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A phylogenetic study of the cecal amphistome *Zygocotyle lunata* (Trematoda: Zygocotylidae), with notes on the molecular systematics of Paramphistomoidea

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Abstract

Zygocotyle lunata inhabits the caecum of birds and mammals from the American continent. This amphistome parasite is easily maintained in the laboratory and serves as a model organism in life-cycle studies, but it has seldom been studied using molecular data. Neither the position of *Z. lunata* in the superfamily Paramphistomoidea nor the monophyly of the Zygocotylidae has been evaluated with molecular phylogenetic methods. In the present study, adult specimens of *Z. lunata* obtained experimentally in mice from Brazil were submitted to molecular studies. Partial sequences of nuclear (1261 bp of 28S and 418 bp of 5.8S-ITS-2) and mitochondrial (1410 bp of cytochrome *c* oxidase 1, *cox*1) markers were compared with published data. In the most well-resolved phylogeny, based on 28S sequences, *Z. lunata* clustered in a well-supported clade with *Wardius zibethicus*, the only other species currently included in the Zygocotylidae, thus confirming the validity of this family. Divergence of 28S sequences between these species was 2.2%, which falls in the range of intergeneric variation (0.9–5.6%) observed in the other two monophyletic groups in the 28S tree, i.e., representatives of Gastrodicidae and Neotropical cladorchiids (Cladorchiidae). Analysis of ITS-2 and two parts of the *cox*1 gene placed *Z. lunata* within poorly resolved clades or large polytomies composed of several paramphistomoid families, without clarifying higher-level phylogenetic relationships. The *cox*1 of a Brazilian isolate of *Z. lunata* is 99.6% similar to a Canadian isolate, confirming the pan-American distribution of the species. Finally, our phylogenetic reconstructions of Paramphistomoidea revealed a complex scenario in the taxonomic composition of some amphistome families, which highlights a need for further integrative studies that will likely result in rearrangements of traditional morphology-based classifications.

Keywords Amphistomes · Phylogeny · Taxonomy · Trematodes · *Zygocotyle*

Introduction

The amphistome trematodes (superfamily Paramphistomoidea) are parasites of all classes of vertebrates. Several species of veterinary importance (members of *Paramphistomum* Fischoeder, 1901; *Calicophoron* Näsmark, 1937; *Cotylophoron* Stiles & Goldberger, 1910; and *Balanorchis* Fischoeder, 1901) cause paramphistomosis in domestic ruminants (Sargison et al. 2016; Huson et al. 2017; Tandon et al. 2019). Other species (in *Fischoederius* Stiles & Goldberger, 1910; *Gastrodiscoides* Leiper, 1913; and *Watsonius* Stiles & Goldberger, 1910) are sporadically reported as zoonotic agents in humans (Mas-Coma et al. 2006; Chai 2019; Chai and Jung 2019). A large number of paramphistomoids are reported in wild animals, indicating the importance of this cosmopolitan group in

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terms of biodiversity, although the actual number of species of these flukes is still unknown (Yamaguti 1971; Sey 1991; Tandon et al. 2019). The classification of amphistomes is also complex, with numerous morphology-based systems proposed over time (reviewed by Sey 1988, 1991; Jones 2005a; Tandon et al. 2019).

In recent decades, molecular data have become available from representatives of paramphistomoids, mainly from species infecting ruminants. Most of these recent works focused on identification of species using DNA sequences, especially the second internal transcribed spacer of the ribosomal DNA (ITS-2) (e.g., Rinaldi et al. 2005; Lotfy et al. 2010; Dube et al. 2016; Chamuah et al. 2016; Mohanta et al. 2017; Firdausy et al. 2019). A smaller number of integrative works have focused on paramphistomoids from particular regions or hosts (Ghatani et al. 2014; Laidemitt et al. 2017; Besprozvannykh et al. 2018; Pantoja et al. 2019). In studies of higher-level systematic relationships in the digeneans, partial sequences of rDNA subunits from about 35 paramphistomoid species have supported the status (monophyly) of the superfamily Paramphistomoidea (Pérez-Ponce de Léon and Hernández-Mena 2019). Complete mitochondrial genomes have been sequenced in several paramphistomoids (e.g., Yang et al. 2016), but most studies using these data concentrate on other taxa (e.g., Le et al. 2019). Focused phylogenetic studies of higher-level classifications among paramphistomoid trematodes are lacking. The scarcity of molecular data, particularly from key genera and families, hinders the advancement of knowledge of the interrelationships within this superfamily.

