



# Phylogenetic position of *Diplostomum* spp. from New World herons based on complete mitogenomes, rDNA operons, and DNA barcodes, including a new species with partially elucidated life cycle

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## Abstract

*Diplostomum ardeae* Dubois, 1969 has seldom been reported since its description from the great blue heron (*Ardea herodias* L., 1758) in the USA. Sequences obtained in this study from the barcode region of cytochrome *c* oxidase 1 (CO1) in diplostomids from black-crowned night heron (*Nycticorax nycticorax* (L., 1758)) in Puerto Rico matched data from *D. ardeae* from *A. herodias* in the type region. We also obtained DNA barcodes from morphologically similar diplostomids from a rufescent tiger heron (*Tigrisoma lineatum* (Boddaert, 1783)) and from metacercariae from eye lenses of *Trachelyopterus galeatus* (Linnaeus, 1766) from the Paraná River basin in Argentina and Brazil, respectively. Barcodes matched (97–100% identity) in these South American adult and larval specimens as well as in recently published sequences from metacercariae from 11 other siluriform fishes from the same region. Barcodes from the South American species, which we describe as *Diplostomum lunaschiae* n. sp., differed from those of *D. ardeae* by 7.2–9.8%, and the new species differs from *D. ardeae* in its size, pharynx:oral sucker length ratio, egg:body length ratio, and distribution of vitellaria. As in prior phylogenetic analysis of CO1 sequences, both *D. ardeae* and *D. lunaschiae* n. sp. were not associated with *Diplostomum*. In more character-rich analyses of nuclear rDNA and of mitochondrial genomes, *D. ardeae* was an early divergent member of clades of species of *Diplostomum*. Consequently, we continue to consider *D. ardeae* and *D. lunaschiae* n. sp. members of *Diplostomum*, in contrast to recent suggestions that these species may belong to a different genus.

**Keywords** Eye fluke · Catfish · Neotropic · Diplostomoidea · Phylogeny · Genomics

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## Introduction

Dubois (1969) described *Diplostomum ardeae* from three specimens from a great blue heron (*Ardea herodias* L., 1758, Ardeidae, Pelecaniformes) collected in Hampden County, Massachusetts. El-Naffar et al. (1980) used the pre-occupied name of *D. ardeae* to describe a species from *Ardea goliath* Cretzschmar, 1829 in Egypt, but this species bears little resemblance to *D. ardeae* Dubois, 1969, differing markedly in the morphology of eggs, prepharynx, pseudosuckers, and relative lengths of the fore- and hindbody. *Diplostomum ardeae* was next reported from 3 of 13 *Egretta caerulea* (L., 1758) (Ardeidae) examined by Dronen and Chen 2002 in coastal Texas, about 2500 km southeast of the type locality. Locke et al. (2015) published a partial sequence of cytochrome *c* oxidase I (CO1) from *D. ardeae* from *A. herodias* collected approximately 400 km northwest of the type locality. Pelegrini et al. (2019) sequenced the same (barcode) region

of CO1 in metacercariae resembling the adult form of *D. ardeae* from the eye lenses of 11 species in three families of siluriform fishes in Brazil. The CO1 sequences of these larval parasites diverged by 6.9–7.6% from *D. ardeae*, leading Pelegrini et al. (2019) to conclude they belong to a species closely related to *D. ardeae*. Phylogenetic analyses of CO1 sequences suggest both the Brazilian species and *D. ardeae* are not allied with *Diplostomum* or other diplostomid genera from which data are available (Locke et al. 2015; Hernández-Mena et al. 2017; Pelegrini et al. 2019). Pelegrini et al. (2019) also noted that the metacercariae they examined presented morphological inconsistencies with species of *Diplostomum*, particularly the relative lengths of the fore- and hindbody and the distribution of vitellaria. We also note that most species of *Diplostomum* mature in members of the Charadriiformes, rather than peleciform hosts such as ardeids (Dubois 1970; Dubois and Angel 1972).

In this study, we conducted additional molecular analysis to further evaluate the generic affiliation of *D. ardeae* and we report additional sequence-based records that enlarge the geographic and host range of this species. We describe the adult form of the species Pelegrini et al. (2019) encountered as metacercariae and report additional records of its larval forms.

## Materials and methods

### Specimen collection and identification

Immature adult diplostomids were collected from the small intestine of a road-killed black-crowned night heron (*Nycticorax nycticorax* (L., 1758)) found near Yauco, Puerto Rico (18.0131, –66.8963) in June, 2017. Mature adult diplostomids were collected from the small intestine of a rufescent tiger heron (*Tigrisoma lineatum* (Boddaert, 1783)) shot in 2016 at Marcela Farm, Formosa Province, Argentina (–26.2930, –59.1439) with authorization of the Ministerio de la Producción, Dirección de Fauna y Parques of Formosa. Specimens were preserved in 95% ethanol, later rehydrated, stained in dilute acetocarmine, dehydrated in ethanol, cleared in clove oil or xylol, and mounted on a slide in Canada balsam, and studied and drawn with the aid of an ocular micrometer and camera lucida. Two paragenophores of a specimen of *D. ardeae* from *A. herodias* in Montreal, Quebec, in which CO1 was sequenced by Locke et al. (2015) (KR271033), were also studied morphologically and are newly deposited in the Museum of Southwestern Biology (MSB:Para: 30692). Metacercariae were collected from the eyes of 837 fish in 56 species caught with nets at several localities (Ivinhema, Baía, and Paraná Rivers) in the upper Paraná River floodplain along the borders of states Paraná and Mato Grosso do Sul (–22.8230, –53.4378), Brazil, in

June, 2011 (Table 1). Fish were identified according to Graça and Pavanelli (2007) and sacrificed by spinal section with the authorization of the Ethics Council of the State University of Maringá (CEAE - Opinion 123/2010). In specimens of *Trachelyopterus galeatus* (Linnaeus, 1766), one eye was fixed in 10% formalin, embedded in paraffin, and sectioned with a microtome into 5- $\mu$ m slices that were stained with hematoxylin and eosin and photographed on slides with phase contrast using a Nikon Eclipse E200 microscope coupled to a Samsung DV100 camera. Parasites in the other eye of the same individual *T. galeatus* were preserved in 70% ethanol and refrigerated in 95% ethanol until DNA extraction.

