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Molecular phylogeny of *Diplostomum*, *Tylodelphys*, *Austrodiplostomum* and *Paralaria* (Digenea: Diplostomidae) necessitates systematic changes and reveals a history of evolutionary host switching events ☆

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ABSTRACT

The Diplostomidae Poirier, 1886 is a large, globally distributed family of digeneans parasitic in intestines of their definitive hosts. *Diplostomum* and *Tylodelphys* spp. are broadly distributed, commonly reported, and the most often sequenced diplostomid genera. The majority of published DNA sequences from these genera originated from larval stages only, which typically cannot be identified to the species level based on morphology alone. We generated partial large ribosomal subunit (28S) rRNA and cytochrome *c* oxidase subunit 1 (*cox*1) mtDNA gene sequences from 14 species/species-level lineages of *Diplostomum*, six species/species-level lineages of *Tylodelphys*, two species/species-level lineages of *Austrodiplostomum*, one species previously assigned to *Paralaria*, two species/species-level lineages of *Dolichorchis* and one unknown diplostomid. Our DNA sequences of 11 species/species-level lineages of *Diplostomum* (all identified to species), four species/species-level lineages of *Tylodelphys* (all identified to species), *Austrodiplostomum compactum*, *Paralaria alarioides* and *Dolichorchis lacombeensis* originated from adult specimens. 28S sequences were used for phylogenetic inference to demonstrate the position of *Paralaria alarioides* and *Dolichorchis* spp. within the Diplostomoidea and study the interrelationships of *Diplostomum*, *Tylodelphys* and *Austrodiplostomum*. Our results demonstrate that two diplostomids from the North American river otter (*P. alarioides* and a likely undescribed taxon) belong within *Diplostomum*. Further, our results demonstrate the non-monophyly of *Tylodelphys* due to the position of *Austrodiplostomum* spp., based on our phylogenetic analyses and morphology. Furthermore, the results of phylogenetic analysis of 28S confirmed the status of *Dolichorchis* as a separate genus. The phylogenies suggest multiple definitive host-switching events (birds to otters and among major avian groups) and a New World origin of *Diplostomum* and *Tylodelphys* spp. Our DNA sequences from adult digeneans revealed identities of 10 previously published lineages of *Diplostomum* and *Tylodelphys*, which were previously identified to genus only. The novel DNA data from this work provide opportunities for future comparisons of larval diplostomines collected in ecological studies.

1. Introduction

The Diplostomidae Poirier, 1886 is a large, globally distributed family of digeneans, typically parasites in the intestines of their tetrapod definitive hosts. At present, the family includes 42 genera split among four subfamilies (Niewiadomska, 2002; Heneberg et al., 2020). The type-genus *Diplostomum* von Nordmann, 1932

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™ under accession numbers **MZ314149–MZ314189** (28S) and **MZ323246–MZ323308** (*cox*1).

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(subfamily Diplostominae Poirier, 1886) is highly speciose and globally distributed (Shigin, 1986, 1993; Galazzo et al., 2002; Niewiadomska, 2010; Behrmann-Godel, 2013; Georgieva et al., 2013; Locke et al., 2015; Gordy et al., 2016; Hoogendoorn et al., 2020). Members of *Diplostomum* have been the focus of numerous studies related to their ecology, host-parasite relationships, systematics and taxonomy (e.g., Shigin, 1986, 1993; Galazzo et al., 2002; Karvonen et al., 2004, 2006; Seppälä et al., 2008; Locke et al., 2010a,b, 2015; Niewiadomska, 2010; Georgieva et al., 2013; Pérez-del-Olmo et al., 2014; Kudlai et al., 2017; Enabulele et al., 2018; Rudko et al., 2018; Hoogendoorn et al., 2020; Vivas Muñoz et al., 2021). Complete mitochondrial genome sequences have been generated and studied for the type-species *Diplostomum spathaceum* (Rudolphi, 1819), *Diplostomum pseudospathaceum* Niewiadomska, 1984, *Diplostomum ardeae* Dubois, 1969 and *Diplostomum baeri* (Dubois, 1937) (Brabec et al., 2015; Landeryou et al., 2020; Locke et al., 2020). Brabec et al. (2015) demonstrated discordance between phylogenies generated from complete mitochondrial genomes and the nuclear rRNA operons of *Diplostomum* spp. in relation to other members of the order Diplostomida Olson, Cribb, Tkach, Bray & Littlewood, 2003 and Plagiorchiida La Rue (1927).