Among the poorly studied amphistome species is *Zygocotyle lunata* (Diesing, 1836), which is currently placed in the family Zygocotylidae Ward, 1917. This family includes just two genera, both monotypic: *Zygocotyle* Stunkard, 1916 (the type genus of the family), and *Wardius* Barker & East, 1915 (Sey 1991; Jones 2005b). *Zygocotyle lunata* has been reported in natural infections of the caecum and large intestine of about 40 species of birds and mammals (revised by Fried et al. 2009). Interestingly, it is the only species of paramphistomoid that infects avian hosts (Jones 2005b; Fried et al. 2009). Among paramphistomoids, *Z. lunata* is unusually easy to maintain under laboratory conditions, which has led to decades of studies of its biology, life cycle, pathology, immunology, biochemistry, and more recently pharmacotherapy (Willey 1941; Ostrowski de Núñez et al. 2003; Fried et al. 2009; Pinto et al. 2019). Nevertheless, molecular data from this parasite are scant (only 3 public sequences) and have only been employed in taxonomically narrow studies related to the identification of the parasite (Van Steenkiste et al. 2015; Pinto et al. 2019). Neither the phylogenetic position of *Z. lunata* among members of the superfamily Paramphistomoidea nor the monophyly of the Zygocotylidae has been evaluated.

In the present study, we obtained partial sequence data of the large subunit nuclear rDNA (28S) and the 5.8S-ITS2 as well as of the mitochondrial cytochrome *c* oxidase subunit I (*cox*1) genes for a Brazilian isolate of *Z. lunata*. Newly generated sequences were used for the first time in a phylogenetic context, which, besides confirming the identification of the parasite, supported the distinctive status of the family Zygocotylidae. The validity of other families within Paramphistomoidea is also briefly discussed based on the phylogenetic results here presented.

Material and methods

The isolate of *Z. lunata* used in the present study was obtained from an experimental infection initiated from cercariae emerged from a naturally infected specimen of *Biomphalaria straminea* (Dunker, 1848) collected from an urban lake in Belo Horizonte (19° 47′ 06.20″ S and 43° 57′11.41″ W), Minas Gerais, Brazil, in July 2017. Briefly, metacercariae encysted on the wall of the plastic container where the naturally infected snail was kept were carefully removed with the aid of metal needles. The metacercariae were counted and forced-fed to five mice (50 metacercariae/animal). Experimental infection of the mice performed in this study was carried out in accordance with the procedures recommended by the local ethics committee on animal experimentation, CEUA, UFMG (protocol 21/2016). Adult parasites obtained in the cecum of rodents between 15 and 45 days post-infection were identified as *Z. lunata* (Fig. 1) according to morphological criteria described by different authors (Sey 1991; Ostrowski de Núñez et al. 2003, 2011; Jones 2005b; Barbosa et al. 2011). As isolates of *Z. lunata* from the same geographical area are well characterized (Travassos et al. 1969; Ostrowski de Núñez et al. 2003; Barbosa et al. 2011), detailed morphological analysis is not included in the present account. Voucher specimens (isogenophores sensu Pleijel et al. 2008) were deposited in the Collection of Trematodes, Taxonomic Collections Center of the Universidade Federal de Minas Gerais (UFMG-TRE 118).

DNA was extracted from one adult specimen from an experimentally infected mouse using Wizard Genomic DNA Purification kit (Promega), according to the protocol presented by the manufacturer. Extracted DNA was quantified using a microvolume spectrophotometer (NanoDrop Lite; Thermo Fisher Scientific). Partial regions of the genes 28S rDNA (~ 1200 bp) and 5.8S-ITS2 (~ 400 bp) were amplified using the primer pairs Dig12/1500R (Tkach et al. 2003) and 3S/BD2 (Bowles et al. 1995), respectively. For *cox*1, we amplified two regions widely used in the study of trematodes; one of them (~ 800 bp of the post-barcoding region) was obtained using the primer pair JB3/COI-RTrema (Miura et al. 2005), and the other (~ 600 bp including a fragment of the barcoding region) with the primers 123F/858R (Laidemitt et al. 2017). PCR reactions were performed in a final volume of 25 μl, which included 12.5 μl Platinum Hot Start PCR Master Mix,