### DNA extraction, PCR amplification, and sequencing

DNA extracted from individual worms (or worm subsamples) was analyzed with the aim of determining whether the samples collected in different localities and hosts represented the same species, and whether they could be placed in a known genus. Barcode sequences of CO1 were obtained using the extraction methods, diplostomid-specific primers, and protocols of Moszczyńska et al. (2009). DNA from a specimen of *D. ardeae* from *N. nycticorax* from Puerto Rico was shotgun sequenced in a tenth of a lane on an Illumina HiSeq 4000, and 150-bp paired-end libraries were built with Nextera adapters at Genewiz (NJ). To assemble Illumina reads into a mitochondrial genome, data from *D. spathaceum* (Rudolphi, 1819) (KR269763) were used as a scaffold in Geneious Prime 2019 ([www.geneious.com](http://www.geneious.com)) using default parameters. The longest resulting fragment assembled with good coverage (3407 bp,  $\geq 183$  reads per site) was then used to seed iterative assemblies from the total read pool, extending to the whole mitochondrion, and the final assembly was annotated using MITOS (Bernt et al. 2013) and by alignment with *D. spathaceum* (KR269763) and *D. pseudospathaceum* Niewiadomska, 1984 (KR269764). The rDNA operon was assembled iteratively using as an initial scaffold a consensus from an alignment of sequences from *Diplostomum* spp. (KR269765-6) and *Tylodelphys immer* Dubois, 1961 (MH521252) with default parameters in Geneious.

Sequences were aligned with representative, published data from diplostomids, including data from Pelegrini et al. (2019). Because of substantial saturation (ISS = 0.573, ISS.cAsym = 0.516, T = 1.198, DF = 115,  $P = 0.23$ ; Xia et al. 2003, Xia and Lemey 2009), the third codon was removed from the phylogenetic analysis of CO1. In the CO1 phylogeny and others (see below), alignments were stripped of gaps and redundant sequences, and models of nucleotide evolution were selected using Bayesian Information Criterion in MEGA-X (Kumar et al. 2018). Genetic distances (uncorrected p) are reported based on all sites and sequences. Phylogenetic trees were constructed with 1000 bootstrap replicates with RAXML (Silvestro and Michalak 2012; Stamatakis 2014) and using

**Table 1** Fish examined in Paraná River floodplain (Paraná and Mato Grosso do Sul, Brazil)

Fish host	N examined
Characiformes	
Acestrorhynchidae	
<i>Acestrorhynchus lacustris</i> (Lütken, 1875)	37
Anostomidae	
<i>Leporinus elongatus</i> Valenciennes, 1850	3
<i>Leporinus friderici</i> (Bloch, 1794)	9
<i>Leporinus lacustris</i> Campos, 1945	30
<i>Leporinus macrocephalus</i> Garavello and Britski, 1987	5
<i>Leporinus obtusidens</i> (Valenciennes, 1836)	31
<i>Schizodon borellii</i> (Boulenger, 1900)	41
<i>Schizodon nasutus</i> Kner, 1858	2
Characidae	
<i>Aphyocharax dentatus</i> Eigenmann and Kennedy, 1903	1
<i>Astyanax altiparanae</i> Garutti and Britski, 2000	34
<i>Brycon orbignyanus</i> (Valenciennes, 1850)	1
<i>Moenkhausia aff intermedia</i> Eigenmann, 1908	2
<i>Piaractus mesopotamicus</i> (Holmberg, 1903)	6
<i>Psellogrammus kennedyi</i> (Eigenmann, 1903)	1
<i>Roebooides descavadensis</i> fowler, 1932	29
<i>Salminus brasiliensis</i> (Cuvier, 1816)	13
<i>Serrasalmus maculatus</i> Kner, 1858	12
<i>Serrasalmus marginatus</i> Valenciennes, 1837	29
Curimatidae	
<i>Cyphocharax nagelii</i> (Steindachner, 1881)	1
<i>Steindachnerina brevipinna</i> (Eigenmann and Eigenmann, 1889)	4
<i>Steindachnerina insculpta</i> (Fernández-Yépez, 1948)	20
Gymnotiformes	
Erythrinidae	
<i>Hoplerythrinus unitaeniatus</i> (Agassiz, 1829)	7
<i>Hoplias aff malabaricus</i> (Block, 1794)	28
Hemiodontidae	
<i>Hemiodus orthonops</i> Eigenmann and Kennedy, 1903	21
Parodontidae	
<i>Apareiodon affinis</i> (Steindachner, 1879)	1
Prochilodontidae	
<i>Prochilodus lineatus</i> (Valenciennes, 1836)	42
Gymnotidae	
<i>Gymnotus inaequilabiatus</i> (Valenciennes, 1839)	3
Rhamphichthyidae	
<i>Rhamphichthys hahni</i> (Meiken, 1937)	2
Stemopygidae	
<i>Eigenmannia trilineata</i> (López and Castello, 1966)	4
Perciformes	
Cichlidae	
<i>Astronotus crassipinnis</i> (Heckel, 1840)	54
<i>Cichla kelberi</i> Kullander and Ferreira, 2006	37
<i>Cichla</i> sp.	2
<i>Crenicichla</i>	
<i>Crenicichla britskii</i> Kullander, 1982	1
<i>Geophagus</i>	
<i>Geophagus cf. proximus</i> (Castelnau, 1855)	14
<i>Satanoperca</i>	
<i>Satanoperca pappaterra</i> (Heckel, 1840)	1
Sciaenidae	
<i>Plagioscion</i>	
<i>Plagioscion squamosissimus</i> (Heckel, 1840)	6
Siluriformes	
Auchenipteridae	
<i>Ageneiosus inermis</i> (Linnaeus, 1766)	1
<i>Auchenipterus osteomystax</i> (Miranda-Ribeiro, 1918)	9
<i>Trachelyopterus galeatus</i> (Linnaeus, 1766)	26