The systematic and taxonomic history of *Diplostomum* is rather complex, with its composition varying greatly among authors (e.g., Dubois, 1968, 1982; Shigin, 1993; Niewiadomska, 2010). Recent molecular phylogenetic studies of *Diplostomum* (e.g., Galazzo et al., 2002; Locke et al., 2010a, 2015; Georgieva et al., 2013; Faltýnková et al., 2014; Pérez-del-Olmo et al., 2014; Selbach et al., 2015; Kudlai et al., 2017; Soldánová et al., 2017; Gordy and Hanington, 2019; Hoogendoorn et al., 2020) have revealed the presence of numerous species or species-level lineages. However, most sequences originate from larval specimens, which often cannot be accurately identified morphologically to species. This prevents the resolution of the complex taxonomy and systematics of *Diplostomum* (e.g., Hoogendoorn et al., 2020). Previous studies have used molecular tools to reveal that some species of *Diplostomum* are distributed in multiple biogeographic realms (Locke et al., 2015, 2020; Hoogendoorn et al., 2020).

Close relationships between members of *Diplostomum* and two other genera of the Diplostominae, *Tylodelphys* Diesing, 1850 and *Austrodiplostomum* Szidat & Nani, 1951 have been repeatedly demonstrated using molecular phylogenies (e.g., Locke et al., 2015, 2018; Selbach et al., 2015; Garcia-Varela et al., 2016; Blasco-Costa and Locke, 2017; Achatz et al., 2019b, 2019c, 2019d, 2020, 2021a; Pelegrini et al., 2019; Sereno-Uribe et al., 2019a, 2019b; Heneberg et al., 2020; Hoogendoorn et al., 2020; Tkach et al., 2020). Members of these three genera utilise a wide range of fish species as second intermediate hosts and typically parasitize fish-eating birds as adults (e.g., Gibson, 1996; Niewiadomska, 2002; Dronen, 2009; Locke et al., 2010a, 2010b, 2015; Georgieva et al., 2013; Rosser et al., 2016a, 2016b). Importantly, some members of these genera are well-known agents of fish diseases, often causing ocular diplostomiasis (e.g., Inchausty et al., 1997; McCloughlin 2016; Rosser et al., 2016a).

In contrast to the well-studied members of *Diplostomum*, species of *Paralaria* Kraus, 1914, parasitic in New World river otters as adults, have received little attention (Kraus, 1914; Dubois, 1944, 1968). Kraus (1914) established *Paralaria* for *Paralaria clathrata* (Diesing, 1850), the type-species, and his newly described *Paralaria pseudoclathrata* (Kraus, 1914). In the concept of Dubois (1938, 1968, 1970, 1982) *Paralaria* was a subgenus of *Alaria* Schrank, 1788 and included species parasitic in mammals other than otters. *Paralaria* is considered a valid, separate genus in the most recent revision of the Diplostomoidea Poirier, 1886 (see Niewiadomska, 2002).

Members of the small genus *Dolichorchis* Dubois, 1961, also a member of the Diplostominae, are rarely reported parasites of birds in the Afrotropical, Australasian, Indomalayan and Neotropical realms (Dubois, 1968; Niewiadomska, 2002; Lunaschi and Drago, 2006). Historically, this taxon was considered as either a subgenus of *Diplostomum* (e.g., Dubois, 1968) or as an independent genus (Niewiadomska, 2002).

More than 1,000 *cox*1 sequences of *Diplostomum*, *Tylodelphys* and *Austrodiplostomum* are currently available in GenBank (19 February 2021), whereas no DNA sequence data have been published for *Paralaria* or *Dolichorchis*. Despite the recent surge in molecular systematic and ecological studies on *Diplostomum* and its close relatives *Tylodelphys* and *Austrodiplostomum*, DNA sequence data are available for only 19 nominal species identified based on adult morphology (e.g., Galazzo et al., 2002; Locke et al., 2010a, b, 2015, 2018, 2020; Georgieva et al., 2013; Pérez-del-Olmo et al., 2014; Chibwana et al., 2015; Sereno-Uribe et al., 2019a, b; Hoogendoorn et al., 2020; Heneberg and Sitko, 2021). Less than 6% of the DNA sequence data for *Diplostomum* spp. currently available in GenBank originates from adult specimens (Hoogendoorn et al., 2020).

