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Filling the gaps in the classification of the Digenea Carus, 1863: systematic position of the Proterodiplostomidae Dubois, 1936 within the superfamily Diplostomoidea Poirier, 1886, inferred from nuclear and mitochondrial DNA sequences

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Abstract The Diplostomida Olson, Cribb, Tkach, Bray & Littlewood, 2003 is the less diverse order of the two orders within the subclass Digenea Carus, 1863 and is currently classified into three superfamilies, i.e. Brachylaimoidea Joyeux & Foley, 1930, Diplostomoidea Poirier, 1886, and Schistosomatoidea Stiles & Hassall, 1898. Although the suprageneric-level relationships have been elucidated with the use of molecular markers, the lack of representation of some groups obscure the phylogenetic relationships among families, rendering the classification unstable. Here, we tested the phylogenetic position of the family Proterodiplostomidae Dubois, 1936 based on partial 28S rDNA and complete 18S rDNA sequences for *Crocodilicola pseudostoma* (Willemoes-Suhm, 1870), a crocodile parasite that has been found as a progenetic metacercaria parasitising the pale catfish *Rhamdia guatemalensis* (Günther) in Mexico and in other siluruforms in the Neotropics. We augmented the representation of the species, genera and families within the Diplostomida, including mostly representatives of the superfamily Diplostomoidea, and assembled a dataset that contains 49 species for the 28S rRNA gene, and 45 species for the 18S rRNA gene. Additionally, we explored the phylogenetic signal of the mitochondrial gene *cox*1 in reconstructing the phylogenetic relationships of selected members of the superfamily. Our analyses showed that the family Proterodiplostomidae is the sister taxon to the paraphyletic Diplostomidae Poirier, 1886 and Strigeidae Railliet, 1919, with Cyathocotylidae Mühling, 1898 + Brauninidae Wolf, 1903 as their sister group. Analysis of concatenated 18S + 28S sequences revealed the Liolopidae Odhner, 1912 as the basal group of the superfamily Diplostomoidea, although analyses of independent datasets showed that the position of this family remains uncertain. Analysis based on *cox*1 unequivocally resolved the Proterodiplostomidae as the sister taxon to the Diplostomidae and Strigeidae, although the Cyathocotylidae was nested in a different clade, along with brachylaimoids and schistosomatoids.

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Introduction

The order Diplostomida Olson, Cribb, Tkach, Bray & Littlewood, 2003 is the less diverse of the two orders of the subclass Digenea Carus, 1863, representing just

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12.7% of all the digenean families, and containing approximately 1,477 species included in 210 genera and 19 families (Littlewood et al., 2015). Species included in the order are parasites of tetrapod vertebrates as definitive hosts, with the exception of the fish blood-flukes belonging to the family Aporocotylidae Odhner, 1912 (see Kostadinova & Pérez-del-Olmo, 2014; Littlewood et al., 2015). Species of the Diplostomida are currently classified into three superfamilies, i.e. the Diplostomoidea Poirier, 1886, Schistosomatoidea Stiles & Hassall, 1898 and Brachylaimoidea Joyeux & Foley, 1930 (see Olson et al., 2003). The Diplostomoidea is the most diverse group of the order, with 797 species parasitising mammals, birds and reptiles (Littlewood et al., 2015). Species of this superfamily are easily differentiated from other groups of digeneans in possessing a unique holdfast organ and a cirrus-sac and cirrus replaced by an atypical copulatory apparatus and terminal genitalia (except for the Cyathocotylidae Mühling, 1898) (Niewiadomska, 2002a). Six families containing 97 genera are currently recognised within the superfamily: the monotypic Brauninidae Wolf, 1903 and Bolbocephalodidae Strand, 1935, and the species-rich Strigeidae Railliet, 1919, Diplostomidae Poirier, 1886, Cyathocotylidae and Proterodiplostomidae Dubois, 1936 (see Niewiadomska, 2002a, b, c, d, e, f, g). Only two of the six families and five out of the 97 genera currently known for the superfamily were represented in the comprehensive phylogenetic analysis of the Digenea conducted by Olson et al. (2003). Kostadinova & Pérez-del-Olmo (2014) marked as important omissions the families Cyathocotylidae and Proterodiplostomidae and concluded that the assessment of the relationships within the superfamily required further exploration based on a wider array of taxa, including the type-genus of the family Strigeidae, *Strigea* Abildgaard, 1790.

The recent expansion of the genetic library of various molecular markers for several groups of trematodes has enhanced our capacity to reconstruct the phylogenetic relationships at different levels of the taxonomic hierarchy of the group. Particularly for diplostomoids, a large number of DNA sequences has been obtained, especially for clinostomids, strigeids and diplostomids, in studies on the phylogenetic relationships within species of a genus (e.g. Bell et al., 2001), studies of species delimitation (e.g. Galazzo et al., 2002; Dzikowski et al., 2003; Locke et al., 2010, 2015; Georgieva et al., 2013; Hernández-Mena et al., 2014; García-Varela et al., 2016a; Pérez-Ponce de León et al., 2016), and studies that link the larval stages in their intermediate fish or amphibian hosts with the adults in fish-eating birds (e.g. Locke et al., 2011; Blasco-Costa et al., 2016a; García-Varela et al., 2016b). In these studies, sequences of the nuclear ITS1-5.8-ITS2 and mitochondrial *cox*1 genes were primarily obtained. However, some studies have also provided 18S and 28S rDNA sequences (e.g. Pulis et al., 2013; Patrelle et al., 2015; Blasco-Costa et al., 2016a). Interestingly, 18S and/or 28S rDNA sequences of several diplostomoids have been added to the GenBank since the publication by Olson et al. (2003), including the cyathocotylid *Holostephanus dubinini* Vojtek & Vojtkova, 1968, from the great cormorant (Dzikowski et al., 2004); *Braunina cordiformis* Wolf, 1903, the only member of the family Brauninidae, a parasite of short beaked common dolphin (Fraija-Fernández et al., 2015); and *Strigea* sp. (mesocercariae), from brown frogs and water frogs (Patrelle et al., 2015).

Here, we contribute to the classification of the Digenea by presenting an updated phylogeny of the group, with a particular focus on the relationships within the superfamily Diplostomoidea. We present a molecular phylogenetic analysis using sequences of the 18S and 28S rRNA genes obtained from GenBank and newly generated sequences for a broad diversity of taxa belonging to the superfamily to investigate the systematic position of the Proterodiplostomidae. We expand the information on both nuclear genes and explore the phylogenetic signal of one additional gene for a higher-level classification, the cytochrome *c* oxidase subunit 1 (*cox*1).

Materials and methods

Specimen collection and identification

Adult specimens of various species belonging to five families (Clinostomidae Lühe, 1901, Cyathocotylidae, Diplostomidae, Proterodiplostomidae, and Strigeidae) were collected in different species of fish-eating birds or freshwater fishes from 13 localities in Mexico between 2010 and 2015 (Table 1). Birds were collected with a shotgun under permission FAUT 0202 issued by the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT) of Mexico to

MGV, and fish were captured using seine nets. The intestines of each individual host were removed and placed in Petri dishes with 0.75% or 0.65% saline solution and observed under a stereomicroscope. For fishes, the body cavity of each individual was also examined. Some of the specimens from each host were fixed in 4% hot formalin for morphological study, and other specimens were placed in vials with 100% ethanol for molecular analysis. For taxonomic identification, specimens were stained with Mayer's paracarmine and mounted on permanent slides with Canada balsam. Vouchers of some of the specimens collected for this study were deposited at the Colección Nacional de Helmintos (CNHE), Biology Institute, National Autonomous University of México, Mexico City, Mexico (Table 1). Host taxonomy follows Froese & Pauly (2017) for fish, Avibase (https://avibase.bsc-eoc.org/) for birds, and Integrated Taxonomic Information System, ITIS (https://www.itis.gov) for all other hosts.