**Table 1** (continued)

Fish host	N examined
Callichthyidae	
<i>Hoplosternum littorale</i> (Hancock, 1828)	5
<i>Leptoplosternum pectorale</i> (Boulenger, 1895)	1
Clariidae	
<i>Clarias gariepinus</i> (Burchell, 1822)	2
Doradidae	
<i>Pterodoras granulosus</i> (Valenciennes, 1821)	20
<i>Trachydoras paraguayensis</i> (Eigenmann and Ward, 1907)	18
Heptapteridae	
<i>Pimelodella avanhandavae</i> Eigenmann, 1917	1
Loricariidae	
<i>Hypostomus aff hermanni</i> (Ihering, 1905)	1
<i>Loricariichthys platymetopon</i> Isbrücker & Nijssen, 1979	34
<i>Pterygoplichthys ambrosettii</i> (Holmberg, 1893)	106
<i>Rhinelepis aspera</i> Spix and Agassiz, 1829	4
Pimelodidae	
<i>Hemisorubim platyrhynchos</i> (Valenciennes, 1840)	3
<i>Iheringichthys labrosus</i> Lütken, 1874	9
<i>Pimelodus maculatus</i> La Cepède, 1803	22
<i>Pimelodus mysteriosus</i> Azpelicueta, 1998	4
<i>Pimelodus ornatus</i> Kner, 1858	1
<i>Pseudoplatystoma corruscans</i> (Spix and Agassiz, 1829)	11
<i>Sorubim lima</i> (Bloch and Schneider, 1801)	25

Bayesian Inference (Huelsenbeck and Ronquist 2001) implemented in Geneious, the latter with four chains of Markov chain Monte Carlo searches sampled every 200 and printed every 1000 generations with 1,100,000 generations and 500 initial trees discarded, yielding posterior probabilities based on 11,002 topologies.

## Results

### Phylogenetic analysis

Three CO1 sequences from immature adult diplostomids from *N. nycticorax* in Puerto Rico (two Sanger and one Illumina sequence) matched KR271033, i.e., that of *D. ardeae* from *A. herodias* in Quebec (mean 99.6%, range 99.5–100% identity over at least 518 base pairs within *D. ardeae*).

The CO1 sequences from four adult diplostomids from *T. lineatum* in Formosa, Argentina, and from 28 metacercariae from eyes of nine *T. galeatus* from Paraná and Mato Grosso do Sul, Brazil, matched two sequences Pelegrini et al. (2019) reported from metacercariae from the eyes of siluriform fish in São Paulo State, Brazil (mean 98.9%, range 97.4–100% identity over at least 420 base pairs). All the foregoing collections were from the Paraná River basin. CO1 sequences from *D. ardeae* and the specimens from South America differed by mean 8.7%, range 7.2–9.8% over at least 420 base pairs of CO1. In phylogenetic analysis, the CO1 sequences from Argentina and Brazil and those from Puerto

Rico and Canada each formed well-supported clades, and both clades were placed in a larger clade containing *Tylodelphys* and *Austrodiplostomum* spp. (Fig. 1). *Diplostomum* was unresolved in this phylogenetic analysis of CO1.

The mitochondrial genome assembly of *D. ardeae* was 14,037 bp in length with mean coverage of 911 (range 542–1134) reads per site (Table 2). The most similar mitochondrial genomes available are those of *D. pseudospathaceum* (KR269764, 76.99% similarity) followed by *T. immer* (MH536513, 74.37%). The rDNA operon assembly of *D. ardeae* was 7744 bp in length with mean coverage of 16,747 (range 1169–27,936) reads per site. Its overall similarity was greatest with *Diplostomum* spp. (KR269765-6, 96.58–96.65%) followed by *Alaria americana* Hall and Wigdor, 1918 (MH521246, 94.61%), *T. immer* (MH521252, 94.31%), and *Hysteromorpha triloba* (Rudolphi, 1819) (MH521250, 93.74%). In phylogenetic analyses of nuclear

rDNA operons or portions thereof, and of mitochondrial genomes, *D. ardeae* was an early divergent member of clades of species of *Diplostomum* (Figs. 2, 3, and 4).

Classification: Platyhelminthes Claus, 1887, Trematoda Rudolphi, 1808, Digenea Carus, 1863, Diplostomida Olson, Cribb, Tkach, Bray, and Littlewood, 2003, Diplostomidae Poirier, 1886, *Diplostomum* von Nordmann, 1832.

*Diplostomum lunaschiae* n. sp.

Type host: *Tigrisoma lineatum* (Boddaert)