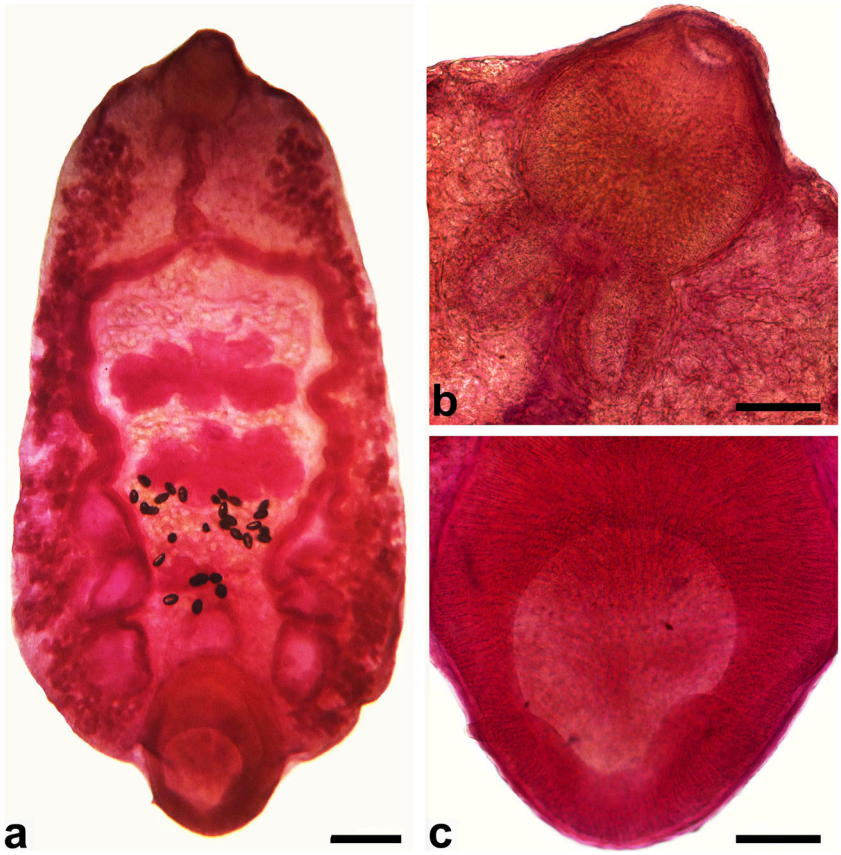

Fig. 1 Isogenophore specimen of *Zygocotyle lunata* obtained in experimentally infected mice (27-day post-infection). **a** Whole view. **b** Details of pharynx with primary pharyngeal sacs. **c** Detail of posterior margin of acetabulum bearing muscular lips. Scale bars: **a** = 500 μm, **b** and **c** = 200 μm

1.25 pmol of each primer, and about 50 ng of template DNA. The PCR conditions used were as described by the authors listed above. Amplification products were visualized in 1% agarose gel electrophoresis stained with UniSafe Dye 20,000 × (Uniscience). PCR products were purified with polyethylene glycol (PEG) 20% PEG 8000 (Promega, USA), resuspended in 20 μl of ultrapure water, and dosed and sequenced in both directions by capillary electrophoresis in an ABI3730 sequencer using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, USA). Chromatograms obtained were edited using the ChromasPro v.2.0.1 (Technelysium Pty Ltd., Australia) and contigs, including a consensus sequence of both partially overlapping *cox*1 fragments, were compared with sequences available to species of the superfamily Paramphistomoidea available in GenBank.

Four alignments of each molecular marker (28S, ITS-2, and two fragments of *cox*1) were created using the newly generated sequences and those of other members of Paramphistomoidea (Table S1) using default parameters of MAFFT (Katoh and Standley 2013) implemented in the Guidance2 web server (http://guidance.tau.ac.il/; Sela et al. 2015). Unreliable positions in the alignments were identified and removed using the Gblock web server (http://phylogeny.lirmm.fr/; Dereeper et al. 2008) with less stringent settings; the 5.8S-ITS2 alignment was further trimmed to only the ITS-2 gene, as some sequences are restricted to this marker. Uncorrected *p* distances were calculated using MEGA 7.0 (Kumar et al. 2016).

Phylogenetic reconstructions were performed with the Bayesian inference (BI) and maximum likelihood (ML) criteria using the evolutionary models selected based on the Akaike Information Criterion corrected for small sample size in ModelFinder (Kalyaanamoorthy et al. 2017) within IQ-TREE (Nguyen et al. 2015); models for BI were restricted to those allowed in MrBayes ver. 3.2 (Ronquist et al. 2012). The models used were as follows: GTR + F + I + G_4 for both BI and ML analyses of 28S; GTR + F + G_4 and TVMe+G_4 for BI and ML analyses, respectively, of ITS-2; GTR + F + I + G_4 for codons 1 + 2 and HKY + F + I + G_4 for codon 3 in BI, and TIM + F + I + G_4 for codons 1 + 2 and TPM2 + F + I + G_4 for codon 3 for ML analysis of barcode-region *cox*1; and GTR + F + G_4 for codons 1 + 2 and HKY + F + I + G_4 for codon 3 for BI, and K3Pu + F + I + G_4 for codons 1 + 2 and TPM2u + F + I + G_4 for codon 3 for ML analysis of post-barcode *cox*1. Outgroups were chosen based on previous studies of 28S (Pérez-Ponce de Léon and Hernández-Mena 2019), ITS-2 (Laidemitt et al. 2017), and mt genome (Le et al. 2019) datasets that include members of Paramphistomoidea and closely related superfamilies.

