. from Mexico (OR537901 and OR537902, bootstrap value = 95). Notably, in the COX II tree (Figure 4), only the clade of *Contracaecum* sp. from Brazil (PQ115182 and PQ115183) was well-supported (PQ115182 and PQ115183, posterior probability = 0.93). Also in both inferences, the clade containing *Contracaecum* sp. from Brazil appeared as a sister group to a clade primarily comprising sequences of *C. multipapilatum* from the Palearctic, Nearctic, and Oriental regions (Figures 3 and 4), beside a sequence from Ecuador, on the Pacific coast (Figure 4).

Molecular distance analyses based on 28S suggest a single species within the clade composed of *Contracaecum* sp. from Brazil and Mexico, with populations differing by approximately 0-0.3% (0-2 base pairs) (Table S2). Conversely, using COX II, higher molecular distances were observed but remained within the range (0-2.1%; 0-14 base pairs) (Table S3), indicating these are likely the same species. This includes *Contracaecum* sp. from Brazil, *C. jorgei* from Colombia and Argentina, and Panamanian *C. multipapilatum* from freshwater environments. Comparatively, molecular variation between Panamanian lineages of *C. multipapilatum* and those lineages of *C. multipapilatum* from Ecuador, Neartic, Paleartic, and Oriental regions, ranged from 10.2-13.8% in COX II and 5.6-9.1% in 28S (Tables S2 and S3).

Table 2. Morphometric analysis (mm) of *Contracaecum* L3 larvae with short ventricular appendix.

	***Contracecum* sp. type II**	***Contracecum jorgei***	***Contracecum jorgei***	***Contracecum* sp. *G. brasiliensis***
	Moravec et al. (1993)	**Sardella et al. (2020)**	**Duarte et al. (2025)**	**Present study**
Body length	15.7-25.7	11.10-12.23 (11.56)	11.17-18.33 (15.64)	19.25-22.98 (20.81)
Body width	0.45-0.84	0.24-0.39 (0.29)	0.27-0.38 (0.31)	0.66-0.77 (0.70)
Excretory pore length		0.04-0.05 (0.05)	0.04-0.05 (0.04)	0.04-0.05 (0.04)
Nerve ring from the anterior end	0.31-0.38	0.18-0.28 (0.25)		0.25-0.28 (0.27)
Oesophagus length	1.97-2.1	1.63-1.89 (1.76)	1.60-1.63 (1.62)	1.94-2.13 (2.03)
Oesophagus width		0.06-0.16 (0.10)	0.06-0.16 (0.11)	0.07-0.15 (0.10)
Ventriculus length	0.06-0.08	0.03-0.05 (0.04)	0.036-0.05 (0.04)	0.05-0.12 (0.09)
Ventriculus width		0.04-0.06 (0.05)	0.049-0.05 (0.05)	0.05-0.13 (0.09)
Ventricular appendix length		0.30-0.37 (0.34)	0.42	0.52-0.59 (0.55)
Ventricular appendix width		0.04-0.07 (0.06)	0.06	0.09-0.13 (0.11)
Ratio ventricular appendix/body length (%)		2.76-3.07		2.5-2.8 (2.67)
Intestinal caecum length	1.5-1.58	0.98-1.30 (1.08)	0.90-1.33 (1.12)	1.46-1.81 (1.62)
Intestinal caecum width		0.10-0.12 (0.12)	0.10-0.15 (0.13)	0.11-0.17 (0.14)
Ratio intestinal caecum length/body length (%)		8.86-11.57		7.00-9.39 (7.82)
Tail length	0.09	0.11-0.13 (0.13)	0.11-0.14 (0.13)	0.09-0.15 (0.12)