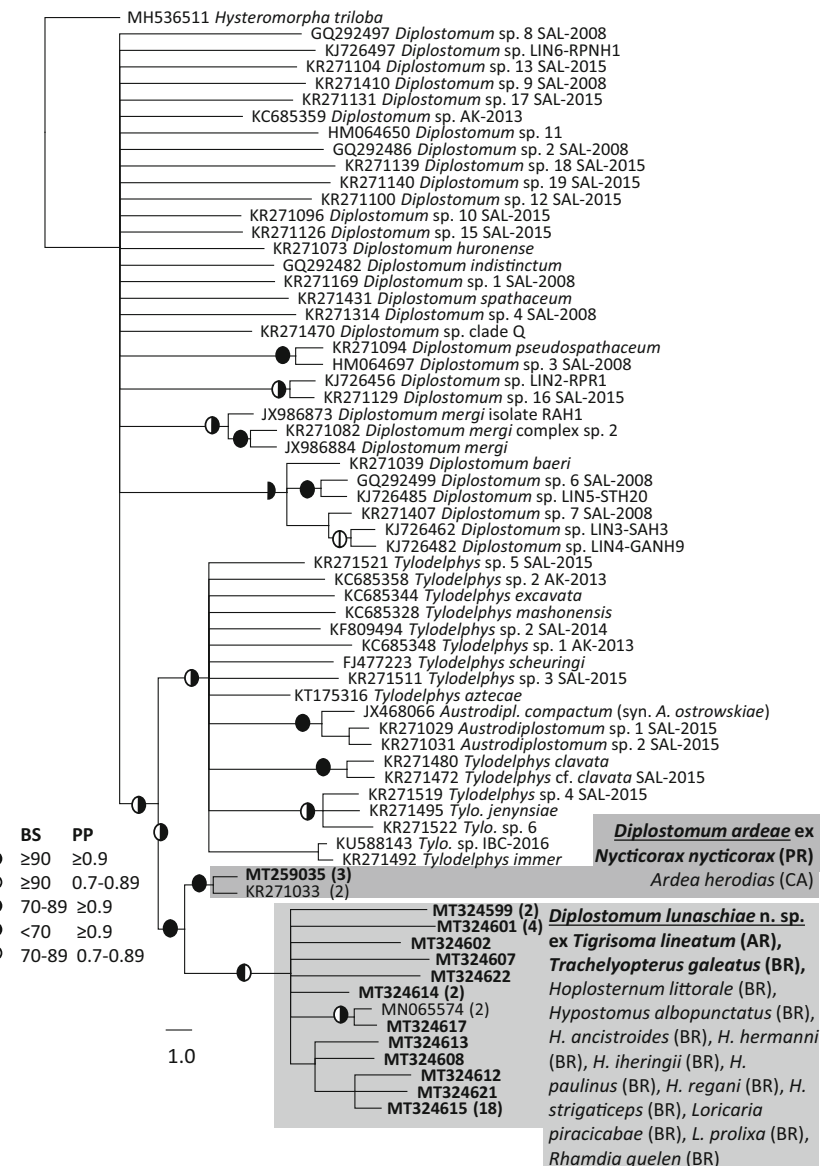
Type locality: La Marcela farm (–26.2931, –59.1439), Pirané, Formosa Province, Argentina.

Other localities: Upper Paraná River basin, Mato Grosso do Sul, Paraná, and São Paulo states, Brazil.

Site: Intestine.

Type material: Holotype and paratype: Museum of Southwestern Biology (MSB:Para: 30693). Other vouchers, Museo de La Plata (MLP-He 7656).

**Fig. 1** Phylogenetic analysis of partial sequences of cytochrome c oxidase I from *Diplostomum ardeae* and *D. lunaschiae* n. sp., with representatives of other members of the Diplostomidae. Nodes in the maximum likelihood topology are annotated with support in 1000 bootstrap replicates and with posterior probability from Bayesian Inference as indicated in the legend. The alignment was 231 bp long and included only first and second codon positions. Trees were generated with HKY+G models of nucleotide substitution (or near equivalents). Numbers of identical haplotypes indicated in parentheses. Hosts and country of origin are listed for sequences in clades in shaded boxes. Data from present study in bold





**Table 2** Mitochondrial genome of *Diplostomum ardeae* Dubois, 1969 from *Nycticorax nycticorax* (L., 1758) found near Yauco, Puerto Rico (GenBank accession MT259035). Terminal codons in parenthesis are completed by the addition of 3' A residues to the mRNA

	Type	Start	End	Length	Initial/ terminal codon
COX3	CDS	1	650	650	ATG/TA(A)
tRNA-His	tRNA	680	744	65	
CYTB	CDS	748	1875	1128	ATG/TAG
ND4L	CDS	1860	2123	264	ATG/TAG
ND4	CDS	2084	3379	1296	GTG/TAG
tRNA-Gln	tRNA	3384	3449	66	
tRNA-Phe	tRNA	3454	3514	61	
tRNA-Met	tRNA	3536	3603	68	
ATP6	CDS	3607	4125	519	ATG/TAG
ND2	CDS	4154	5042	889	ATG/T (AA)
tRNA-Val	tRNA	5043	5105	63	
tRNA-Ala	tRNA	5113	5182	70	
tRNA-Asp	tRNA	5187	5251	65	
ND1	CDS	5249	6146	898	ATG/T (AA)
tRNA-Pro	tRNA	6162	6225	64	
tRNA-Asn	tRNA	6247	6310	64	
tRNA-Ile	tRNA	6329	6398	70	
tRNA-Lys	tRNA	6400	6468	69	
ND3	CDS	6470	6826	357	ATG/TAG
tRNA-Ser	tRNA	6835	6893	59	
tRNA-Trp	tRNA	6897	6960	64	
COX1	CDS	7055	8611	1557	ATG/TAG
tRNA-Thr	tRNA	8658	8724	67	
Large subunit	rRNA	8713	9757	1045	
tRNA-Cys	tRNA	9712	9778	67	
Small subunit	rRNA	9776	10,508	733	
COX2	CDS	10,534	11,151	618	ATG/TAA
ND6	CDS	11,159	11,617	459	ATG/TAG
tRNA-Tyr	tRNA	11,625	11,690	66	
tRNA-Leu	tRNA	11,691	11,755	65	
tRNA-Ser	tRNA	11,756	11,822	67	
tRNA-Leu	tRNA	11,825	11,891	67	
tRNA-Arg	tRNA	11,915	11,985	71	
ND5	CDS	11,985	13,574	1589	GTG/TAG
tRNA-Glu	tRNA	13,579	13,653	75	
tRNA-Gly	tRNA	13,967	14,034	68	