The ML trees were generated via IQ-TREE and nodal supports were estimated with 10,000 UFBoot iterations (Minh

et al. 2013). BI analyses were conducted in MrBayes ver. 3.2 running two independent MC3 runs of 4 chains (one cold, three heated) for 10 million generations, sampling tree topologies every 1000th generation, with the first 25% of samples discarded as burn-in; all above-mentioned analyses were run on the computational resource CIPRES (Miller et al. 2010). Tracer v.1.6 (Rambaut et al. 2014) was used to check the convergence and mixing of different parameters and to confirm that the effective sample size (ESS) of each parameter was adequate to provide reasonable estimates of the variance in model parameters (i.e., ESS values > 200). Some nodes are collapsed to simplify the tree, highlighting the interrelationship within and between families. FigTree ver. 1.4.2 (Rambaut 2012) was used for tree visualization and Adobe® Photoshop® CS5 (Adobe Inc., USA) for additional editing; family-level classification in the trees follows Jones (2005a), except for the Microscaphidiidae and Mesometridae, which have appeared nested within Paramphistomoidea in most recent phylogenies (see Pérez-Ponce de Léon and Hernández-Mena 2019). Sequences here generated were deposited in the GenBank database [MT514525 (28S); MT514521 (5.8S-ITS2); MT511662 (*cox*1)].

Results

We generated sequences of 28S (1261 bp), 5.8S-ITS2 (418 bp), and *cox*1 (610 bp with the primers 123F/858R and 871 bp with JB3/COI-R Trema, resulting in a fragment of 1410 bp) genes for a Brazilian isolate of *Z. lunata*. The alignment of 45 sequences of partial sequences of the gene 28S comprised 1440 positions, of which 462 were excluded, resulting in a final alignment of 978 positions. The final matrix included 399 distinct patterns, 324 parsimony-informative, 102 singletons, and 552 constant sites. The trees resulting from the BI and ML analyses of this markers were congruent in topology (Fig. 2a) and placed *Z. lunata* with *W. zibethicus* in a well-supported clade, thus confirming the monophyly of the Zygocotylidae. While the 28S sequences of both members of the Gastrodiscidae, i.e., *Gastrodiscoides hominis* (Lewis & McConnell, 1876) and *Homalogaster paloniae* Poirier, 1883, exhibited a sister relationship, representatives of Paramphistomidae, Gastrothylacidae, and Olveriidae nested together in a well-supported, polyphyletic clade. The cladorchiids split into two sister clades, one moderately supported and formed by isolates recovered from unrelated hosts in the Palaearctic (*Amurotrema dombrowskajae* Achmerow, 1959), Indomalayan [*Helostomatis* cf. *helostomatis* (MacCallum, 1905)], Nearctic [*Pisciamphistoma stunkardi* (Holl, 1929), *Megalodiscus* spp.], and Australasian (*Solenorchis travassosi* Hilmy, 1949) realms, and the other a strongly supported clade of species that parasitize Neotropical fishes. Although nominally in the same family, the monophyly of the 28S clade containing these two cladorchiid clades was not well supported. The monophyly of the Diplodiscidae could not be assessed as it was represented only by *Diplodiscus*, in which three species formed a well-supported clade, tentatively sister to cladorchiids. The earliest diverging 28S lineage of Paramphistomoidea is represented by isolates of Microscaphidiidae, which is paraphyletic with respect to a single representative of Mesometridae (*Mesometra* sp.). Pairwise comparisons within well-supported monophyletic assemblages (Zygocotylidae, Gastrodiscidae, Neotropical cladorchiids) revealed 28S divergence of 2.2% (22-nt difference) between *Z. lunata* and *W. zibethicus* (Zygocotylidae), 0.9% (9-nt difference) between *G. hominis* and *H. paloniae* (Gastrodicidae), and 1.3% to 5.6% (13–54-nt differences) among nine species in five genera of Neotropical cladorchiids.

The alignment of 33 ITS-2 sequences comprised 551 positions, of which 267 were excluded, resulting in a final alignment of 284 positions. The final matrix included 156 distinct patterns, 141 parsimony-informative, 39 singletons, and 104 constant sites. In the ITS-2 phylogenetic reconstruction (Fig. 2b), *Z. lunata* was nested within a large polytomy including members of the Gastrodiscidae, Gastrothylacidae, Paramphistomidae, and Stephanopharyngidae. Only members of the Neotropical cladorchiids from freshwater fishes formed a strongly supported clade, apparently sister to *Diplodiscus mehrai* Pande, 1937 (Diplodiscidae). The ITS-2 of *Z. lunata* diverged by 4.3 to 20.7% (13–56-nt difference) from other Paramphistomoidea.