DNA extraction, amplification and sequencing

Each specimen was digested and its genomic DNA extracted using the REDExtract-N-Amp Tissue PCR kit (Sigma, St. Louis, USA) following the manufacturer's instructions. The domains D1-D3 of the 28S rDNA and the entire 18S rDNA from nuclear ribosomal DNA were amplified using polymerase chain reaction (PCR). Additionally, the cytochrome *c* oxidase subunit 1 mitochondrial gene (*cox*1) was amplified. For 28S, the primers 139 and 536 were used for amplification. For 18S, two overlapping fragments were amplified with the primers 18S1A plus 32 and 652 plus 28 (Table 2). For *cox*1, we used the primers M13 and BarCoxR (Table 2). PCR reactions were performed with 2 µl of DNA extraction supernatant, 1 µl of each PCR primer (10 µM), 2.5 µl of 10× buffer, 1.5 mM of $MgCl_2$, 0.5 of dNTPs (10 mM), 16 µl of water and 1 U of Taq DNA polymerase. The following thermo-cycling profile was utilized for the three molecular markers: denaturation of DNA (95°C for 5 min); 36 cycles of amplification (94°C for 1 min, 50°C for 1 min and 72°C for 1 min); and a post-amplification incubation at 72°C for 10 min. Sequencing reactions were performed with ABI Big Dye terminator sequencing chemistry (Applied Biosystems, Boston, Massachusetts, USA). To sequence 18S amplicons, we used the four PCR primers; for 28S, we used the two PCR primers plus two internal primers, 503 and 504; and for *cox*1, we used the PCR primers (Table 2). Reaction products were separated and read on an ABI 3730xl Genetic Analyser (Applied Biosystems). The resulting contiguous sequences were assembled in Geneious Pro 4.8.4® (Biomatters Ltd., Auckland, New Zealand) to generate consensus sequences. The new consensus sequences of each molecular marker belonging to the different species included in this study were deposited in the GenBank under the accession numbers MF398316–MF398319 (*cox*1), MF398320–MF398348 (28S), and MF398349–MF398365 (18S) (see Supplementary Table S1 for details).

Alignment and phylogenetic analyses

Sequences for 18S, 28S and *cox*1 were generated for the following species: *Clinostomum marginatum* (Rudolphi, 1819), *C. tataxumui* Sereno-Uribe, Pinacho-Pinacho, García-Varela & Pérez-Ponce de León, 2013 (Clinostomidae), *Mesostephanus microbursa* Caballero, Grocott & Zerecero, 1953 (Cyathocotylidae), *Posthodiplostomum* sp., *Uvulifer* sp., *Hysteromorpha triloba* (Rudolphi, 1819), *Tylodelphys aztecae* García-Varela, Sereno-Uribe, Pinacho-Pinacho, Hernández-Cruz & Pérez-Ponce de León, 2015, *Austrodiplostomum ostrowskiae* Dronen, 2009 (Diplostomidae), *Crocodilicola pseudostoma* (Proterodiplostomidae), *Cardiocephaloides* sp., *Cotylurus gallinulae* (Lutz, 1928), *Australapatemon burti* (Miller, 1923), *Apharyngostrigea cornu* (Zeder, 1800), *Parastrigea plataleae* Hernández-Mena, García-Prieto & García-Varela, 2014, *P. cincta* Brandes, 1888, *P. diovadena* Dubois & Macko, 1972 and *Strigea* sp. (Strigeidae) (Table 1). The newly generated sequences were aligned with other sequences of 18S, 28S and *cox*1, available in the GenBank database, including those for species of the following families of the Diplostomida: Aporocotylidae, Brachylaimidae Joyeux & Foley, 1930, Brauninidae, Clinostomidae, Cyathocotylidae, Diplostomidae, Leucochloridiidae Poche, 1907, Liolopidae Odhner, 1912, Schistosomatidae Stiles & Hassall, 1898 and Spirochiidae Stunkard, 1921 (see Supplementary Table S1). Sequences for *Fasciola hepatica* Linnaeus, 1758 (Plagiorchiida La Rue, 1957) were employed in each dataset to root the phylogenetic trees.

Each dataset (18S, 28S and *cox*1) was individually aligned in the SATé software under the default setting SATé-II-fast (Liu et al., 2009, 2012), implementing

Table 1 Specimens of diplostomoids collected in birds and fish in different areas of Mexico used for sequencing and molecular phylogenetic analyses

Trematode species	Host species	Locality	CNHE numbers
Clinostomidae Lühe, 1901			
Clinostomum marginatum (Rudolphi, 1819)	*Ardea alba* Linnaeus	Ocotes, Oaxaca (16°36′57″N, 96°43′13″W)	8345
Clinostomum tataxumui Sereno-Uribe, Pinacho-Pinacho, García-Varela & Pérez-Ponce de León, 2013	*Ardea alba* Linnaeus	Tlacotalpan, Veracruz (18°36′00″N 95°39′00″W)	8338
Cyathocotylidae Mühling, 1898			
Mesostephanus microbursa Caballero, Grocott & Zerecero, 1953	*Sula nebouxii* Milne-Edwards	Isla Isabel, Nayarit (21°52′00″N, 105°54′00″W)	7286
Diplostomidae Poirier, 1886			
Austrodiplostomum ostrowskiae Dronen, 2009	*Phalacrocorax brasilianus* (Gmelin)	Presa La Angostura, Chiapas (16°11′31″N, 92°59′52″W)	9753
Hysteromorpha triloba (Rudolphi, 1819)	*Phalacrocorax brasilianus* (Gmelin)	Tlacotalpan, Veracruz (18°36′N, 95°39′W)	–
Posthodiplostomum sp.	*Phalacrocorax brasilianus* (Gmelin)	El Espino, Tabasco (18°14′47″N, 92°49′57″W)	–
Tylodelphys aztecae García-Varela, Sereno-Uribe, Pinacho-Pinacho, Hernández-Cruz & Pérez-Ponce de León, 2015	*Podilymbus podiceps* (Linnaeus)	Tlahúac, CDMX (19°15′58″N, 99°00′24″W)	9777
Uvulifer sp.	*Megaceryle alcyon* (Linnaeus)	Ocotes, Oaxaca (16°36′57″N, 96°43′13″W)	–
Proterodiplostomidae Dubois, 1936			
Crocodilicola pseudostoma (Willemoes-Suhm, 1870)	*Rhamdia guatemalensis* (Günther)	Catemaco Lake, Veracruz (18°24′46.7″N, 95°06′33.8″W)	10423
Strigeidae Railliet, 1919			
Apharyngostrigea cornu (Zeder, 1800)	*Nycticorax nycticorax* (Linnaeus)	El Huizache, Sinaloa (23°05′28″N, 106°15′57″W)	–
Apharyngostrigea cornu (Zeder, 1800)	*Ardea alba* Linnaeus	Tlacotalpan, Veracruz (18°36′00″N, 95°39′00″W)	10424
Australapatemon burti (Miller, 1923)	*Anas diazi* Ridgway	Chicnahuapan, Estado de Mexico (19°10′N, 99°29′W)	7176
Cardiocephaloides sp.	*Larus occidentalis* Audubon	Guerrero Negro, Baja California Sur (27°57′32″N, 114°03′22″W)	7175
Cotylurus gallinulae (Lutz, 1928)	*Aythya affinis* (Eyton)	La Esperanza, Sonora	7173
Parastrigea cincta Brandes, 1888	*Eudocimus albus* (Linnaeus)	Caimanero, Sinaloa (25°36′30″N, 108°26′25″W)	10425
Parastrigea diovadena Dubois & Macko, 1972	*Eudocimus albus* (Linnaeus)	Pijijiapan, Chiapas (15°31′54″N, 93°09′39″W)	10426
Parastrigea plataleae Hernández-Mena, García-Prieto & García-Varela, 2014	*Platalea ajaja* (Linnaeus)	El Huizache, Sinaloa (23°05′28″N, 106°15′57″W)	8258
Strigea sp.	*Caracara cheriway* (von Jacquin)	Presa La Angostura, Chiapas (16°11′31″N, 92°59′52″W)	10427