In the present study, we generated sequences of the large ribosomal subunit (28S) rRNA and cytochrome *c* oxidase 1 (*cox*1) mtDNA genes from 14 species/species-level lineages of *Diplostomum* from birds, otter, fish and snails collected in the Nearctic, Neotropics and Palaearctic, six species/species-level lineages of *Tylodelphys* from birds and fish collected in the Nearctic, Neotropics and Palaearctic, two species/species-level lineages of *Austrodiplostomum* from birds collected in the Palaearctic and Neotropics, two species of *Dolichorchis* from birds in the Neotropics, one species of *Paralaria* from otter collected in the Nearctic and an as-yet unidentified diplostomid from a bird in the Neotropics. Sixteen of the 26 studied taxa were identified to the species level based on adult morphology. We used DNA sequence data to explore the interrelationships of these taxa, determine phylogenetic relationships and re-evaluate their systematic placement.

2. Materials and methods

2.1. Morphological study

Adult diplostomids were obtained from the intestines of a variety of avian and mammalian hosts and larval diplostomids were collected from a variety of snail and fish species in Europe as well as North and South America (Table 1). Live digeneans were briefly rinsed in saline, heat-killed with hot water and fixed in 70% ethanol. Dead digeneans were immediately fixed in 70% or 95% ethanol. Specimens for light microscopy were stained with aqueous alum carmine according to Lutz et al. (2017) and studied using a differential interference contrast optics equipped Olympus BX51 compound microscope (Olympus Corp., Tokyo, Japan). Morphological vouchers were deposited in the collection of the H. W. Manter Laboratory, University of Nebraska, Lincoln and Parasitology Collection at the University of Wisconsin - Stevens Point, U.S.A. (Table 1).

Different authors referred to the two distinct body parts in diplostomoideans as prosoma/opisthosoma, or forebody/hindbody, or anterior/posterior segments. The latest revision by Niewiadomska (2002) in the "Keys to the Trematoda" used the terms forebody and hindbody for these body parts, whereas a different meaning was given to the same terms in chapters on all other distome digeneans, which was somewhat confusing. To avoid confusion, we use the terms prosoma and opisthosoma (e.g. Achatz et al., 2019a, 2019c; Tkach et al., 2020) to reflect the fact that these parts of the body in diplostomoideans are not

Table 1
Hosts, geographic origin, GenBank and museum accession numbers of diplostomine taxa used in this study. Site of infection is provided for specimens collected from fish intermediate hosts.