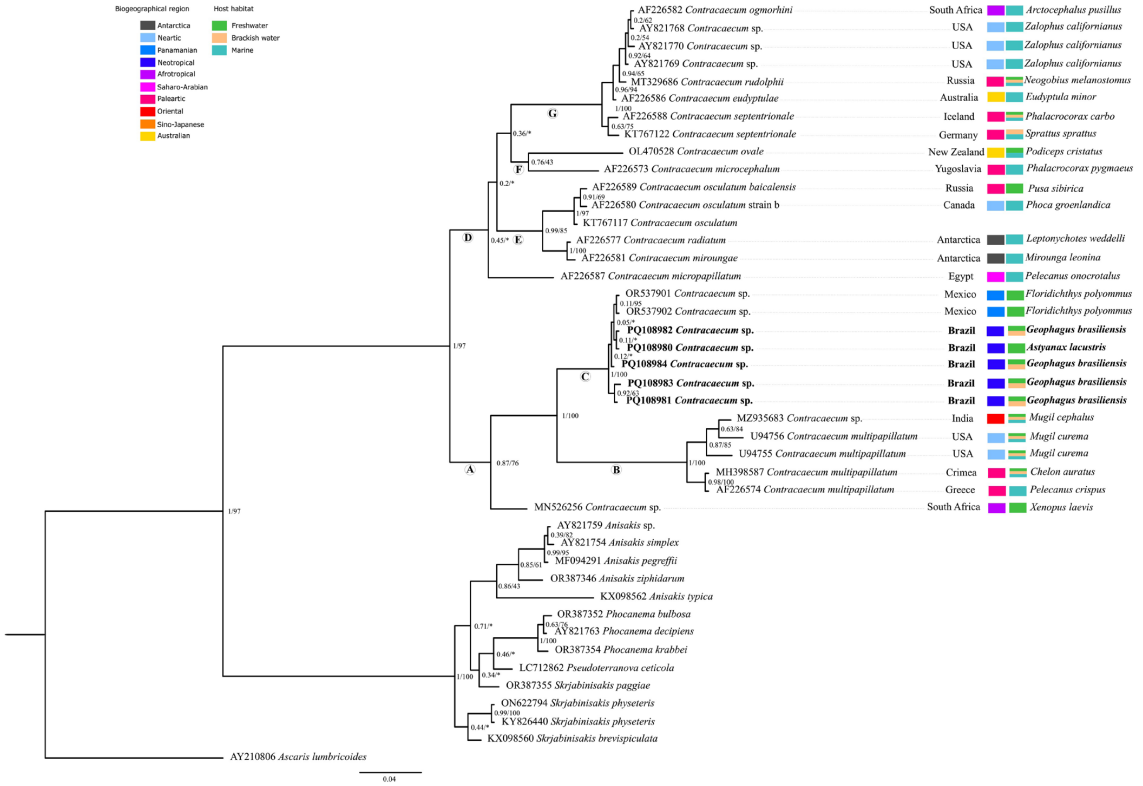

Figure 3. The majority-rule consensus tree inferred for the 28S rDNA gene. The topology presented is the result of Bayesian Inference. Branch supports are indicated by posterior probabilities from the Bayesian inferences and corresponding bootstrap values from the Maximum Likelihood. The colored squares represent the zoogeographic regions where the parasites occur, as well as the habitats in which their hosts and/or the parasites themselves are found. New sequences of *Contracaecum* are highlighted in bold.

Haplotype network analysis, conducted using both pegas and PopART, identified 13 distinct haplotypes (Figure 5). The most frequent haplotype was X, found in Argentina and Costa Rica, followed by haplotype VI, which is exclusive to Brazil. Except for the haplotype X, the remaining haplotypes are not present in different countries. In Brazil, there was the highest haplotype diversity (haplotypes II, IV, VI, and XIII), followed by Costa Rica (haplotypes III, X, and XII). Guatemala (haplotypes VII and VIII) and Argentina (haplotypes V and X) have two haplotypes recognized, while Colombia (haplotype IX), Mexico (haplotype XI) and Ecuador (haplotype I) have one each. While statistical differences between populations from different countries were not significant, substantial variation was noted among subpopulations, indicating the presence of different haplotypes within the same country, or at least differing in part of the subpopulations from each locality. The most divergent haplotype was haplotype I, representing the Pacific lineage of *C. multipapilatum* from Ecuador.