The alignment of 24 barcode-region *cox*1 sequences comprised 1548 positions, of which 864 were excluded (mostly on the flanking regions), resulting in a final alignment of 684 positions. The final matrix included 327 distinct patterns, 289 parsimony-informative, 46 singletons, and 349 constant sites. In phylogenetic analysis (Fig. 3a), Brazilian and Canadian isolates of *Z. lunata* nested within a polytomy formed by members of the Gastrodiscidae, Gastrothylacidae, Paramphistomidae, and Stephanopharyngidae. Four of five species of *Calicophoron* clustered in a well-supported clade, as did species of three genera of gastrothylacid amphistomes, suggesting the monophyly of the latter group. A single representative of the Cladorchiidae, *Megalodiscus* sp., was sister to the remaining Paramphistomoidea, but support for its position was not strong. Barcode *cox*1 sequences of Brazilian and Canadian isolates of *Z. lunata* differed by two nucleotide substitutions (0.4% of divergence, corresponding to G/A and T/C transitions), and by 14.4 to 20.2% (67–94-nt differences) from other paramphistomoids.

The alignment of 22 post-barcode-region *cox*1 sequences comprised 1548 positions of which 1155 were excluded (mostly on the flanking regions), resulting in a final alignment of 393 positions. The final matrix included 200 distinct patterns, 155 parsimony-informative, 31 singletons, and 207

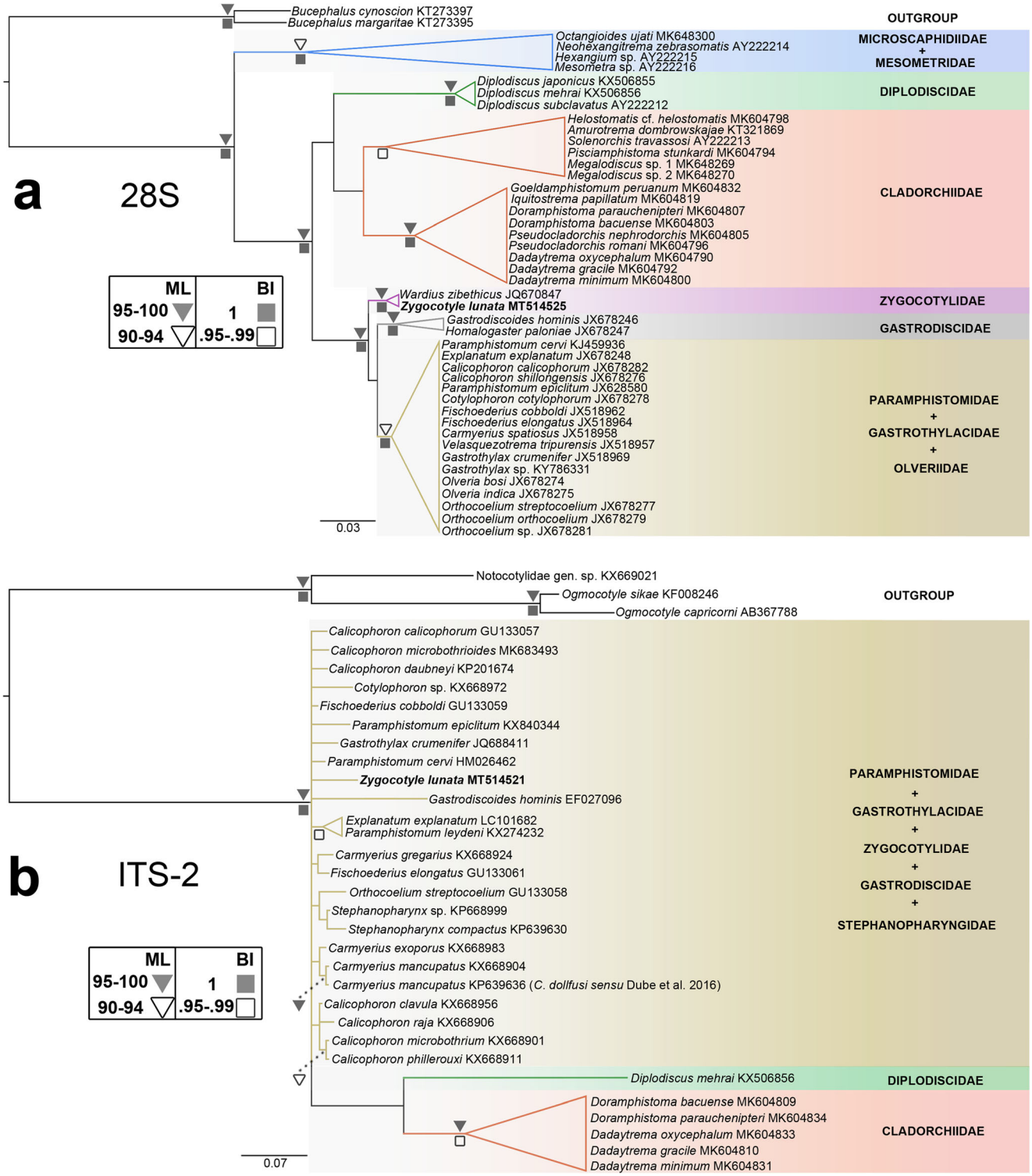

Fig. 2 Bayesian inference (BI) phylograms of the relationships of *Zygocotyle lunata* (in bold) among selected Paramphistomoidea as inferred from partial sequences of 28S (**a**), and nearly complete ITS-2 (**b**). BI posterior probabilities (≥ 0.95) and maximum likelihood (ML) bootstrap support values (≥ 90%) are shown below and above nodes, respectively (see inset). Branch length scale bar indicates number of substitutions per site