Digenean taxa	Host species	Geographical origin	Museum No.	GenBank accession numbers	
				28S	*cox1*
Austrodiplostomum compactum	*Phalacrocorax brasilianus*	Brazil (Pantanal)	HWML-216519	MZ314149	MZ323246
Austrodiplostomum sp. VVT1	*Pelecanus onocrotalus*	Ukraine (Odessa oblast)	HWML-216520	MZ314150	MZ323247
Diplostomidae gen. sp. VVT1	*Tigrisoma lineatum*	Brazil (Pantanal)	–	MZ314151	MZ323248
Diplostomum alarioides[a]	*Lontra canadensis*	U.S.A. (Mississippi)	HWML-216521	MZ314152	MZ323249
Diplostomum alascense n. comb.	*Mergus merganser*	U.S.A. (Minnesota)	HWML-216522	MZ314153	MZ323250
Diplostomum gavium	*Gavia immer*	U.S.A. (North Dakota)	HWML-216523	MZ314154	MZ323251
D. gavium	*Hypentelium nigricans* (eye)	U.S.A. (Minnesota)	–	MZ314155	MZ323252
D. gavium	*Lymnaea stagnalis*	U.S.A. (North Dakota)	–	MZ314156	MZ323253
D. gavium	*Lymnaea* sp.	U.S.A. (North Dakota)	–	MZ314157	MZ323254, MZ323255
D. gavium	*Stagnicola elodes*	U.S.A. (North Dakota)	–	MZ314158	MZ323256
Diplostomum huronense	*Larus delawarensis*	U.S.A. (Illinois)	UWSP-P-8634	MZ314159	MZ323257
D. huronense	*Larus delawarensis*	U.S.A. (North Dakota)	–	–	MZ323258, MZ323259
D. huronense	*Larus dominicanus*	Chile (Concepción)	HWML-216524	MZ314160	MZ323260
D. huronense	*Leucophaeus pipixcan*	U.S.A. (North Dakota)	–	–	MZ323261
Diplostomum indistinctum	*Larus argentatus*	U.S.A. (North Dakota)	–	MZ314161	MZ323262
D. indistinctum	*Larus delawarensis*	U.S.A. (North Dakota)	HWML-216525	MZ314162 – MZ314164	MZ323263
D. indistinctum	*Leucophaeus pipixcan*	U.S.A. (North Dakota)	–	–	MZ323264
D. indistinctum	*Recurvirostra americana*	U.S.A. (North Dakota)	–	MZ314165	MZ323265, MZ323266
D. indistinctum	*Stagnicola elodes*	U.S.A. (North Dakota)	–	MZ314166	MZ323267
Diplostomum marshalli	*Tringa melanoleuca*	U.S.A. (North Dakota)	HWML-216526	MZ314167	MZ323268
Diplostomum pseudospathaceum	*Chroicocephalus genei*	Ukraine (Kherson oblast)	HWML-216527	MZ314168	MZ323269
Diplostomum rauschi	*Chroicocephalus genei*	Ukraine (Kherson oblast)	–	MZ314169	MZ323270
D. rauschi	*Hydroprogne caspia*	Ukraine (Kherson oblast)	HWML-216528 – HWML-216529	–	MZ323271, MZ323272
Diplostomum scudderi	*Lophodytes cucullatus*	U.S.A. (North Dakota)	HWML-216530	MZ314170	MZ323273
Diplostomum spathaceum	*Chroicocephalus genei*	Ukraine (Kherson oblast)	–	–	MZ323274
D. spathaceum	*Larus argentatus*	Ukraine (Kherson oblast)	HWML-216533 – HWML-216535	MZ314171	MZ323275 – MZ323277
D. spathaceum	*Larus cachinnans*	Ukraine (Chernihiv oblast)	HWML-216531	MZ314172	MZ323278 – MZ323280
D. spathaceum	*Spatula querquedula*	Ukraine (Kherson oblast)	HWML-216532	–	MZ323281
Diplostomum sp. VVT1	*Lymnaea stagnalis*	U.S.A. (Minnesota)	–	MZ314173, MZ314174	MZ323282, MZ323283
Diplostomum sp. VVT1	*Umbra limi* (brain)	U.S.A. (Minnesota)	–	MZ314175	MZ323284, MZ323285
Diplostomum sp. VVT2	*Lepomis cyanellus* (skin)	U.S.A. (Minnesota)	–	–	MZ323286
Diplostomum sp. VVT2	*Lepomis gibbosus* (eye)	U.S.A. (Minnesota)	–	–	MZ323287
Diplostomum sp. VVT2	*Perca flavescens* (skin)	U.S.A. (Minnesota)	–	MZ314176	MZ323288, MZ323289
Diplostomum sp. VVT2	*Perca flavescens* (eye)	U.S.A. (Minnesota)	–	–	MZ323290 – MZ323293
Diplostomum sp. VVT3	*Lontra canadensis*	U.S.A. (Wisconsin)	UWSP-P-8635–8637	MZ314177	MZ323294
Diplostomum sp. VVT4	*Lymnaea stagnalis*	U.S.A. (Minnesota)	–	MZ314178	MZ323295
Diplostomum sp. VVT5	*Egretta caerulea*	U.S.A. (Mississippi)	HWML-216536	MZ314179	MZ323296
Dolichorchis lacombeensis	*Ardea cocoi*	Brazil (Pantanal)	HWML-216537	MZ314180	MZ323297
Do. lacombeensis	*Busarellus nigricollis*	Brazil (Pantanal)	–	MZ314181	MZ323298
Dolichorchis sp. VVT1	*Phimosus infuscatus*	Brazil (Pantanal)	–	MZ314182	MZ323299
Tylodelphys cf. *americana*	*Jabiru mycteria*	Brazil (Pantanal)	HWML-216538	MZ314183, MZ314184	MZ323300, MZ323301
Tylodelphys conifera	*Podiceps grisegena*	U.S.A. (Minnesota)	HWML-216539	MZ314185	MZ323302
Tylodelphys immer	*Gavia immer*	U.S.A. (North Dakota)	HWML-216540	MZ314186	MZ323303
Tylodelphys robrauschi n. comb.	*Podilymbus podiceps*	U.S.A. (Minnesota)	HWML-216541	MZ314187	MZ323304
Tylodelphys scheuringi	*Lepomis gibbosus* (eye)	U.S.A. (Minnesota)	–	–	MZ323305
T. scheuringi	*Lepomis macrochirus* (eye)	U.S.A. (Minnesota)	–	–	MZ323306
T. scheuringi	*Umbra limi* (brain)	U.S.A. (Minnesota)	–	MZ314188	MZ323307
Tylodelphys sp. VVT1	*Ambystoma talpoideum*	U.S.A. (Mississippi)	–	MZ314189	MZ323308

Museum abbreviations: HWML, Harold W. Manter Laboratory, U.S.A.; UWSP – PARA, University of Wisconsin – Stevens Point Parasitology Collection, U.S.A.
[a] Formerly included in *Paralaria*.

segments (e.g. unlike segments or proglottides in cestodes) and the terms forebody and hindbody are universally used to designate the parts of body posterior and anterior to the ventral sucker in distome digeneans. Our use of this terminology is also consistent with its use in similar situations among other invertebrates, e.g. arachnids.