Representative DNA sequences: MT324594-626

**Etymology:** The species is named after Lía Lunaschi, for her contributions to parasitology.

**Other hosts:** metacercariae in eye lenses of siluriform fishes, *Trachelyopterus galeatus*, *Hypostomus regani* (Ihering, 1905), *Hypostomus strigaticeps* (Regan, 1908), *Hypostomus hermanni* (Ihering, 1905), *Hypostomus iheringii* (Regan,

1908), *Hypostomus ancistroides* (Ihering, 1911), *Hypostomus albobunctatus* (Regan, 1908), *Hypostomus paulinus* (Regan, 1905), *Loricaria proluxa* (Isbrücker & Nijssen, 1978), *Loricaria piracicabae* (Ihering, 1907) (Loricariidae); *Rhamdia quelen* (Quoy & Gaimard, 1824) (Heptapteridae); *Hoplosternum littorale* (Hancock, 1828) (Callichthyidae).

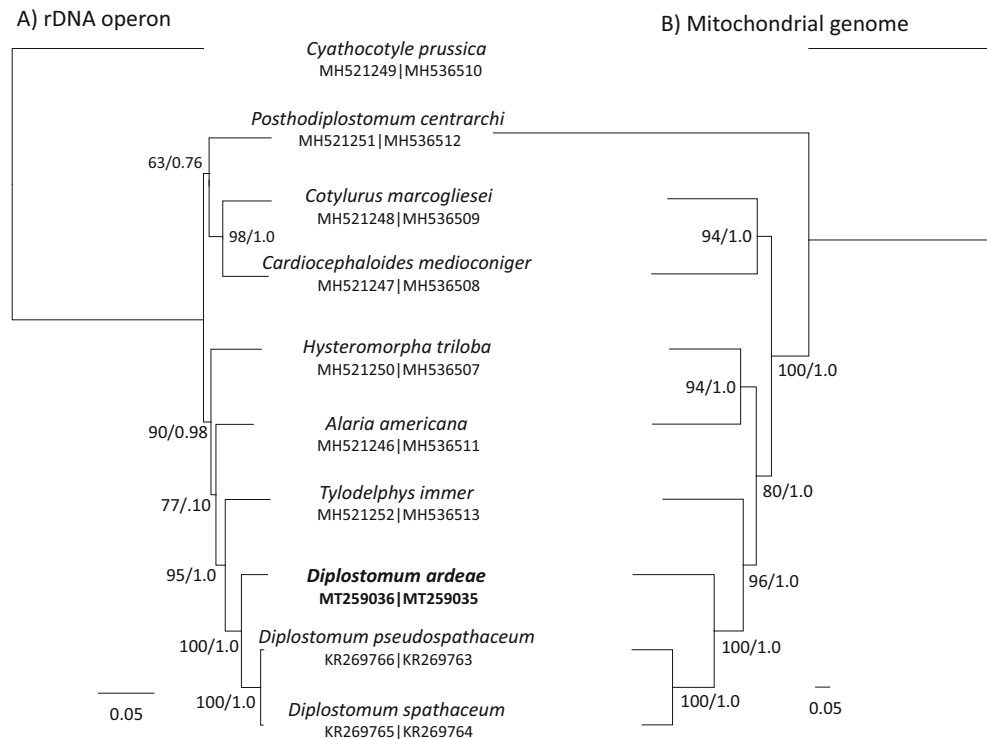
Description based on 11 mounted specimens from small intestine of *Tigrisoma lineatum* (Fig. 5, Table 3).

**Description:** Body fusiform, slightly bipartite. Anterior extremity trilobated, middle lobe well differentiated, trapezoidal in shape, occupied by oral sucker, and part of pseudosuckers. Forebody spatulate, hindbody conical. Forebody length 1.5 to 2.5 (mean 1.9) times hindbody length. Fine spines covering anterior part of forebody to intestinal bifurcation or ventral sucker. Oral sucker subterminal. Ventral sucker larger than oral sucker, located in middle third of a body, at 61–68% (mean 65%) of forebody length from anterior extremity. Pseudosuckers prominent, larger than suckers. Holdfast organ oval, almost immediately posterior to ventral sucker, located 66–77% (mean 73%) of forebody length from anterior extremity. Prepharynx long. Pharynx well developed, larger than oral sucker; esophagus short, intestinal caeca almost reaching posterior extremity. Testes tandem, anterior testis asymmetrical, cuneiform, posterior testis bilobed, with lobes directed forward. Seminal vesicle posterior to testes. Copulatory bursa not delimited, with genital pore terminal. Genital cone absent. Ovary median, ellipsoidal, pretesticular. Anterior margin of vitellarium 37–55% (mean 48%) of forebody length from anterior extremity. Vitellaria distributed in both parts of body, forming a narrow ventral band and bifurcating into two fields near posterior end of hindbody, co-extensive with intestinal caeca. Uterus with 2–4 large eggs. Excretory pore terminal. Vesicle not seen.

**Remarks:** *Diplostomum lunaschiae* n. sp. resembles *D. ardeae* in its possession of well-developed, chromatophorous pseudosuckers, its overall shape, including the strongly trilobed anterior extremity, indistinct constriction between and relative lengths of the forebody and hindbody, and in its unusually long prepharynx. However, in *D. lunaschiae* n. sp., body length, pseudosuckers, holdfast organ, and the esophagus are smaller than in *D. ardeae*. In *D. lunaschiae* n. sp., the pharynx is larger than the oral sucker (Ph/OS 1.3–1.5), but in *D. ardeae*, the pharynx is smaller than the oral sucker (Ph/OS 0.52–0.86). Eggs in *D. lunaschiae* n. sp. and *D. ardeae* are of similar size, but in the smaller bodied *D. lunaschiae* n. sp., the total length is only up to 10.8 times the egg length, while body lengths are 11–14 times egg lengths in *D. ardeae*. The vitellaria do not extend as far along the anterior axis of the forebody in *D. lunaschiae* n. sp. as in *D. ardeae* (37–55% versus 28% of forebody distance to anterior extremity).