Table 2 Primers used for amplification and sequencing of ribosomal and mitochondrial DNA of the digenean species used in this study

Gene	Primer	Primer sequence (5′-3′)	Direction	Application	References
Ribosomal					
18S	18S1A	GGCGATCGAAAAGATTAAGCCATGCA	Forward	Amplification and sequencing	Nadler et al. (2000)
18S	32	CGAAGTCCTATTCCATTATTC	Reverse	Amplification and sequencing	This study
18S	652	GCAGCCGCGGTAATTCCAGCTC	Forward	Amplification and sequencing	Nadler et al. (2000)
18S	28	AGCGACGGGCGGTGTGT	Reverse	Amplification and sequencing	This study
28S	391	AGCGGAGGAAAAGAAACTAA	Forward	Amplification and sequencing	Nadler & Hudspeth (1998)
28S	536	CAGCTATCCTGAGGGAAAC	Reverse	Amplification and sequencing	García-Varela & Nadler (2005)
28S	503	CCTTGGTCCGTGTTTCAAGACG	Reverse	Amplification	Stock et al. (2001)
28S	504	CGTCTTGAAACACGGACTAAGG	Forward	Amplification	García-Varela & Nadler (2005)
Mitochondrial					
*cox*1	M13F	TGTAAAACGACGGCCAGT	Forward	Amplification and sequencing	Messing (1983)
*cox*1	BarCoxR	ATAAACCTCAGGATGCCCAAAAAA	Reverse	Amplification and sequencing	Razo-Mendivil (pers. com.)

100 iterations for each dataset. A concatenated dataset of 18S + 28S was also assembled. For the concatenated alignment, only the taxa with sequences available for both molecular markers were considered. All phylogenetic analyses were run under Maximum Likelihood (ML) and Bayesian inference (BI), employing the substitution model GTR + Γ for 18S and 28S and GTR + Γ + I for *cox*1. The models of nucleotide evolution were estimated in jModelTest v2 (Darriba et al., 2012). ML inference (100 replicates), model parameters and bootstrap support (1,000 replicates) were estimated with RAxML v. 7.0.4 (Stamatakis, 2006). MrBayes v. 3.2.1 (Ronquist et al., 2012) was used to perform BI analysis, running four independent MCMC runs of four chains each run (a heating parameter value of 0.5) for 20 million generations and sampling tree topologies every 1,000 generations (printfreq = 1,000; samplefreq = 1,000; diagnfreq = 10,000). 'Burn-in' periods were set to the first 1,500 generations. A 50% majority-rule consensus tree and nodal support estimated as posterior probability values were calculated from the remaining trees. The phylogenetic trees obtained from both analyses were visualized in FigTree v.1.4.3. (http://tree.bio.ed.ac.uk/software/figtree/).

Results

28S and 18S rDNA datasets

In total, 29 new partial 28S rDNA sequences were generated for 17 species of diplostomoids (Table 1). The alignment of the 28S rRNA gene sequences was 1,437 bp long and consisted of 64 sequences representing 49 species of digeneans, of which 31 were members of the Diplostomoidea. The nucleotide frequencies were as follows: A = 0.204, C = 0.217, G = 0.319, T = 0.259. The phylogenetic tree obtained from the ML analysis had a log-likelihood of -13,971.057555. The ML and BI trees yielded similar topologies (Fig 1A). These trees unequivocally resolved the Proterodiplostomidae as the sister group of the Strigeidae and Diplostomidae, a relationship supported by high bootstrap and posterior probability values.

Eighteen new sequences of the 18S rRNA gene were generated for 15 species of diplostomoids

(Table 1). The 18S dataset was 2,022 bp long and consisted of 50 sequences of 45 species, of which 13 were species of the superfamily Diplostomoidea. The nucleotide frequencies were as follows: A = 0.239, C = 0.220, G = 0.286, T = 0.256. The ML analysis yielded a single tree with a log-likelihood of -11,315.496983. The phylogenetic trees of ML and the consensus of the BI resulted in similar topologies (Fig. 1B). These trees showed the Proterodiplostomidae as the sister group of the Strigeidae and Diplostomidae. These relationships were also supported by high bootstrap and posterior probability values.

The resulting phylogenetic hypotheses of both nuclear genes analysed independently showed very similar topologies regarding the relationships among families (and superfamilies) of the order Diplostomida (Fig. 1A, B). Relationships were confirmed through an ML and BI analysis of the concatenated dataset of 18S + 28S (Fig. 2). For instance, all trees showed that the Diplostomoidea is monophyletic, with high bootstrap and posterior probability support values. Within diplostomoids, two clades were formed, one containing the Brauninidae (*B. cordiformis*) as the sister group of the Cyathocotylidae and a second clade containing the newly generated sequences for the Proterodiplostomidae (*Crocodilicola pseudostoma*) as the sister group of the Diplostomidae and Strigeidae; both clades were highly supported by bootstrap support and posterior probability values. Furthermore, the Diplostomidae and Strigeidae were paraphyletic, and relationships among them remain unclear. However, the Strigeidae was clearly divided into two