constant sites. Trees resulting from the ML and BI analyses revealed the distinctive status of *Z. lunata*, although with unclear affiliation among other amphistomes, and with some variation in node support (Fig. 3b). As in the analysis of the barcode-region *cox*1 (Fig. 3a), post-barcode *cox*1 sequences from gastrothylacid amphistomes clustered in a well-

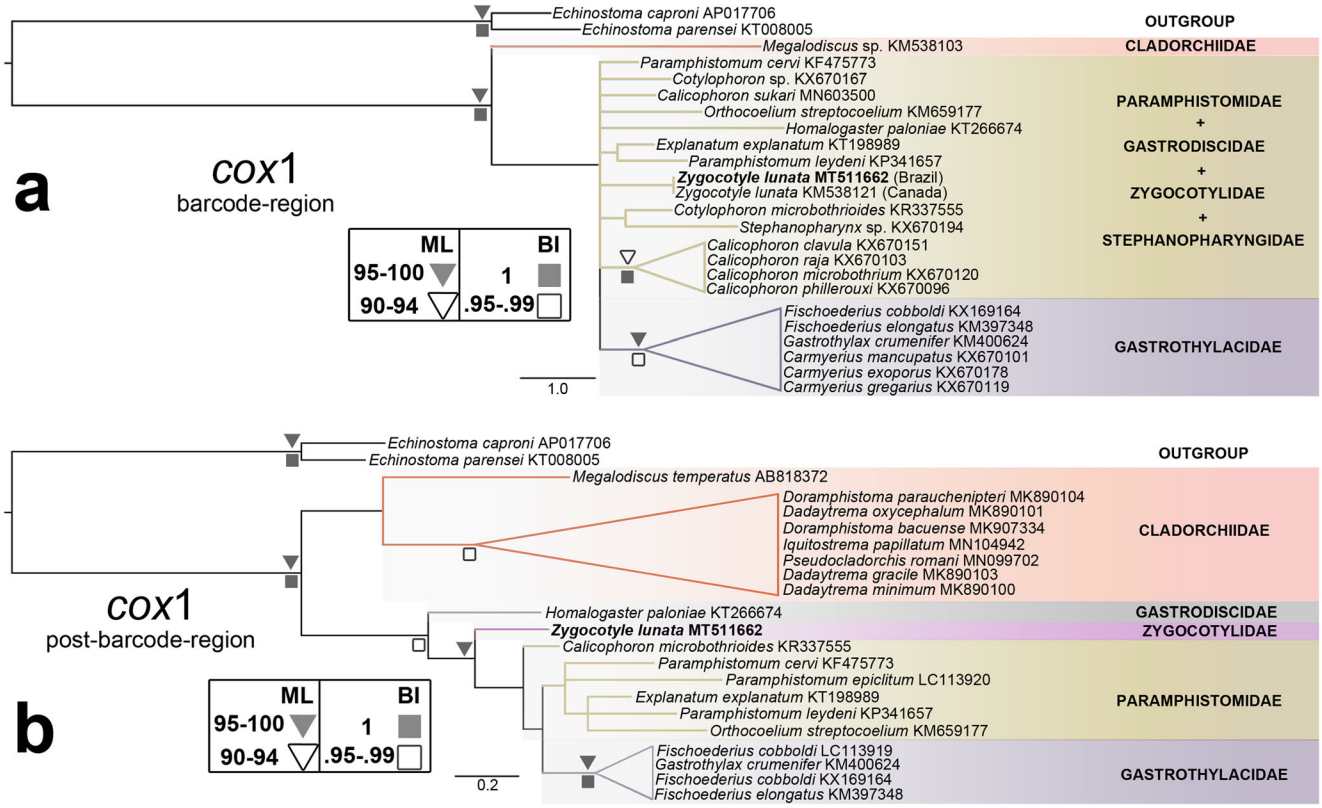

Fig. 3 Bayesian inference (BI) phylograms of the relationships of *Zygocotyle lunata* (new sequence in bold) among selected Paramphistomoidea as inferred from sequences of the barcoding region (**a**) and post-barcoding region of cytochrome *c* oxidase 1 (*cox*1) (**b**). BI posterior probabilities (≥ 0.95) and maximum likelihood (ML) bootstrap support values (≥ 90%) are shown below and above nodes, respectively. Branch length scale bar indicates number of substitutions per site

supported clade within a virtual polytomy (clades lacking strong statistical support) formed also by members of Paramphistomidae and *Z. lunata*. Post-barcode *cox*1 sequences from Neotropical cladorchiids parasitizing fish clustered in a weakly supported clade with another cladorchiid species, *Megalodiscus temperatus* (Stafford, 1905). Divergence of post-barcode *cox*1 sequence of *Z. lunata* in relation to other Paramphistomoidea ranged from 11.2 to 22.3% (36–85-nt differences).