2.2. Molecular study

Genomic DNA was extracted following the protocol described by Tkach and Pawlowski (1999) or using a ZR Genomic DNA Tissue Micro Prep kit (Zymo Research, Irvine, California, U.S.A.) following the manufacturer's protocol. A fragment of the nuclear ribosomal

28S rRNA gene was amplified by PCR using the forward primer digL2 (5′-AAG CAT ATC ACT AAG CGG-3′) and reverse primer 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) (Tkach et al., 2003). Fragments of *cox*1 were amplified by PCR using the forward primers Plat-diploCOX1F (5′-CGT TTR AAT TAT ACG GAT CC-3′), Cox1_Schist_5′ (5′-TCT TTR GAT CAT AAG CG-3′), Dipl_Cox_5′ (5′-ACK TTR GAW CAT AAG CG-3′) and BS_CO1_INT_F (5′-ATT AAC CCT CAC TAA ATG ATT TTT TTY TTT YTR ATG CC-3′) with reverse primers Plat-diploCOX1R (5′-AGC ATA GTA ATM GCA GCA GC-3′), acox650R (5′-CCA AAA AAC CAA AAC ATA TGC TG-3′), JB5 (5′-AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG-3′), Dipl650R (5′-CCA AAR AAY CAR AAY AWR TGY TG-3′), Dipl_Cox_3′ (5′-WAR TGC ATN GGA AAA AAA CA-3′) and BS_CO1_INT_R (5′-TAA TAC GAC TCA CTA TAA AAA AAA MAM AGA AGA RAA MAC MGT AGT AAT-3′) (Lockyer et al., 2003; Derycke et al., 2005; Moszczynska et al., 2009; Kudlai et al., 2015; Achatz et al., 2021a,b). PCR amplifications were performed in a total volume of 25 µl using GoTaq G2 DNA Polymerase from Promega (Madison, Wisconsin, U.S.A.) according to the manufacturer's instructions and using an annealing temperature of 53 °C for nuclear rDNA amplifications and 45 °C for *cox*1 amplifications.

An ExoSAP-IT PCR clean-up enzymatic kit from Affymetrix (Santa Clara, California, U.S.A.) was used to purify PCR products. PCR products were cycle-sequenced directly using a BrightDye Terminator Cycle Sequencing Kit (MCLAB, California, U.S.A.), purified using a BigDye Sequencing Clean Up Kit from MCLAB and run on an ABI 3130 automated capillary sequencer (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.).

PCR primers and the previously published 28S internal forward primer DPL600F (5′-CGG AGT GGT CAC CAC GAC CG-3′) and reverse primer DPL700R (5′-CAG CTG ATT ACA CCC AAA G-3′), together with new 28S internal forward primer DPL250F (5′-GGG TTG TTT GTG AAT GCA GCC C-3′) and internal reverse primers DPL350R (5′-GTT TAC CTC TGA GCG GTT TCA CG-3′), DPL1300R (5′-GCC TTT GGG TTT CGT AAC GCC-3′) and DPL1450R (5′-GAC GGG CCG GTG ATG CGC C-3′), designed by T.J. Achatz and V.V. Tkach , were used for sequencing reactions (Achatz et al., 2019d). Contiguous sequences were assembled using Sequencher version 4.2 software (GeneCodes Corp., Ann Arbor, Michigan, U.S.A.). Newly obtained sequences were deposited in the GenBank database (Table 1).

Sequences were initially aligned using ClustalW as implemented in MEGA7 software (Kumar et al., 2016). Initially, the phylogenetic positions of *Diplostomum*, *Paralaria*, *Tylodelphys*, *Austrodiplostomum*, *Dolichorchis* and one unidentified diplostomid within the Diplostomoidea Poirier, 1886 were determined using a 28S alignment with *Suchocyathocotyle crocodili* (Yamaguti, 1954) (Cyathocotylidae Mühling, 1896) as the outgroup based on the phylogeny published by Achatz et al. (2019d). This alignment included newly obtained sequences of species of *Diplostomum* ($n = 3$), *Paralaria* ($n = 1$), *Tylodelphys* ($n = 2$), *Austrodiplostomum* ($n = 2$), *Dolichorchis* ($n = 2$) and the unidentified diplostomid ($n = 1$) together with previously published sequences of *Diplostomum* ($n = 3$), *Tylodelphys* ($n = 1$) and *Austrodiplostomum* ($n = 1$), together with 15 other representatives of the Diplostomidae, 10 representatives of the Strigeidae Railliet, 1919 and two representatives of the Proterodiplostomidae Dubois, 1936.