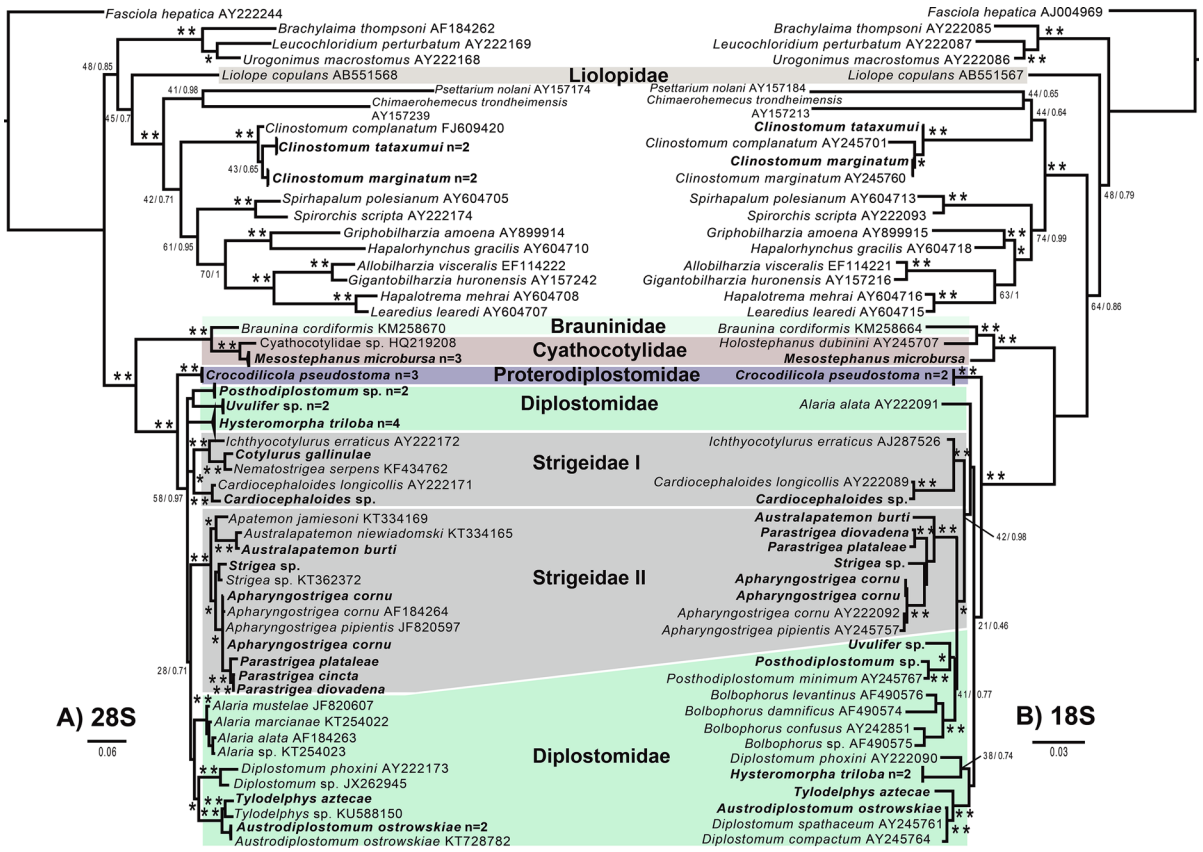

Fig. 1 Phylogenetic relationships among the taxa of the Diplostomoidea resulting from Maximum Likelihood and Bayesian inference analyses based on the partial sequences of the 28S rRNA gene (A) and the 18S rRNA gene (B). Bootstrap support values and posterior probability values are shown near the nodes. *Key*: **, bootstrap support of 80–100% and posterior probabilities of 0.80–1.00; *, bootstrap support of 60–79% and posterior probabilities of 0.60–0.79. The newly generated sequences are highlighted in bold and are presented without GenBank accession numbers. For some of the species analyses in this study more than one specimen was sequenced; the "n" in parentheses indicates the number of isolates obtained for each species. The scale-bar indicates the number of substitutions per site

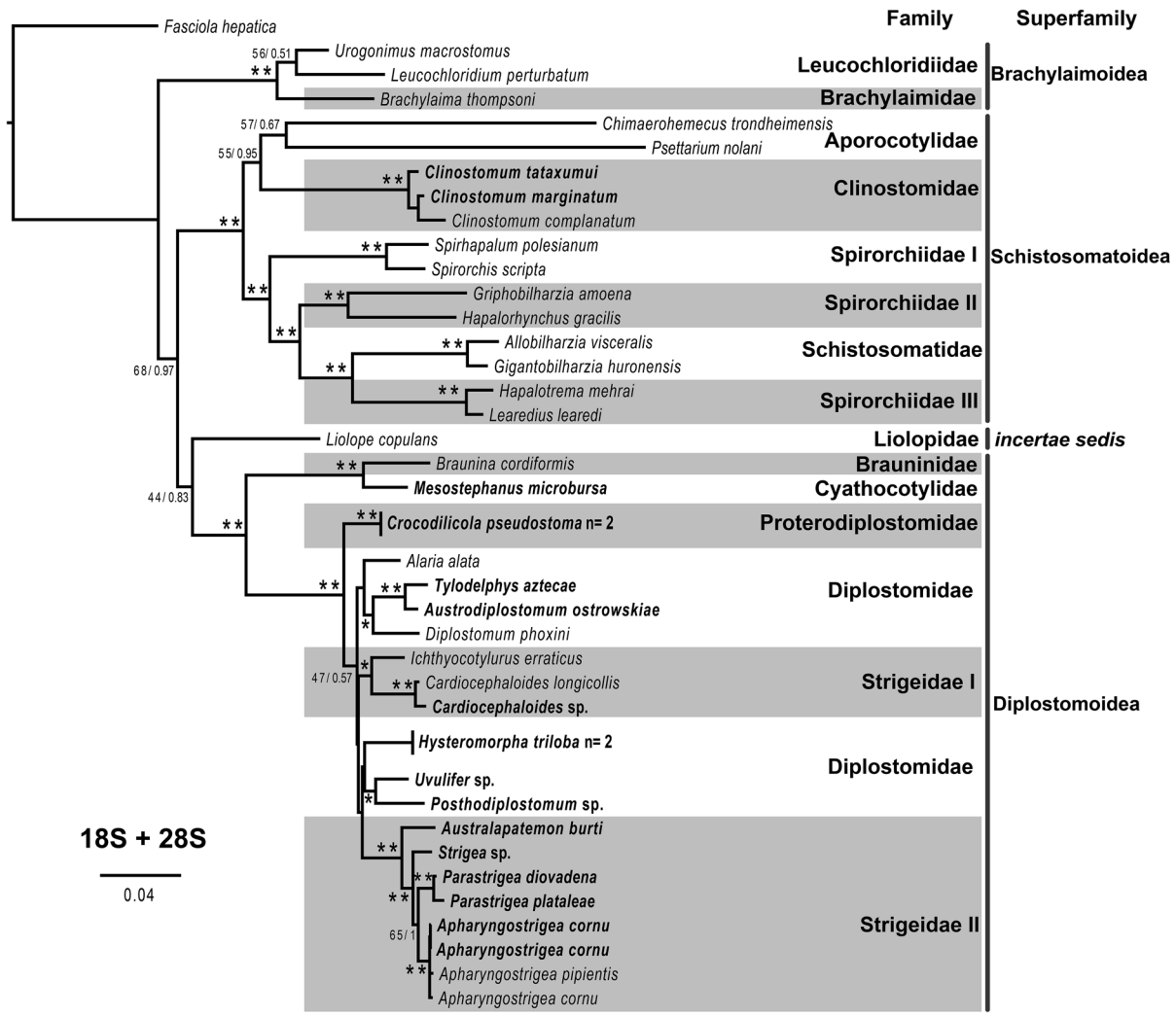

Fig. 2 Phylogenetic tree inferred with the combined nuclear datasets (18S + 28S), using Maximum Likelihood and Bayesian inference analyses. Bootstrap values and posterior probability values are shown near the nodes. *Key*: **, bootstrap support of 80–100% and posterior probabilities of 0.80–1.00; *, bootstrap support of 60–79% and posterior probabilities of 0.60–0.79. Labels along the vertical bars indicate the classification of the species at the level of family and superfamily