*Diplostomum lunaschiae* n. sp. can be distinguished by its longer prepharynx from all species of *Diplostomum* except *D. ardeae*. In comparison to many species in the genus,

**Fig. 2** Phylogenetic analysis of (A) rDNA operons and (B) mitochondrial genomes from *Diplostomum ardeae* and other members of the Diplostomoidea. Nodes in the maximum likelihood topology are annotated with support in 1000 bootstrap replicates and with posterior probability from Bayesian Inference. Both trees were generated with a GTR+G+I model of nucleotide substitution. The analysis in (A) was based on 8402 ungapped sites in an 8509-bp alignment and in (B) on 12,881 ungapped sites in a 17,193-bp alignment



*D. lunaschiae* n. sp. has a relatively short hindbody (i.e., smaller hindbody/forebody length ratio). It can be differentiated from species of *Diplostomum* with similar hindbody/forebody length ratios (*D. amygdalum* Dubois and Pearson, 1965, *D. baeri baeri* Dubois, 1937, *D. baeri buculentum* Dubois and Rausch, 1948, *D. gavium* (Guberlet, 1922), *D. mergi alascense* Dubois, 1969, *D. mergi mergi* Dubois, 1932, *D. oediconemum* Singh, 1956, *D. phoxini* (Faust, 1918), *D. pusillum* (Dubois, 1928), *D. scudderi* (Olivier, 1941)) by (in most cases) its larger pseudosuckers or larger pharynx/oral sucker length ratio.

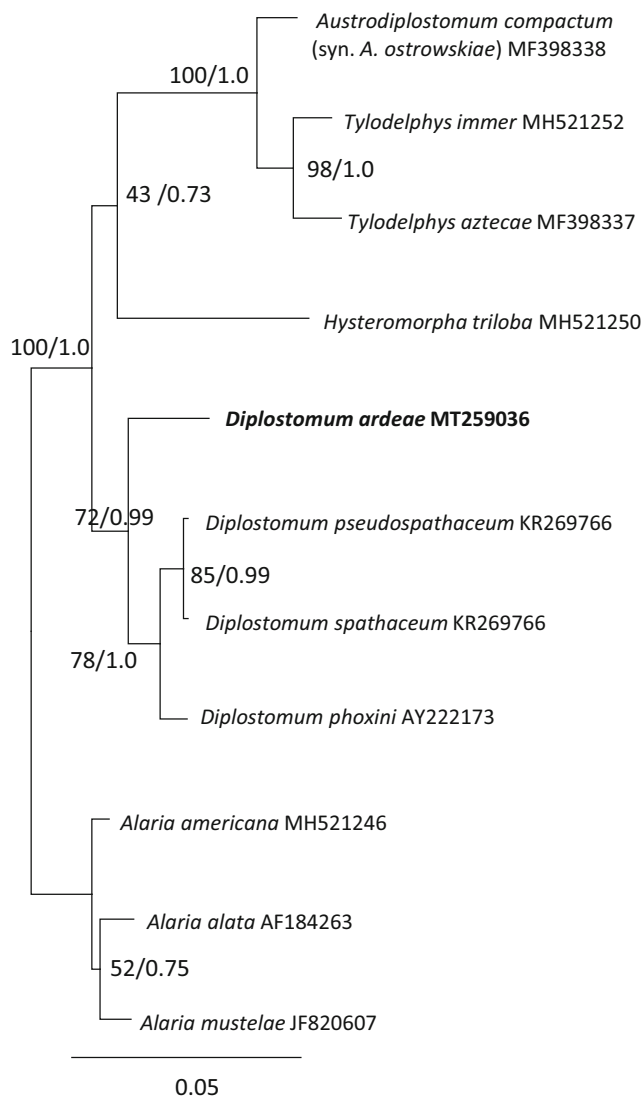
In the present work, metacercariae of *D. lunaschiae* n. sp. were found in the cortex of lenses of 9 of 26 *T. galeatus* examined (Fig. 6); the mean intensity of infection was 25.6 (s.d. = 21.8); mean abundance was 8.8. Fifty-five other fish species were negative for *D. lunaschiae* n. sp. (Table 1). See Pelegrini et al. (2019) for morphological characterization of metacercariae of *D. lunaschiae* n. sp.

## Discussion

As in prior studies (Locke et al. 2015; Hernández-Mena et al. 2017; Pelegrini et al. 2019), *D. ardeae* and *D. lunaschiae* n. sp. were not associated with *Diplostomum* in phylogenetic analysis of partial CO1 sequences, but *D. ardeae* was within a clade of *Diplostomum* spp. with moderate to strong support in all other analyses. We find no compelling non-molecular evidence for