Discussion

Zygocotyle lunata has been accommodated in different schemes of classification since its original description as *Amphistoma lunatum* based on parasites collected by the naturalist Johann Natterer from wild birds and deer in the early nineteenth century (Jones 2005b; Fried et al. 2009). The species was included in its own subfamily, Zygocotylinae, within the Paramphistomidae for decades (Yamaguti 1971). Later, Sey (1988, 1991) retained *Z. lunata* within the Zygocotylinae, but treated the taxon under the family Zygocotylidae, together with representatives of the Olveriinae, Stephanopharynginae, Pseudodiscinae, and Watsoniinae. In the most recent morphology-based treatment, the familial status of Zygocotylidae was sustained in order to include only the monotypic genera *Zygocotyle* and *Wardius*, but four former subfamilies were excluded: Olveriinae and Stephanopharynginae were elevated to family rank, and Pseudodiscinae and Watsoniinae were treated as synonyms of the Gastrodiscidae (Jones 2005b). Surprisingly, the relationship of *Z. lunata* with other paramphistomoids has never been assessed in a molecular phylogenetic context, which may raise doubts about the systematic value of some morphological characters used in the traditional classification.

Analyses of nuclear and mitochondrial sequences generated herein shed light on the relationships of *Z. lunata* with other paramphistomoids. The most well-resolved phylogeny, based on 28S, supports the placement of *Z. lunata* within the Zygocotylidae along with only *W. zibethicus*, which corroborates the scheme proposed by Jones (2005b) and refutes the five zygocotylid subfamilies suggested by Sey (1988, 1991). Unfortunately, the validity of the Zygocotylidae could not be tested using additional markers due to the lack of data from *W. zibethicus*. Both representatives of the Zygocotylidae possess a pair of extramural primary pharyngeal sacs and lack a cirrus-sac (Jones 2005b), but unique morphological synapomorphies for the family remain unclear.

Natural infections with *Z. lunata* in both intermediate and definitive hosts have been recorded across the Americas, but most of these reports are from morphologically based surveys or experimental infections of planorbid snails in *Helisoma* (in North America) and *Biomphalaria* (in South America) (Fried et al. 2009 and references therein; Barbosa et al. 2011; Spatz et al. 2012; Silva et al. 2016). Both the wide geographic distribution of *Z. lunata* and its presence in different first intermediate hosts may raise a suspicion of cryptic speciation (Nadler and Pérez-Ponce de León 2011). However, the virtual identity of *cox*1 sequence in the Brazilian and Canadian isolates of *Z. lunata* confirms the wide distribution of this species in Nearctic and Neotropical realms, and the range of its first intermediate hosts. Sey (1991) also listed reports of *Z. lunata* from Asia and Africa, but molecular data are required to confirm this wider pan-continental distribution. The compatibility of *Z. lunata* with members of both *Helisoma* and *Biomphalaria* may be related to the close relationship between these planorbids (Morgan et al. 2002; Jørgensen et al. 2004).

The few attempts that assess the systematic status of families within Paramphistomoidea have mostly focused on amphistome parasites of particular groups of hosts (Besprozvannykh et al. 2018; Pantoja et al. 2019). Although not the main focus here, our phylogenetic reconstructions suggest the non-monophyly of several traditional morphology-based families. Historically, the systematic position of the two earliest diverging families in our 28S analyses, i.e., Microscaphidiidae and Mesometridae, has been controversial (Sey 1991; Blair 2005; Jones and Blair 2005). Despite remarkable morphological differences between these families, which are considered to constitute a superfamily, Microscaphidioidea, by some authors (see Jones and Blair 2005), molecular data indicate both families belong within Paramphistomoidea. The 28S phylogeny also indicate the Microscaphidiidae to be paraphyletic with respect to a single representative of the Mesometridae (Acc. No. AY222216) (Olson et al. 2003; Pérez-Ponce de Léon and Hernández-Mena 2019; present data). Future phylogenetic studies of these monostome flukes will need broader taxonomic sampling, mainly with species of the poorly represented Mesometridae, along with detailed morphological evaluations, which may clarify their actual interrelationships.