clades, one including the genera *Cardiocephaloides* Sudarikov, 1959, *Ichthyocotylurus* Odening, 1969, *Cotylurus* Szidat, 1928, and *Nematostrigea* Sandground, 1924 (the last two only present in the 28S dataset), and a second clade comprising the genera *Apatemon* Szidat, 1928 (only present in the 28S dataset), *Australapatemon* Sudarikov, 1959, *Apharyngostrigea* Ciurea, 1927, *Parastrigea* Szidat, 1928, and *Strigea* (Figs. 1A, B, 2). However, for diplostomids, even though two clades were formed, the relationships among the genera were inconsistent, and different topologies were obtained in the analysis of the concatenated 18S and 28S data. The systematic position of the Liolopidae was also inconsistent. *Liolope copulans* Cohn, 1902 was nested as the sister taxon of the Schistosomatoidea in both nuclear datasets analysed separately. However, in the concatenated dataset, *L. copulans* was nested as the basal member of the Diplostomoidea, although the relationships were supported by a very low bootstrap support value (44%) and a moderate posterior probability support value (0.83) (Fig. 2).

*cox*1 dataset

We obtained *cox*1 sequences for three species of the Diplostomoidea, i.e. *Mesostephanus microbursa* Caballero, Grocott & Zerecero, 1953 (n = 1), *Crocodilicola pseudostoma* (n = 2), and *Strigea* sp. (n = 1). The *cox*1 dataset contained 684 bp and comprised 80 sequences, of which 64 were for species of the Diplostomoidea. The nucleotide frequencies were as follows: A = 0.190, C = 0.127, G = 0.206, T = 0.477. The ML analysis exhibited a single tree with a log-likelihood of -16,187.659230. The phylogenetic trees from ML and BI analyses showed a similar topology (Fig 3). The Proterodiplostomidae was unequivocally nested as the sister group of the clade formed by the Diplostomidae and Strigeidae, with high bootstrap (95%) and posterior probability (1) support values. In the *cox*1 tree, the family Strigeidae was recovered as monophyletic, albeit with very low bootstrap and posterior probability support values (18% and 0.65, respectively). Instead, the Diplostomidae was paraphyletic, because the Strigeidae was included in the same clade. Within the Strigeidae, as in the 18S and 28S trees, the same two clades were formed, one with *Cardiocephaloides*, *Cotylurus* and *Ichthyocotylurus* (bootstrap: 67%; posterior probability: 0.98) and a second clade containing *Apatemon*, *Australapatemon*, *Apharyngostrigea*, *Parastrigea* and *Strigea* (bootstrap: 94%; posterior probability: 1). No *cox*1 sequences are available for *B. cordiformis* or *L. copulans*, and, interestingly, the *cox*1 dataset showed an unexpected relationship for the Cyathocotylidae because the family was not closely related to the proterodiplostomids, diplostomids or strigeids but was apparently nested within the Schistosomatoidea.

Discussion

The results of the phylogenetic analyses based on sequences of the nuclear genes 18S and 28S, the mitochondrial *cox*1 gene, and the concatenated dataset for the two nuclear markers unequivocally showed that the Proterodiplostomidae is the sister group of the Diplostomidae and Strigeidae, within the superfamily Diplostomoidea. Our study demonstrates the value of steadily adding new sequence data to a growing genetic library of several molecular markers for digeneans, searching for a stable and useful classification that represents an accurate account of interrelationships among digenean taxa. Littlewood (2008) noted that 18S and 28S rRNA genes provide the foundation of molecular systematics for the parasitic platyhelminths, having been used extensively for revealing interrelationships within and between families and across the phylum (e.g. Cribb et al., 2001; Olson et al., 2003). Additionally, the use of both nuclear genes in combined analyses may provide better resolution than each of the genes analysed separately, when they do not provide stability of taxa across the trees (see Lockyer et al., 2003; Waeschenbach et al., 2007; Littlewood, 2008). Our study further corroborates Littlewood's (2008) contention, since both 18S and 28S trees are congruent relative the position of the Proterodiplostomidae as the sister group of the Strigeidae and Diplostomidae, and this topology is also recovered by the combined phylogenetic analysis of both nuclear genes. Actually, in this study we explored the phylogenetic signal of the *cox*1 mitochondrial gene at deeper levels of the hierarchy of the digenean classification, and we found that the position of Proterodiplostomidae is also congruent with the topology of the trees recovered by the nuclear genes. Mitochondrial genes are not commonly used to reconstruct phylogenetic relationships at deeper levels because they evolve considerably faster than nuclear markers. For this reason, some authors have advocated that these genes would be better used to resolve younger clades resulting from recent radiations, although the analysis of multiple mitochondrial genes has demonstrated usefulness to resolve deeper level phylogenies in other platyhelminths (see Littlewood, 2008 and references therein; Littlewood et al., 2015).

The current tendency actually shows that the complete mitochondrial genomes of digeneans provide a phylogenetic signal because they offer a wealth of homologous markers. Therefore, complete mitochondrial genomes have been used to resolve deeper-level phylogenetic relationships of digeneans (e.g. Littlewood et al., 2006; Webster & Littlewood, 2012; Briscoe et al., 2016). In this study, we followed Littlewood's (2008) suggestion to conduct empirical tests of the performance of some popular mitochondrial genes, in this case, *cox*1, to reconstruct the phylogenetic relationships between major trematode lineages. Littlewood et al. (2015) and Blasco-Costa et al. (2016b) argued that the application of next-