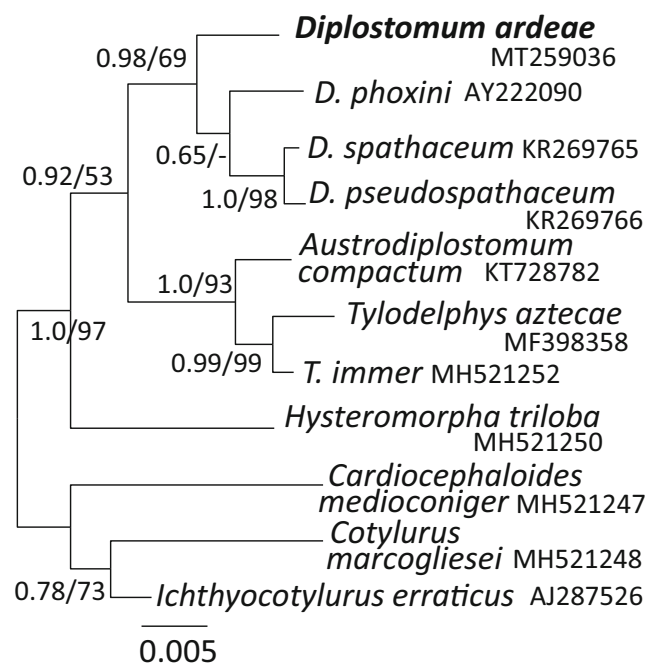
considering *D. ardeae* and *D. lunaschiae* n. sp. members of a genus other than *Diplostomum*. In the adult form of *D. ardeae* and *D. lunaschiae* n. sp., the distribution of the vitellaria is typical of members of *Diplostomum*, in contrast to the more restricted vitelline fields Pelegrini et al. (2019) observed in metacercariae. These authors also noted that the relative length of the forebody is unusually great in *D. lunaschiae* n. sp., but adults of many species of *Diplostomum* (e.g., *D. pusillum*, *D. oediconemum*, *D. minutum*) possess adult forebodies and hindbodies with relative lengths similar to *D. ardeae* and *D. lunaschiae* n. sp., as do the metacercariae of *D. scudderi* (syn. *D. baeri eucaliae*) (Hoffman and Hundley, 1957; Dubois, 1970). In this light, and given the lack of resolution in the CO1 analysis (e.g., of *Diplostomum*) and the much larger number of characters in other molecular phylogenies (Figs. 2, 3, and 4), we continue to classify *D. ardeae* and *D. lunaschiae* n. sp. within *Diplostomum*. Nonetheless, the basal position and large branching distance of *D. ardeae* in the more resolved phylogenies of mitochondrial genomes, rDNA operons, 28S, and 18S, suggest that this classification could change as markers that are more phylogenetically informative than the partial CO1 barcode fragment are sequenced in additional diplostomid species and genera.

The broad sampling of fish in this study was undertaken to assess the host specificity of metacercariae encountered in the upper Paraná River. We recovered *D. lunaschiae* n. sp. only in the lenses of *T. galeatus*, but the same parasite was found in 11 additional siluriform fishes by Pelegrini et al. (2019), who



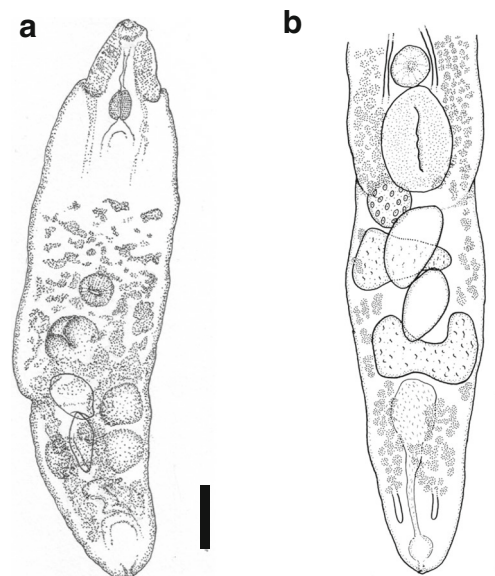
**Fig. 3** Phylogenetic analysis of partial 28S rDNA from *Diplostomum ardeae* and other members of the Diplostomoidea. Nodes in maximum likelihood topology are annotated with support in 1000 bootstrap replicates and with posterior probability from Bayesian Inference. Trees were generated with a HKY+G model of nucleotide substitution (or near equivalent) from 1390 ungapped sites in a 1407-bp alignment

sampled about 600 river km away in the same drainage basin. Infection levels recorded by Pelegrini et al. (2019) were higher than in our survey and the differences in host range could reflect geographic variation in the abundance of *D. lunaschiae* n. sp. The narrow host range we observed could also be related to differences in sampling effort (15 fish/species in the present study, 30 fish/species in Pelegrini et al. 2019). Acosta et al. (2020) surveyed macroparasites in 405 fish in eight siluriform species in the mouth of a tributary to the Paraná River 250 km upstream from our sampling area, roughly half the river distance to the locality surveyed by Pelegrini et al. (2019), but Acosta et al. (2020) reported only *Austrodiplostomum* sp. from the eyes of seven of the eight fish species sampled. However, the photos of *Austrodiplostomum*



**Fig. 4** Phylogenetic analysis of partial 18S rDNA from *Diplostomum ardeae* and other members of the Diplostomoidea. Nodes in a Bayesian Inference topology are annotated with posterior probability and support in maximum likelihood (1000 replicates). Trees were generated with K2+G+I models of nucleotide substitution (or near equivalents) from 1854 ungapped sites in an 1890-bp alignment

from *Trachydoras paraguayensis* (Eigenmann and Ward, 1907) and *Pimelodella avanhandavae* Eigenmann, 1917 (supplementary figures 2g and 3c in Acosta et al. 2020) resemble metacercariae of *D. lunaschiae* n. sp. In the present study, in Pelegrini et al. (2019), and in Acosta et al. (2020),



**Fig. 5** *Diplostomum lunaschiae* n. sp. from intestine of *Tigrisoma lineatum* (Boddaert), Pirané, Formosa Province, Argentina. (A) Entire worm, ventral view, scale = 100 μm. (B) Ventral view of hindbody, scale = 200 μm

**Table 3** Morphometrics of *Diplostomum lunaschiae* n. sp. and *Diplostomum ardeae* in micrometers ( $\mu\text{m}$ ), with range followed by mean in parentheses