4. Discussion

The morphometric data indicated that the *Contracaecum* sp. larvae examined in this study, collected from *A. lacustris* and *G. brasiliensis* resemble the L3 larvae of *Contracaecum* sp. Type 2 of Moravec, Kohn & Fernandes (Moravec, 1998) and *Contracaecum jorgei* Sardella, Mancini, Salinas, Simões & Luque, 2020, such as the ones described by Sardella et al. (2020), mainly owing the relatively short ventricular appendix. This is backed up by the molecular data, which consistently placed our samples within highly supported clades (Figure 3, Clade C and Figure 4, Clade F) containing sequences of *C. jorgei* and others of *Contracaecum multipapilatum* (Drasche, 1882). The phylogenetic relationships inside these clades, however, were poorly supported, which could be the result of using samples of suboptimal length (Rosenberg and Kumar, 2001) or an insufficient number of *loci* for the task at hand (Rokas and Carroll, 2005), or yet, to the persistence of ancestral haplotypes (Posada and Crandall, 2001).

Each tree searches successfully reconstructed clades exclusively composed of freshwater and brackish Neotropical and Panamanian *Contracaecum* lineages (Figure 3, Clade C and Figure 4, Clade F), where our sequences were clustered. The minimal molecular distance among members of these clades suggests that they could be conspecific, pointing towards intraspecific variation. However, the possibility of recent divergence or ongoing gene flow cannot be ruled out.

In 28S (Table S2), the molecular distances ranged from 0.0%, as seen between *Contracaecum* sp. from Mexico (OR537901 and OR537902) and Brazil (PQ108982, PQ108980, and PQ108984), to 0.1-0.3% between sequences