Based on the results of the initial broader analysis, the interrelationships of *Diplostomum*, *Paralaria*, *Tylodelphys* and *Austrodiplostomum*, as currently recognised, were studied using two additional 28S alignments and two *cox*1 alignments with *Alaria mustelae* Bosma, 1931 used as the outgroup in all three analyses. One of these two 28S alignments included all newly obtained sequences of *Diplostomum* spp. ($n = 14$) and *Paralaria* spp. ($n = 1$), together with previously published sequences of *Diplostomum* spp. ($n = 8$). The other additional 28S alignment included newly obtained sequences of *Tylodelphys* spp. ($n = 5$) and *Austrodiplostomum* spp. ($n = 2$) as well as previously published sequences of *Tylodelphys* spp. ($n = 2$), *Austrodiplostomum* spp. ($n = 3$) and an unidentified diplostomid ($n = 1$). The first *cox*1 alignment included newly generated sequences of *Diplostomum* spp. ($n = 27$) and *Paralaria* spp. ($n = 1$), together with previously published sequences of species of *Diplostomum* ($n = 53$). The second *cox*1 alignment included newly generated sequences of *Tylodelphys* spp. ($n = 9$) and *Austrodiplostomum* spp. ($n = 2$) as well as previously published sequences of *Tylodelphys* spp. ($n = 21$), *Austrodiplostomum* spp. ($n = 5$) and an unidentified diplostomid ($n = 1$). Although numerous other *cox*1 sequences are available, we opted to include only a limited number of representatives from each of the previously published species/species-level lineages. Recent studies on *Diplostomum* (e.g., Georgieva et al., 2013; Blasco-Costa et al., 2014; Locke et al., 2015; Selbach et al., 2015; Kudlai et al., 2017; Hoogendoorn et al., 2020) have already explored relationships among most of these lineages. However, we ensured that all major lineages within these genera were present in our analyses.

Bayesian inference (BI) as implemented in MrBayes v3.2.6 software was used for all phylogenetic analyses (Ronquist and Huelsenbeck, 2003). The general time-reversible model with estimates of invariant sites and gamma-distributed among-site variation (GTR + G + I) model was identified as the best-fitting nucleotide substitution model for the 28S and *cox*1 alignments using MEGA7 (Kumar et al., 2016). BI analyses for all datasets were performed using MrBayes software as follows: Markov chain Monte Carlo (MCMC) chains were run for 3,000,000 generations with sample frequency set at 1,000. Log-likelihood scores were plotted and only the final 75% of trees were used to produce the consensus trees. The number of generations for each analysis was determined as sufficient because the standard deviation stabilised below 0.01. Pairwise comparisons for each locus were carried out using MEGA7.

To accurately and consistently identify which species-level lineage is referred to throughout the text and supplementary materials, a reference to the origin of designations of species-level lineages is provided for non-nominal species that previously were assigned a lineage identification. The following abbreviations for references for species-level lineages were used: B, Blasco-Costa et al. (2014); C, Chibwana et al. (2013); Ch, Chaudhary et al. (unpublished); Ge, Georgieva et al. (2013); Go, Gordy and Hanington (2019); H, Hoogendoorn et al. (2020); Ko, Komatsu et al. (2019); Ku, Kudlai et al. (2017); L, Locke et al. (2010a, b; 2015); M, Moszczynska et al. (2009); N, Nakao and Sasaki (2021); P, Pelegrini et al. (2019); R, Rosser et al. (2016a); Se, Sereno-Uribe et al. (2019a); Sl, Selbach et al. (2015); So, Soldánová et al. (2017).

3. Results

3.1. Molecular phylogenies

The broader 28S alignment of the Diplostomoidea was 1,118 bp long; two nucleotide positions were excluded due to indels. Similar to several recent molecular phylogenetic studies (e.g., Blasco-Costa and Locke, 2017; Hernandez-Mena et al., 2017; Locke et al., 2018; Achatz et al., 2019b, 2019c, 2019d, 2020, 2021a; Queiroz et al., 2020; Tkach et al., 2020), our broader 28S phylogeny (Fig. 1) demonstrated the non-monophyletic nature of the Diplostomidae and Strigeidae.