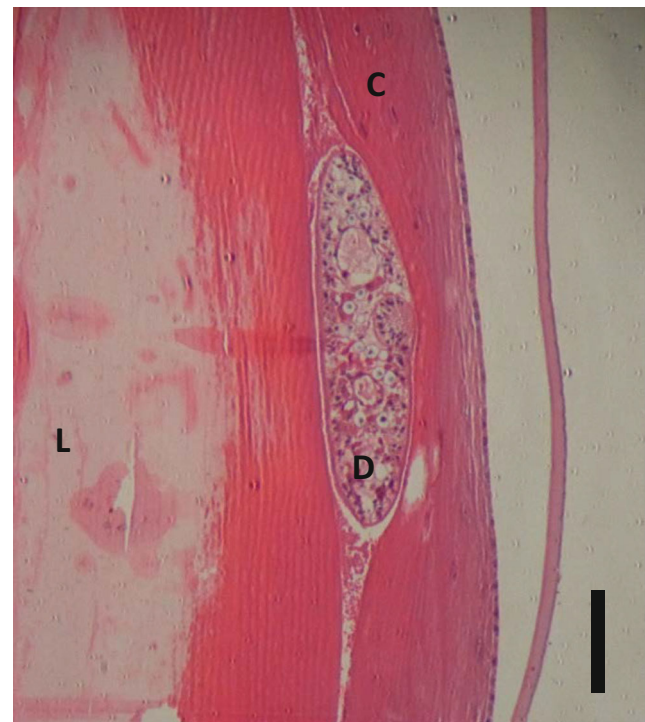
	<i>Diplostomum lunaschiae</i> n. sp. (n = 11)	<i>Diplostomum ardeae</i> (n = 2)	<i>D. ardeae</i> (n = 3)
Host	<i>Tigrisoma lineatum</i>	<i>Ardea herodias</i>	<i>Ardea herodias</i>
Locality	Formosa Province, Argentina	Montreal area, Canada	Hampden County, USA
Body length	503–986 (786)	1192–1222	1069–1190
Forebody (Fb)	423–636 × 176–275 (512 × 229)	848–869 × 415–457	720–850 × 220–300
Hindbody (Hb)	170–430 × 160–176 (300 × 168)	287–351 × 280–287	370–400 × 160–220
Oral sucker (Os)	26–53 × 20–58 (40 × 42)	72–76 × 64–74	50–52 × 47–48
Ventral sucker (Vs)	42–56 × 38–55 (50 × 49)	94–101 × 96–100	52–55 × 50–60
Pseudosuckers (Ps)	80–119 × 14–52 (103 × 37)	152–189 × 64–67	130–150 × 55–65
Holdfast organ (Ho)	81–112 × 55–102 (93 × 75)	179–199 × 119	125–140 × 110–145
Prepharynx (length)	49–83 (71)	76–84	65–95
Pharynx (Ph)	38–55 × 24–34 (44 × 30)	40–62 × 44–50	32 × 26
Esophagus (length)	8–19 (14)	40	30–37
Ovary	50 × 48		42–47 × 63–78
Anterior testis	70–71 × 58–107 (71 × 83)		70–80 × 85–90
Posterior testis	56–71 × 119–140 (64 × 130)	72 × 119–179	60–78 × 130–135
Eggs	88–105 × 36–57 (97 × 48)	90–98 × 48–54	90–96 × 57–66
Egg number	1–4 (2)	0–10	2
Ratios:			
Hb/Fb length	0.4–0.67 (0.52)	0.33–0.41	0.47–0.51
Sucker width (Vs/Os)	1.15–2.3 (1.5)	1.30–1.56	1.06–1.25*
Ph/Os length	1.3–1.5 (1.4)	0.52–0.86	0.62–0.64
Body/Ps length	6.3–10.8 (7.9)	6.5–7.8	7.1–9.2*
Ps/Os length	2–3.2 (2.7)	2.1–2.5	2.5–3*
Ps/Ph length	1.9–2.8 (2.4)	2.5–4.7	4.1–4.7*
Body/egg length	4.9–10.8 (8.7)	12.5–13.6	11–13*
Source	Present study	Present study	Dubois (1969)

\*Calculated from descriptions by Dubois (1969)

metacercariae of *D. lunaschiae* n. sp. are thus limited to siluriform fishes. However, the variation in host ranges in these different surveys suggests this could change in future surveys. For example, molecular and morphological work might reveal diplostomids that Leite et al. (2018) collected from the eyes of a characiform host in the same locality sampled by Acosta et al. (2020) to be *D. lunaschiae* n. sp. A wide host range for metacercariae of *D. lunaschiae* n. sp. would be consistent with other species of *Diplostomum* in which metacercariae infect the lens (Locke et al. 2015). Similarly, although both adults and metacercariae of *D. lunaschiae* n. sp. are currently known only from the Paraná River basin, the geographic ranges of its second intermediate and definitive hosts suggest its distribution could be considerably wider.

The present data expand the latitudinal range of *D. ardeae* to the Greater Antilles. The immaturity of the specimens of *D. ardeae* found in Puerto Rico suggests that the life cycle is locally established, and that these worms were not the result of an infection acquired when the bird host fed elsewhere. No eye-infecting diplostomids are known on the island (Bunkley-Williams and Williams 1994), but Puerto Rico could be a propitious place to search for the unknown larval forms of *D. ardeae* because of its relatively depauperate fish (Neal et al. 2009) and snail faunas (Van der Schalie 1948). Potential intermediate hosts occurring across the known range of *D. ardeae* (records in Dubois 1969; Dronen and Chen 2002;

Locke et al. 2015; present study) include members of the genera *Physa*, *Lepomis*, *Micropterus*, *Anguilla*, *Mugil*, and *Ictalurus*.



**Fig. 6** Sectioned eye of *Trachelyopterus galeatus*. L = lens, C = cortex, D = *Diplostomum lunaschiae* n. sp. Scale = 8  $\mu\text{m}$



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**Data availability** Nucleotide sequences reported here are available in GenBank (*D. ardeae* mitochondrial genome MT259035 and rDNA operon MT259036; *D. ardeae* and *D. lunaschiae* n. sp. CO1 DNA barcodes MT324592-626), and vouchers have been deposited at the Museum of Southwestern Biology (MSB: Para:30692-3) and Museo de La Plata (MLP-He 7656).

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

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