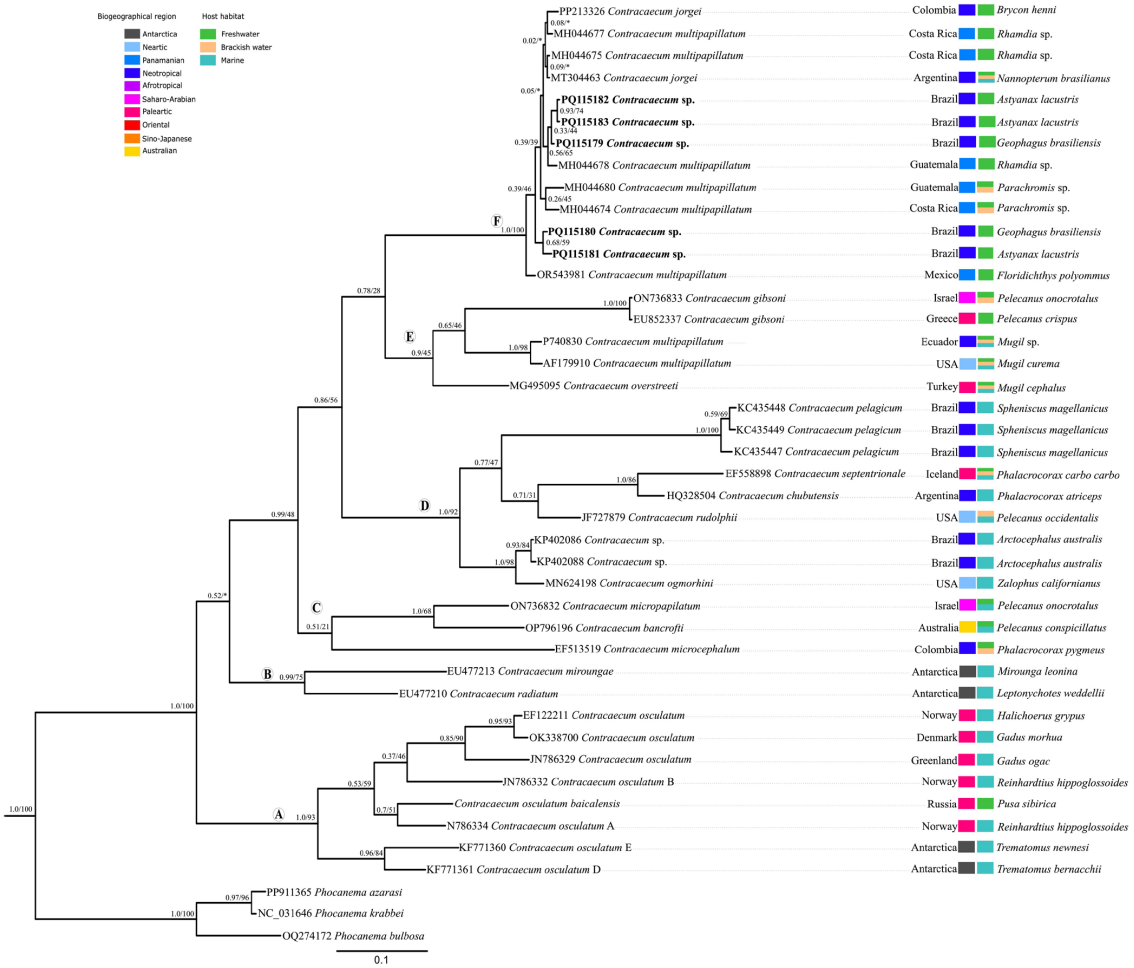

Figure 4. The majority-rule consensus tree inferred for the cytochrome oxidase II (COXII) gene. The topology presented is the result of Bayesian Inference. Branch supports are indicated by posterior probabilities from the Bayesian inferences and corresponding bootstrap values from the Maximum Likelihood. The colored squares represent the zoogeographic regions where the parasites occur, as well as the habitats in which their hosts and/or the parasites themselves are found. New sequences of *Contracaecum* are highlighted in bold.

from Brazil (PQ108981 and PQ108983) and the remaining *Contracaecum* species of this clade (Figure 3, Clade C). This finding highlights two key points. First, it reinforces the robustness of the clade formed by *Contracaecum* sp. from Brazil (PQ108981 and PQ108983), as identified in the phylogenetic analysis (Figure 3), suggesting a composition of more divergent lineages. Second, it supports the idea that these lineages correspond to a single species.

For the COX II gene (Table S3), which exhibited greater molecular distances than 28S, divergence ranged from 0.0% (0 nucleotides) between *C. multipapillatum* (MH044677) and *C. jorgei* (MT304463), and between *Contracaecum* sp. from Brazil (PQ115182 and PQ115183), to 2.1% (10 nucleotides) between *C. multipapillatum* from Guatemala (MH044680) and Mexico (OR543981). Notably, *Contracaecum* sp. from Brazil (PQ115182 and PQ115183) diverged by 1.3-1.5% (6-7 nucleotides) from *C. multipapillatum* from Guatemala (MH044680), Mexico (OR543981), Costa Rica (MH044674), and other *Contracaecum* sp. from Brazil (PQ108980 and PQ108981). This further reinforces the robustness of the clade recovered in the phylogenetic inference (Figure 4), highlighting this more divergent lineage within Brazil. Additionally, it suggests that the entire clade is composed of a single species.

On the other hand, molecular distance analysis focusing on the sister clades (Figure 3, Clade B and Figure 4, Clade E) of the Neotropical-Panamanian freshwater clade yielded higher values of divergence. In the 28S tree (Figure 3), Clade B includes sequences from *C. multipapillatum* found in the Nearctic (U94755, U94756) and Palearctic regions (MH398587, AF226574), along with a sequence of *Contracaecum* sp. from the Oriental region (MZ935683). While the Palearctic lineage of *C. multipapillatum* from Crimea (MH398587) was identical to the one from Greece (AF226574) (Table S2), *C. multipapillatum* from the USA (U94756) in the Nearctic region exhibited a divergence of 2.3-2.5% from the remaining *C. multipapillatum* sequences and 1.2% from *Contracaecum* sp. in the Oriental region (Table S2). These results are relevant as the divergence between sequences of the same species,