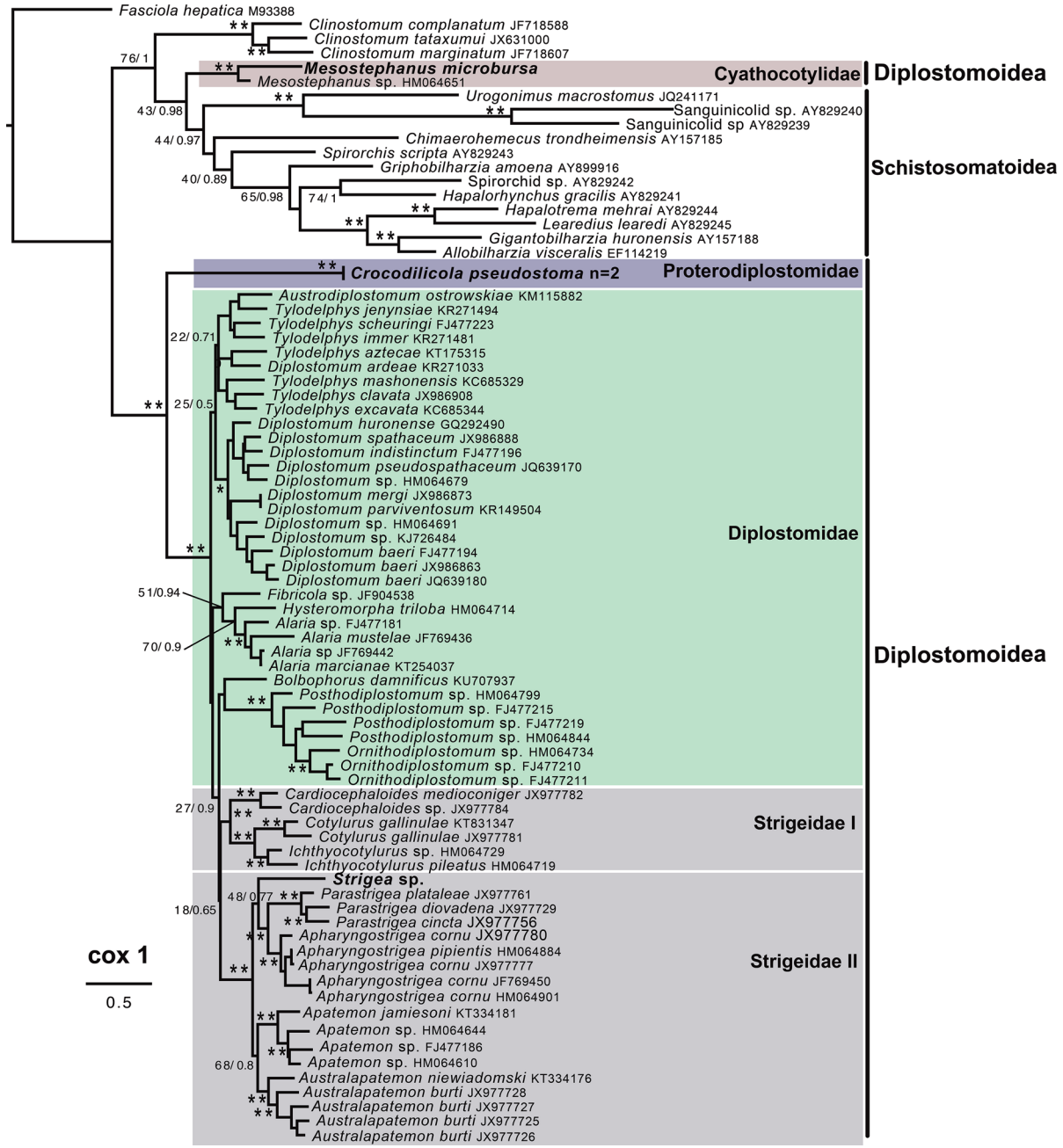

Fig. 3 Phylogenetic tree inferred from *cox* 1 dataset with Maximum Likelihood and Bayesian inference analyses. Bootstrap values and posterior probability values are shown near the nodes; **, bootstrap support of 80–100% and posterior probabilities of 0.80–1.00. The newly generated sequences are highlighted in bold and without GenBank accession numbers. Labels along the vertical bars indicate the classification of the species at the level of family and superfamily

generation sequencing and comparative mitogenomics will offer an unprecedented opportunity to provide better nodal support for phylogenetic relationship inference and that sequencing complete mitochondrial genomes has the potential to provide new insights into the systematics across Trematoda as a whole.

Phylogenetic position of the Proterodiplostomidae

The Proterodiplostomidae is a relatively small group of diplostomoids found exclusively in reptiles and not in birds and mammals, as are the other diplostomoids. The following is a diagnosis of the family according to Niewiadomska (2002e): "Body more or less distinctly bipartite; forebody flattened, with or without pseudosuckers; hindbody cylindrical, claviform or conical, sometimes with thick walled capsule or series of suckers. Holdfast organ variable in size, may be provided with papillae. Parasites exclusively in reptiles. *Type-genus*: *Proterodiplostomum* Dubois, 1936". Members of the family are mainly characterised by the presence of a paraprostatic gland or paraprostate, an independent organ with the shape of a thin- or thick-walled tubule or pouch, surrounded by gland-cells; species are included in 17 genera (Niewiadomska, 2002e). The classification of the Proterodiplostomidae was first elaborated by Dubois (1936) and is mainly based on host-specificity, holdfast organ shape and size, the presence or absence of papillae, and the distribution of the vitellarium. Apparently, the classification of the family remained unchanged until 1970, when Dubois presented a taxonomic revision (Dubois, 1970); two main groups were separated, with species parasitising crocodiles and chelonians in one and those in snakes in the other. The first attempt to establish the systematic position of the family in reference to a phylogeny-based classification was made by Brooks et al. (1985) through a morphological cladistic analysis of the Digenea, where the so-called "Superfamily Strigeoidea Railliet, 1919" was comprised of the Liolopidae, Cyathocotylidae (including Brauninidae), Diplostomidae (including Proterodiplostomidae) and Strigeidae. Based on this classification scheme, Shoop (1989) used Proterodiplostomidae as the outgroup in a systematic analysis of the Diplostomidae and Strigeidae. Brooks et al. (1992) proposed a cladistic classification of the genera of the Proterodiplostomidae based on the arrangement of the terminal genitalia, particularly the way the uterus and paraprostate open into the genital pore, confirming the previously recognised division into two groups.

The proterodiplostomids are morphologically very similar and share several morphological synapomorphies with strigeids and diplostomids. As a result, their phylogenetic position as the sister clade of these two groups of diplostomoids, as discovered in molecular phylogenetic analysis in our study, is not an unexpected result. Likewise, our molecular analyses, including DNA sequences for a proterodiplostomid for the first time, provide the empirical test of the position of the family within the phylogeny of the superfamily Diplostomoidea. The proterodiplostomids and the ancestor of diplostomids and strigeids experienced a diversification process in different groups of tetrapods, with the former diversifying in reptiles and the latter diversifying in birds and mammals. The presence of a paraprostate in species of the Proterodiplostomatidae can be then regarded as a morphological autapomorphy of the family (Shoop, 1989). Although the proterodiplostomid used in the phylogenetic analyses, *C. pseudostoma*, was found parasitising the body cavity of a siluriform freshwater fish, *Rhamdia guatemalensis* (Günther), in Catemaco Lake, Veracruz, Mexico, as a progenetic metacercaria (Pérez-Ponce de León et al., 1992), the first record of the species was made by Caballero (1948) as a parasite of the intestine of *Crocodylus moreletti* (Dumeril & Bibron) from the same locality. Actually, *C. pseudostoma* was originally recorded as a parasite of *Alligator mississippiensis* (Daudin) in the USA (Willemoes-Suhm, 1871). Species of *Crocodilicola*, like many other proterodiplostomids, are considered to represent parasites of the digestive tract of alligators and crocodiles in the Americas (Conroy, 1986). However, progenetic metacercariae of *C. pseudostoma* have also been recorded in another three species of siluriforms in Brazil: Conroy (1986) found *C. pseudostoma* in *Rhamdia quelen* (Quoy & Gaimard, 1824) [as *Rhamdia hilarii* (Valenciennes)] in the State of Sao Paulo; Guidelli et al. (2003) found it in *Hemisorubim platyrhynchus* (Valenciennes) in the upper Paraná River floodplain; and Ferrari-Hoeinghaus et al. (2007) found this species in *Loricariichthys platymetopon* Isbrücker & Nijssen, 1979, also in the Paraná River floodplain.