The two sequences of the Proterodiplostomidae, *Archaeodiplostomum overstreeti* Tkach, Achatz & Pulis, 2020 and *Neocrocodilicola georgiana* (Byrd & Reiber, 1942), formed a monophyletic clade, similar to recent detailed phylogenetic analyses of the family (Tkach et al., 2020). *Diplostomum*, *Paralaria*, *Tylodelphys* and *Austrodiplostomum* formed a weakly supported clade. However, the inter-

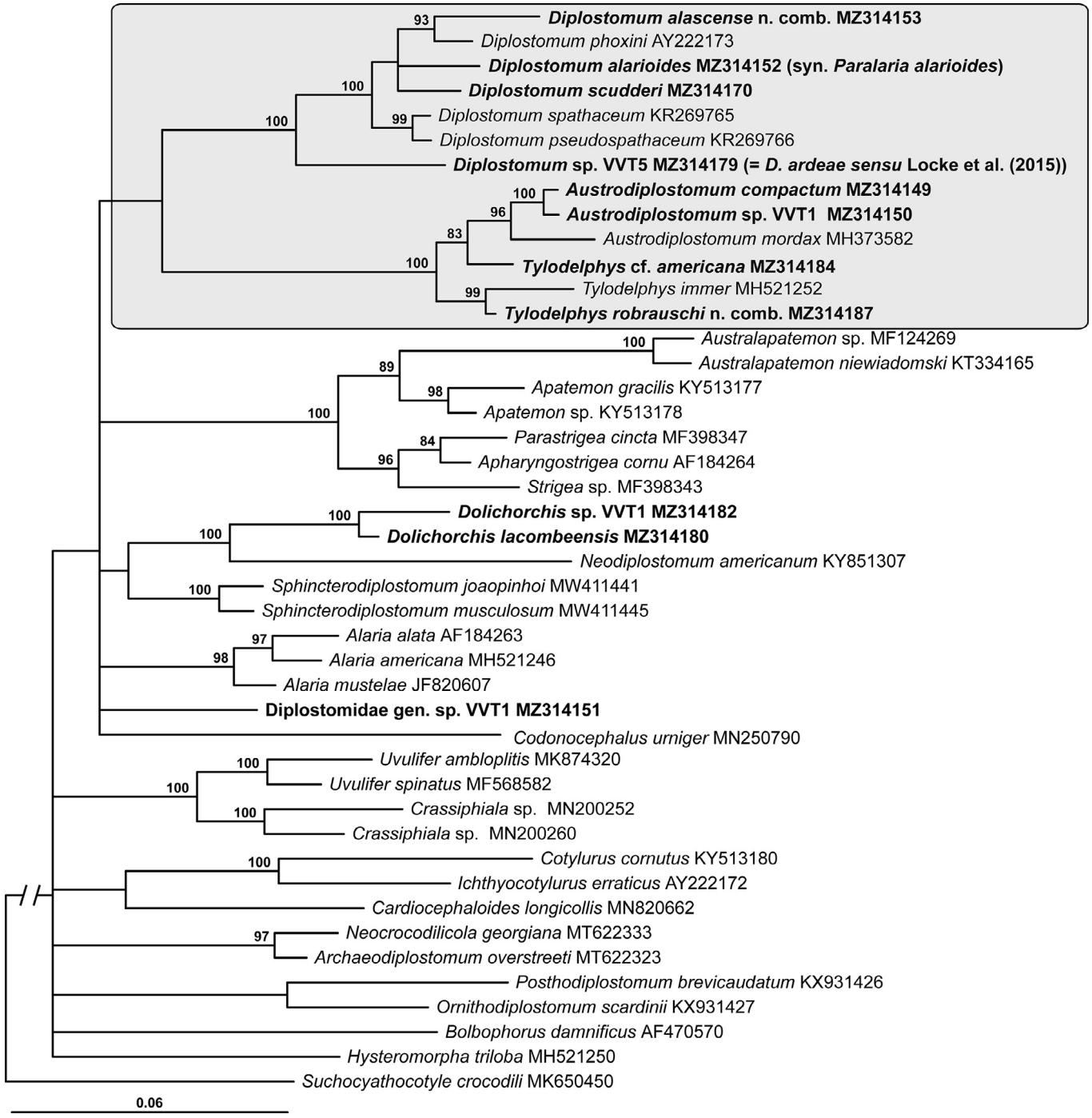

Fig. 1. Phylogenetic interrelationships among 43 diplostomoidean taxa including 13 members of *Diplostomum*, *Tylodelphys* and *Austrodiplostomum* (including a former *Paralaria* sp.), two species-level lineages of *Dolichorchis* and an unknown diplostomid based on Bayesian inference analysis of partial 28S rRNA gene sequences. Members of *Diplostomum* + *Tylodelphys* are indicated by the shaded rectangle. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale bar indicates the number of substitutions per site. GenBank accession numbers are provided after the names of species. The previously accepted/published names are provided in parentheses after GenBank accession numbers.