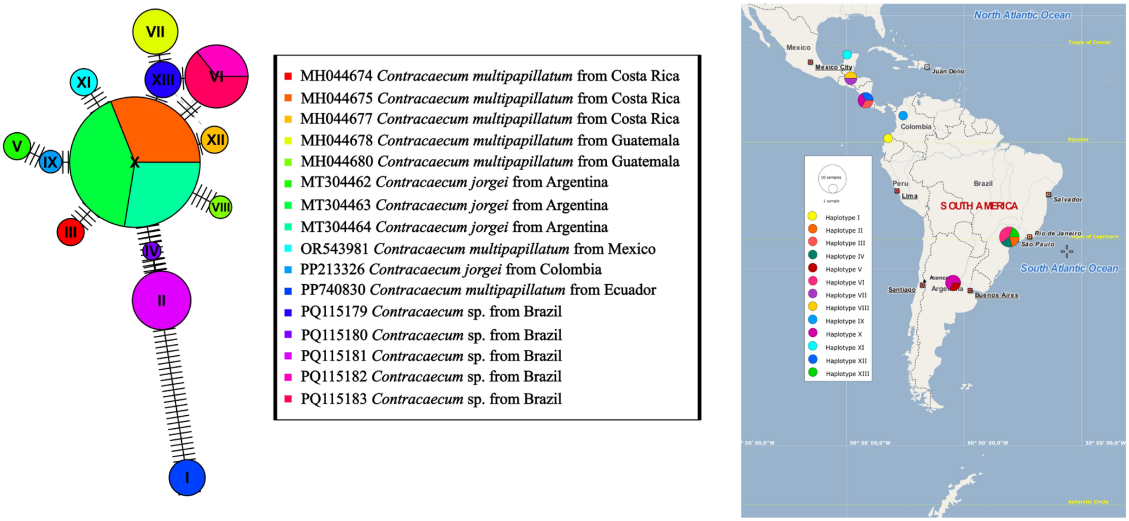

Figure 5. Haplotype network on Neotropical *Contracaecum* spp. phylogenetically and molecularly close to the Brazilian sequences of *Contracaecum* sp. On the left, a haplotype network of the 13 (I-XIII) identified haplotypes, with its corresponding legend in the center, and to the right, a map showing the geographical distribution and the diversity of Neotropical haplotypes close to *Contracaecum* sp. of this study. Each circle on the left represents a haplotype, while on the right, a haplotype, or a group of them, is/are depicted, with the diameter of the circles proportional to the frequency of the haplotype occurrence. Hash marks over the lines connecting the circles represent the number of mutations which distinguish one haplotype from other. Statistical analysis revealed high genetic differentiation among populations (Phi_ST = 0.93571), although not statistically significant ($p = 0.077$). However, there was nearly complete genetic differentiation among subpopulations within groups (Phi_SC = 0.98695), with strong statistical significance ($p < 0.001$).

such as *C. multipapillatum* from the USA (U94755) and Greece (AF226574), was higher than the divergence observed between different species, which was as low as 0.2% between *Contracaecum miroungae* (AF226581) and *Contracaecum radiatum* (AF226577) in Antarctica (Figure 3, Clade E, Table S2).

In the COX II, the sister clades (Figure 4, Clade E) of the Neotropical-Panamanian freshwater clade, including *C. multipapillatum* from the Palearctic (EU852337), Neotropical (PP740830), and Nearctic regions (AF179910), exhibited divergences ranging from 9.7-9.8% (49-50 nucleotides) among themselves and 10.5-12% from the *C. multipapillatum* of the Neotropical-Panamanian freshwater clade (Figure 4, Clade F, Table S3). The distance matrix for COX II, as presented by Garbin et al. (2023), showed that the smallest divergence between sequences of different species was 6%, while the largest divergence within the same species was 1%. Therefore, the molecular distances observed between the different lineages of *C. multipapillatum* suggest that there is more than one species into this group.

To explain the unexpected molecular distance patterns, where distances between different species are lower and distances within the same species are higher, we must consider the likelihood of species misidentification. Our COX II analysis (Figure 4, Table S3) suggests that *C. multipapillatum* is not monophyletic, since the Neotropical-Panamanian freshwater *C. multipapillatum* sequences clustered more closely with *C. jorgei* samples from Argentina and Colombia (Figure 4, Clade F), while the other two samples of *C. multipapillatum* from Ecuador and the USA (both from coastal waters) clustered more closely with *Contracaecum gibsoni* Mattiucci, Paoletti, Solorzano and Nascetti, 2010 and *Contracaecum overstreeti* Mattiucci, Paoletti, Solorzano and Nascetti, 2010 (Figure 4, Clade E). This evidence strongly suggests that the *C. jorgei* from Argentina and Colombia (Clade F, Figure 4) may indeed be conspecific with *C. multipapillatum* from Costa Rica, Guatemala, and Mexico and with *Contracaecum* sp. from Brazil.