Cladorchiidae is one of the most diverse groups of paramphistomoids. In South America, for instance, it is the most species-rich family of trematodes from freshwater fishes (Choudhury et al. 2016; Pantoja et al. 2019), yet phylogenetic studies of cladorchiids are rare (Sokolov et al. 2016; Pantoja et al. 2019). In the most comprehensive analysis, Pantoja et al. (2019) focused on the subfamilial classifications and found evidence for the monophyly of the Neotropical fish cladorchiids, but could not confirm the monophyletic status of the family. Our results agree with these findings since the sister relationship between taxa from unrelated hosts and zoogeographical regions, and the cladorchiids from Neotropical freshwater fishes, is not statistically supported (see Figs. 1a and 2b). We speculate that data from members of the type genus *Cladorchis* Fischoeder, 1901, may result in a narrower concept of the Cladorchiidae as well as in elevation to family rank of the Neotropical cladorchiid parasites of freshwater fishes.

An even more complex scenario emerges among amphistomes from terrestrial mammals, especially those of veterinary importance. The non-monophyly of the speciose family Paramphistomidae was observed in all phylogenetic analyses (see Figs. 2 and 3), as in previous studies (Ichikawa et al. 2013; Shylla et al. 2013; Laidemitt et al. 2017; Pérez-Ponce de Léon and Hernández-Mena 2019). Representatives of three out of four genera of Gastrothylacidae (only *Velasquezotrema* Eduardo & Javellana, 1987 is missing) formed a monophyletic assemblage in analyses of both *cox*1 fragments (see Fig. 3) that confirms the presumptive validity of the family (Laidemitt et al. 2017). However, nuclear markers did not support the Gastrothylacidae (see Fig. 2).

The monophyly of the Stephanopharyngidae and Olveriidae seems plausible as both families comprise a single genus, *Stephanopharynx* Fischoeder, 1901 and *Olveria* Thapar & Sinha, 1945, respectively (Jones 2005c, 2005d). In our analyses based on the nuclear genes, *S. compactus* Fischoeder, 1901 (type and only known species) showed a sister relationship with an unnamed species of *Stephanopharynx* (see Fig. 1b), but 28S from *O. indica* Thapar & Sinha, 1945 (type species) and *O. bosi* Tandon, 1951 did not cluster together in a single clade (see Fig. 1a). Instead, *Olveria* spp. clustered in a large polytomy, also including representatives of the Paramphistomidae and Gastrothylacidae. Despite the unique morphology of both known species of *Olveria*, which have lent weight to the erection of the Olveriidae (Jones 2005d), their sister relationship should be assessed using additional molecular markers. The monophyly of the Gastrodiscidae was recovered in the analysis of 28S, in which *G. hominis* and *H. paloniae* exhibit a strongly supported sister relationship. However, more representatives of the other six genera within Gastrodiscidae (see Jones 2005e) should be sequenced to evaluate the status of this family.

Some of these results differ from recent analyses of mitochondrial genomes. For example, the Paramphistomidae and Gastrothylacidae emerge as monophyletic in Yang et al. (2016), Suleman et al. (2019), Le et al. (2019), and Rajapakse et al. (2020), which collectively include representatives of *Paramphistomum*, *Explanatum*, *Calicophoron*, *Orthocoelium* (Paramphistomidae), *Fischoederius*, and *Gastrothylax* (Gastrothylacidae), and *Homalogaster* (Gastrodiscidae). However, Fu et al. (2019) and Li et al. (2020), who analyzed the same sequences, present mitochondrial genome phylogenies in which the Paramphistomidae forms a paraphyletic group with Gastrothylacidae nested within it.

Clearly, systematic classification in the Paramphistomoidea does not reflect the evolutionary history of the group. A meticulous framework for the morphological identification of these organisms has been proposed (see Eduardo 1982; Sey 1991; Jones 2005a, and references therein), but the relative importance of these characters for paramphistomoid systematics is not clear. Another obstacle is the frequent and exclusive use of ITS-2 sequences (Lotfy et al. 2010; Ichikawa et al. 2013; Dube et al. 2016), which may not display enough phylogenetic signal either to differentiate closely related species (Laidemitt et al. 2017) or unravel the interrelationships among the paramphistomoids, as indicated in the present study. Therefore, we advocate that future integrative studies on paramphistome flukes should prioritize expansion of taxonomic coverage and, secondly, should obtain mitochondrial genomic data or at least two independently evolving loci (e.g., 28S and *cox*1), and finally, should be accompanied by thorough morphological study.

Our molecular phylogenetic analysis of *Z. lunata* will help in future, wider efforts to organize taxonomy in the Paramphistomoidea. Besides confirming the wide distribution of the species in the Americas, our work shows the Zygocotylidae to be one of few traditional paramphistomoid families with robust molecular support.

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Compliance with ethical standards

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

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