Phylogenetic relationships within the Diplostomoidea

Shoop (1989) conducted a phylogenetic analysis of the Diplostomidae and Strigeidae based on morphology and concluded that the classification of the Diplostomoidea at the time did not reflect the real evolutionary relationships and needs to be reconstructed with reference to an accumulation of data from different

sources. More recently, Niewiadomska (2002a) pointed out that patterns of host specificity of adults and some morphological traits such as the structure and shape of the forebody and holdfast organ, the distribution of the vitellarium (in whole body, forebody or hindbody), the presence or absence of bi-segmentation of the body, the presence or absence of a cirrus-sac or paraprostate and the structure of the copulatory apparatus, are commonly used for the general division of the Diplostomoidea. Based on the topology of the phylogenetic trees from our analyses, we present further comments on five particular groups belonging to the order Diplostomida: Cyathocotylidae, Brauninidae, Liolopidae, Diplostomidae and Strigeidae.

Cyathocotylidae. Members of the Diplostomoidea possess an atypical copulatory apparatus and terminal genitalia instead of a cirrus-sac and cirrus (Niewiadomska, 2002a). However, members of the family Cyathocotylidae possess a body generally undivided and a cirrus-sac, but they also have a holdfast organ and a terminal genital pore (Niewiadomska, 2002d). Cyathocotylids (and brauninids) thus have characteristics both of diplostomoids and other digeneans (Niewiadomska, 2002c, d). As adults, cyathocotylids are parasites of reptiles, birds and mammals, while the metacercariae are found in fishes, amphibians and aquatic invertebrates (Niewiadomska, 2002d). In our phylogenetic analyses based on the nuclear genes analysed either separately or concatenated, the Cyathocotylidae and Brauninidae are nested together and represent the sister group of the remaining diplostomoids, i.e. the Proterodiplostomidae, Diplostomidae and Strigeidae (Figs. 1, 2). It is plausible, then, to postulate that the holdfast organ and the terminal genital pore evolved in the ancestor of all diplostomoids. This ancestor possessed cirrus-sac and an undivided body. Both, an atypical cirrus-sac, and body bi-segmentation appeared in the ancestor of the Proterodiplostomidae plus Strigeidae and Diplostomidae. Interestingly, the topology of the trees obtained in the *cox*1 analyses showed a different position of the sequenced cyathocotylids, i.e. *Mesostephanus microbursa* from the blue-footed booby *Sula neuboxii* Milne-Edwards from Mexico, and *Mesostephanus* sp. (metacercariae) from the pumpkinseed *Lepomis gibbosus* (L.) from Canada. In the *cox*1 analyses, the two cyathocotylids are closely related to the Schistosomatoidea and not to the Diplostomoidea, with which they share a close morphological resemblance. This unexpected relationship is probably due to insufficient sampling for that molecular marker, the high nucleotide substitution rate of *cox*1, and/or the lack of *cox*1 sequences for *Braunina cordiformis*. The fact that *cox*1 sequences may not resolve suprageneric relationships cannot be ruled out.

Brauninidae. The monotypic Brauninidae (type-species: *Braunina cordiformis*) is characterised by an uncommon host association, since the type-species is found in cetaceans in seas across the globe (Niewiadomska, 2002c). The taxonomic relationships of this species with other digeneans was first demonstrated by Fraija-Fernández et al. (2015) in a phylogenetic analysis of nuclear DNA sequences (18S and 28S), where samples of *B. cordiformis* from the short beaked common dolphin *Delphinus delphis* L. from Argentina were included in a phylogenetic analysis of 177 taxa representing the broad diversity of the subclass Digenea. Within the order Diplostomida, the species nested as the sister clade of the Strigeidae + Diplostomidae, and this relationship was highly supported by bootstrap and posterior probability values (Fraija-Fernández et al., 2015). However, although these authors included representative sequences of other members of the order Diplostomida, no sequences of cyathocotylids were considered in their analyses. Our phylogenetic trees generated from nuclear gene datasets showed that *B. cordiformis* is grouped with the Cyathocotylidae. Still, the host association (in cetaceans, fish-eating), the body shape (cordiform), the presence of a cirrus-sac but the lack of oral and ventral suckers are considered autapomorphies of the family.

Liolopidae. The taxonomic placement of the family Liolopidae has been controversial, and it has been assigned to the Clinostomidae, Brachylaimidae and Harmostomidae Braun, 1900 (see Niewiadomska, 2002g, and references therein). Brooks & Overstreet (1978) considered the family Liolopidae valid, with 12 species described, one parasite of amphibians and 11 parasitising reptiles, including crocodiles, freshwater and marine snakes, lizards and turtles. Kanev et al. (2002) considered the Liolopidae a valid family within the Clinostomoidea Lühe, 1901 on the basis of adult morphology only, but this superfamily was not accepted by Olson et al. (2003). The family Liolopidae

currently comprises four genera with species parasitic in amphibians and reptiles, and they have, as primary morphological characteristics, an intertesticular ovary, a uterus mostly posterior to terminal genitalia, and a 'strigeid-like' excretory system (Niewiadomska, 2002g). Moreover, they actually possess a tegument covered with minute and slender spines (see redescription of *L. copulans* in Baba et al., 2011). Baba et al. (2011) studied the life-cycle of *L. copulans*, a parasite of the salamander *Andrias japonicus* (Temmink) in Japan, and provided the first molecular evidence, using sequences of the 18S and 28S rRNA genes, of the systematic position of the family within the order Diplostomida. Their molecular phylogenetic analyses suggested that *L. copulans* could be one of the basal members of the order, but they also found inconsistent results between the 18S and 28S trees. The topology of their trees based on 28S placed *L. copulans* as the sister group of Brachylaimoidea + Schistosomatoidea, with Diplostomoidea as their sister group. Instead, the topology of the 18S tree placed *L. copulans* as the basal group of all members of the order Diplostomida. Our phylogenetic analysis, based on an expanded dataset for both 18S and 28S rRNA genes, showed a concordant placement in the trees (Fig. 1A, B). In both cases, *L. copulans* was the basal group of all of the Schistosomatoidea (including the Aporocotylidae, Clinostomidae, Spirorchiidae and Schistosomatidae), and all of them appeared as the sister group of a clade formed by the Brachylaimoidea. Interestingly, the analysis of the concatenated 18S and 28S datasets yielded a topology that we cannot explain at the moment, where *L. copulans* appears as the basal member of the entire superfamily Diplostomoidea, albeit with very low bootstrap and posterior probability support values (Fig. 2). We tentatively consider Liolopidae as *incertae sedis* because tree topology shows they should be considered a member of the Diplostomoidea, but the relationships are unresolved. Baba et al. (2011) concluded that *L. copulans* belongs in the order Diplostomida, but its phylogenetic position remained unclear. Tentatively, these authors placed the family Liolopidae within the superfamily Diplostomoidea on morphological grounds. It is worth noting that no phylogenetic analysis has shown that the family Liolopidae is closely related to the Clinostomidae, as previously considered (see Kanev et al., 2002). Despite the morphological resemblance of these two families, particularly in the intertesticular position of the ovary and the fact that metacercariae are found in fishes, Baba et al. (2011) showed that cercarial morphology is very different. In *L. copulans*, the cercaria is of the non-oculate longifurcate pharyngeate type, while the cercaria of the species of the Clinostomatidae is of the oculate brevifurcate apharyngeate type. Baba et al. (2011) concluded that to treat both families as members of the same superfamily was untenable, and the results of our phylogenetic analyses provide further support to this idea. Further, these authors suggested that adults of *L. copulans* shared some morphological features with the members of the Cyathocotylidae, e.g. the body is not distinctly divided into two segments, the seminal vesicle is enclosed in a cirrus-sac, and the ovary is intertesticular. However, they also recognised several differences, such as the lack of a holdfast organ and the pretesticular position of the cirrus-sac in *L. copulans*. Additionally, the position of the ovary in Cyathocotylidae is quite variable, and in some species, the ovary is pretesticular (Niewiadomska, 2002d). DNA sequences of other species of liolopids are necessary to infer their true phylogenetic relationships with other diplostomoids.