In 2020, Sardella et al. described *C. jorgei*, revealing genetic distances of 10.5–10.7% compared to *C. overstreeti* and 11.8% when compared to *C. gibsoni*, both using COX II gene. Interestingly, these authors did not include comparisons with *C. multipapillatum* in their phylogenetic inferences. Later that same year, Choc-Martinez et al. (2020), made available sequences of COX II for *C. multipapillatum* from Costa Rica and Guatemala, identifying conspecific larvae in *Rhamdia* sp. (MH044678), *Parachromis* sp. (MH044674), and *Hoplias* sp., and reporting genetic distances of 12% and 6% compared to *C. multipapillatum* from the USA (AF179910) and Greece (EU852344), respectively. More recently, another sequence of *C. multipapillatum* (PP740830) was made available from Ecuador, where the larva was found in *Mugil* sp. (Hernandez-Alomia et al., 2024). This sequence likely corresponds to the same species as the one reported as *C. multipapillatum* from the USA (AF179910), given their minimal divergence of just 1.2% in the COX II gene. It, however, diverges significantly by 10.7%, 11–11.5%, and 10.9–12% from C. *multipapillatum* samples from Mexico (OR543981), Guatemala (MH044678 and MH044680), and Costa Rica (MH044677, MH044674, and MH044675), respectively.

It is likely that *C. jorgei*, the Neotropical and Panamanian lineages of *C. multipapillatum*, and *Contracaecum* sp. from Mexico and Brazil are conspecific. Although Li et al. (2016) suggested Cuba as the type locality of *C. multipapillatum*, Drasche (1882) explicitly stated that

his description was based on specimens collected by Natterer in Brazil. Following Drasche's description, Brazil was also recognized as a distribution area for this species by other authors, including Lucker (1941) and Yamaguti (1961), who listed the country as the primary geographic reference for this parasite.

It remains challenging to determine whether *C. jorgei* is a junior synonym of *C. multipapillatum* or if any of these sequences truly correspond to *C. multipapillatum*, as none originate from the type host and locality (*i. e. Mycteria americana* Linnaeus, 1758 from Brazil). Molecular and phylogenetic analyses based on 28S rDNA identified four distinct lineages in the clades of *C. multipapillatum*: i) *C. multipapillatum* from Crimea (MH398587) and Greece (AF226574); ii) a USA lineage of *C. multipapillatum* (U94755); another USA lineage of *C. multipapillatum* (U94756); and iv) *Contracaecum* sp. from India (MZ935683). Additionally, COX II analyses revealed two primary lineages of *C. multipapillatum*: i) one comprising specimens from the USA (AF179910) and Ecuador (PP740830); another grouping the Panamanian and Neotropical lineages of *C. multipapillatum*, including *C. jorgei* and *Contracaecum* sp. from Brazil. The low molecular distance values (0–0.1% in the COX II) between *C. jorgei* from Brazil and *C. jorgei* from Argentina, recently reported by Duarte et al. (2025), indicate that this Brazilian lineage, despite inhabiting a brackish lagoon, likely belongs to the Neotropical and Panamanian lineage here recovered. These findings highlight the urgent need for a taxonomic reassessment to clarify species boundaries within this complex and resolve the status of *C. multipapillatum* across its range.

The broad geographic range of haplotype X, its central position in the haplotype network, and its numerous connections (Figure 5) strongly indicate that it is likely the most ancestral haplotype (Posada and Crandall, 2001), a pattern commonly observed in other organisms (Loretán et al., 2020; Qi et al., 2023). The remaining haplotypes are likely experiencing different levels of divergence, as also indicated by our phylogenetic (Figures 3 and 4) and molecular distance analyses (Tables S2 and S3). However, these divergence events seem to be relatively recent, or perhaps some level of gene flow continues between these populations.

Twelve out of the thirteen haplotypes identified in this study (Figure 5) are geographically confined. At least eight of these haplotypes (III, IV, VI, VIII, XII, IX, XI, and XIII) appear to have directly evolved from haplotype X. Nevertheless, not all haplotypes trace their origins back to haplotype X, and within each country they do not all descend from a single ancestral haplotype. For example, in Brazil, haplotypes VI and VIII are more closely related to each other and to haplotype VII from Guatemala than to haplotypes II and IV from Brazil (Figure 5), a relationship that aligns with the phylogenetic analysis, although with limited support (Figure 4). A similar pattern emerges in Argentina, where haplotype V is more closely associated with haplotype IX from Colombia than with haplotype X from Argentina. Conversely, in Costa Rica, haplotype X is closely linked to haplotypes III and XII, presenting a different scenario altogether.

Geographic isolation is a well-known driver of genetic divergence, often resulting in the emergence of distinct haplotypes (Templeton et al., 1992; Avise, 2000). However, the divergence observed within the Panamanian-Neotropical freshwater *Contracaecum* clade lacks sufficient statistical support to clearly define these as distinct species. Despite this, there appears to be a degree of isolation contributing to the formation of haplotypes VII from Guatemala, II from Brazil, and V from Argentina. These haplotypes have originated from others and, therefore, are not directly derived from haplotype X.

The correct identification of the lineage of *Contracaecum* from the Neotropical and Panamanian regions is important from a public health perspective. There is evidence of zoonotic potential in *Contracaecum* larvae from marine, coastal, and freshwater environments since they are parasites of low host specificity (Shamsi et al., 2018), which could explain how several studies of experimental infection with L3 *Contracaecum* have successfully infected animals which are not typically part of their life cycle: *Contracaecum osculatum* (Rudolphi, 1802) from baltic cod can parasitize rats (Fagerholm, 1988) and pigs (Strøm et al., 2015), L3 larvae of the *Contracaecum ogmorhini* complex obtained from *Cynoscion guatucupa* (Cuvier, 1830) from Argentina are capable of infecting mice (Galeano et al., 2022). Two studies using *Contracaecum multipapillatum* resulted in infection: in one, the larvae obtained from one euryhaline fish from Mexico called *Mayaheros urophthalmus* (Günther, 1862) parasitized a domestic cat (Vidal-Martinez et al., 1994); In the other, the helminths from a freshwater fish from Brazil called *Hoplias malabaricus* (Bloch, 1794) managed to parasitize rabbits (Barros et al., 2004). It has been proven that *Contracaecum* larvae can escape from the fish's digestive tract and migrate to muscular tissue and also survive cooking, freezing, salting, and marinating if some standards are not properly followed (Buchmann and Mehrdana, 2016; Hamouda and Younis, 2022). Marinating the fish meat was proven to be especially inefficient in killing anisakid larvae. It is important to note that accidentally eating dead larvae in well-prepared fishes is also not completely safe. Dead or alive, these helminths still contain allergens that may trigger an immune response. There have been a few examples of real zoonotic infection of *Contracaecum* in humans, in France (Dei–Cas et al., 1986), the Baltic region (Schaum and Müller, 1967), Korea (Im et al., 1995), Japan (Nagasawa, 2012), and Australia (Shamsi and Butcher, 2011), neither of these cases, referring to lineages of *Contracaecum* from freshwater environments.

This study highlights three important conclusions: I) A distinct clade of *Contracaecum* from the Panamanian-Neotropical region has been identified, encompassing *C. multipapillatum* from Guatemala, Mexico, and Costa Rica, *C. jorgei* from Argentina and Colombia and *Contracaecum* sp. from Brazil; II) *C. multipapillatum* is currently non-monophyletic, with distinct lineages found in the Palearctic region, others in Nearctic, Neartic extending to the Pacific Coast of Ecuador and Oriental region; III) To date, no cases of *Contracaecum* species infecting humans have been reported in the Americas, which raises important questions: Do these clade members truly possess zoonotic potential or is the absence of documented cases simply due to underreporting?

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Data Availability Statement

The entire data set that supports the results of this study was published in the article itself or in the supplementary material.

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Supplementary Material

Supplementary material accompanies this paper.

Table S1. Sequences used in molecular analysis and phylogenetic inferences.

Table S2. Molecular distance analysis of 28S rDNA. Data in the lower triangle are the percentage of base differences per site between sequences. Absolute number of nucleotides are in the upper triangle. CSV file.

Table S3. Molecular distance analysis of COX II. Data in the lower triangle are the percentage of base differences per site between sequences. Absolute number of nucleotides are in the upper triangle CSV file.

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