Diplostomidae. The case of the paraphyly of the Strigeidae and Diplostomidae is noteworthy and requires further studies where a denser sampling is conducted before proposing the required nomenclatural changes. Shoop (1989) showed in his morphological phylogenetic analysis that the Diplostomidae was a paraphyletic assemblage and considered the Strigeidae as monophyletic. The paraphyletic relationships between these two families was also recognised by Olson et al. (2003) and Kostadinova & Pérez-del-Olmo (2014), since both families were represented by a single branch as Diplostomidae + Strigeidae. Our phylogenetic analyses using nuclear DNA sequences showed that at least two groups of apparently unrelated members of the Diplostomidae are formed, with some apparent subgroupings, but these groups are in conflict when each nuclear gene is analysed separately, probably because the same genera are not represented in both datasets. The following interpretation is then based on the analysis of concatenated data (Fig. 2). Of the two groups, one includes the genera *Tylodelphys* Diesing, 1850, *Diplostomum* Nordmann, 1832 and *Austrodiplostomum* Szidat & Nani, 1951, *Alaria* Schrank, 1788; the second group includes *Posthodiplostomum* Dubois, 1936 and

Uvulifer Yamaguti, 1934 + *Hysteromorpha* Lutz, 1931. Instead, the tree obtained with the *cox*1 sequences showed that three unrelated groups are formed (Fig. 3), one containing *Diplostomum, Tylodelphys* and *Austrodiplostomum*; one containing *Fibricola* Dubois, 1932, *Hysteromorpha* and *Alaria*; and the other containing *Posthodiplostomum* and *Ornithodiplostomum* Dubois, 1936 + *Bolbophorus* Dubois, 1935. The placement of these genera in the phylogenetic tree is in conflict with the traditional classification of the Diplostomidae, where two groups are recognised according to host associations: Alariinae Hall & Wigdor, 1918 in mammals and Diplostominae Poirier, 1886 in birds (Dubois, 1936). Niewiadomska (2002b) recognised the division of Diplostomidae according to host specificity and metacercarial types into four subfamilies: one in mammals, the Alariinae, and three in birds, the Diplostominae, Crassiphialinae Sudarikov, 1960 and Codonocephalinae Sudarikov, 1959. This classification is almost concordant with the ML and BI *cox*1 phylogenetic trees obtained in this study, except for the conflicting placement of *Hysteromorpha*. The monotypic Codonocephalinae, whose members are parasitic in ardeids in the Palaearctic, is not represented in the phylogenetic tree, since no sequences are yet available. If this phylogeny is accurate, pending the inclusion of other representatives of the family Diplostomidae, the subfamily Crassiphalinae could be elevated to family level to include *Posthodiplostomum*, *Uvulifer*, *Ornithodiplostomum* and *Bolbophorus* (and possibly at least 11 other genera possessing a 'neascus' type of metacercariae and the vitellarium restricted to the hindbody). This nomenclatural change necessarily requires the inclusion of sequences of the type-species of the type-genus, *Crassiphiala bulboglosa* Van Haitsma, 1925, which is found, as an adult, in the intestine of alcedines in North America (Yamaguti, 1971; Niewiadomska, 2002b). The Alariinae could be also elevated to the family level to include *Alaria* and *Fibricola*, but this also requires more sequence data. The inclusion of *Hysteromorpha triloba* in this family is in conflict with the fact that the species shares all characteristics of species of the Diplostomidae, such as vitellarium in both fore- and hindbody, pseudosuckers, and a 'diplostomulum' type of metacercaria. The position of this species is ambiguous in trees obtained with each dataset analysed separately. The concatenated tree of two nuclear genes (18S and 28S, Fig. 2) actually shows a conflicting position of *H. triloba*, since it was resolved as the sister taxon of either *Diplostomum*, *Posthodiplostomum* or *Uvulifer* + *Posthodiplostomum*. In this case, it is also premature to propose nomenclatural changes, since classification is still unstable.

Strigeidae. In the case of the Strigeidae, a family that includes a distinct group of species with a characteristic cup-shaped forebody and a holdfast organ in the form of two (ventral and dorsal) lobes (Niewiadomska, 2002f), two main groups were consistently formed, one including species of *Ichthyocotylurus*, *Cardiocephaloides* (but also *Cotylurus* and *Nematostrigea*), and a second group that includes representatives of *Apatemon* + *Australapatemon* and *Strigea* + *Parastrigea* + *Apharyngostrigea*. These groupings are congruent with those obtained in previous studies where mitochondrial and ribosomal markers were used (Hernández-Mena et al., 2014; Blasco-Costa et al., 2016a). In our molecular phylogenetic analyses based on the nuclear genes, these two groups were not closely related. However, in the *cox*1 analysis, the two clades of the Strigeidae are grouped together, albeit with very low bootstrap and posterior probability support values (18/0.65). Both clades are included within a paraphyletic Diplostomidae (Fig. 3). The original classification by Dubois (1938) separated the family Strigeidae into two subfamilies, the Strigeinae Railliet, 1919 and Duboisiellinae Baer, 1938, according to their host group, i.e. birds and mammals, respectively, with two tribes (Strigeini and Cotylurini) within the Strigeinae (see Niewiadomska, 2002f, for further details about the taxonomic history). No sequences are yet available for the monotypic *Duboisiella* Baer, 1938, occurring in marsupial mammals in the Neotropical region. Therefore, the traditional classification of the Strigeidae cannot be tested at present. Considering the conflict in the topology of the trees obtained in the present study, we conclude that it would be premature to propose any nomenclatural changes, because the phylogenetic position of dipolostomids and strigeids is still unclear. More sequencing work, and the inclusion of other taxa of the Diplostomidae and Strigeidae, will be necessary to accomplish this task and obtain a stable classification

of both families within the superfamily Diplostomatoidea.

Final considerations

To conclude, Cribb et al. (2003) noted that the classification of Digenea was evolving quite rapidly. Interestingly, 15 years later, the classification is still evolving as a result of the development of molecular tools and methodological approaches, as well as the increase in taxon sampling, even though some conflict has been found between the molecular phylogenetic classifications and that followed in the two major taxonomic treatments of trematodes, i.e. *Synopsis of Digenetic Trematodes of Vertebrates* (Yamaguti, 1971) and the *Keys to the Trematoda* (Gibson et al., 2002; Jones et al., 2005; Bray et al., 2008). These bibliographical sources remain the cornerstones for trematode identification. Still, a thorough sampling is required to expand the representation of species belonging to different families and superfamilies. Strigeids and diplostomids are just one example of the lack of representation needed to reconstruct the phylogenetic relationships upon which a stable classification can be assessed.

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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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