nal topology within this clade was well-resolved. *Diplostomum* + *Paralaria* formed a 100% supported clade; similarly, *Tylodelphys* + *Austrodiplostomum* also formed a 100% supported clade. Both of these 100% supported clades had well-supported internal topologies. *Tylodelphys*, as currently recognised, was non-monophyletic because *Tylodelphys* cf. *americana* (Dubois, 1936), a digenean with typical *Tylodelphys* morphology, appeared to be more closely related to *Austrodiplostomum* than to other *Tylodelphys* spp. Members of *Austrodiplostomum* formed a 96% supported clade. Both *Dolichorchis* species-level lineages clustered together with 100% support within a 100% supported clade, which also contained *Neodiplostomum* Railliet, 1919. These two genera showed a very weakly supported relatedness to *Sphincterodiplostomum*. The unidentified diplostomid lineage (Diplostomidae gen. sp. VVT1) formed a separate branch as a part of the extensive basal polytomy of the Diplostomoidea (Fig. 1).

Upon trimming to the length of the shortest sequence, the second 28S alignment limited to *Diplostomum* and *Paralaria* as cur-

rently recognised, was 1,106 bp long. The internal topology within this tree was overall moderately resolved (Fig. 2). Similar to the broader 28S phylogeny (Fig. 1), *Diplostomum* sp. VVT5 (=*D. ardeae sensu* Locke et al. (2015); see section 4.3 below) appeared as a sister branch to a weakly supported clade which contained all other species of *Diplostomum* included in the analysis (Fig. 2); admittedly, this relationship was not well supported. A number of internal topologies were much better resolved. The two *Diplostomum* spp. (sp. A and B (N)) from Japan formed a 100% supported clade, which was positioned as a sister clade to the remainder of the *Diplostomum* taxa. The latter clade included three sub-clades: (i) *Diplostomum* sp. DTS1R (H); (ii) an 80% supported clade of *Diplostomum* sp. VVT2 + an 86% supported clade of (*Diplostomum phoxini* (Faust, 1918) + *Diplostomum alascense* Dubois, 1969 n. comb.; see section 4.3 below); and (iii) a weakly supported large clade consisting of two sub-clades (Fig. 2). The first of these sub-clades included a 100% supported cluster of (*Diplostomum* sp. VVT1 + *Diplostomum scudderi* Olivier, 1941 (94% supported) and *Diplostomum marshalli* Chandler, 1954 + *Diplostomum* sp. VVT4 (87% supported)) and a 100% supported clade of *Diplostomum alarioides* Dubois, 1937 + *Diplostomum* sp. VVT3, both from the North American river otter *Lontra canadensis* (Schreber). The second sub-clade (96% supported) was characterised by largely unresolved internal topology. It included a weakly supported clade of *D. pseudospathaceum* + *Diplostomum gavium* (Guberlet, 1922) and a weakly supported clade of *Diplostomum indistinctum* (Guberlet, 1922) + *Diplostomum rauschi* Shigin, 1993 + *Diplostomum* sp. 16 (L) + a 92% supported clade of (*D. spathaceum* + *Diplostomum huronense* (La Rue, 1927) + *Diplostomum* sp. 14 (L)) (Fig. 2).

The third 28S alignment was 1,106 bp long and limited to members of *Tylodelphys* and *Austrodiplostomum* taxa, as currently recognised. The phylogenetic tree resulting from the analysis of this alignment contained two clusters (Fig. 3). *Tylodelphys* appeared non-monophyletic because, similar to the first 28S-based phylogeny, *Tylodelphys* cf. *americana* appeared to be more closely related to *Austrodiplostomum* or at least to form an independent clade in the basal polytomy. The first clade of *Tylodelphys* was 85% supported and contained *Tylodelphys scheuringi* (Hughes, 1929) and an 89% supported clade of *Tylodelphys conifera* (Mehlis, 1846) + *Tylodelphys robrauschi* Dubois, 1969 n. comb. (see section 4.5 below) + an 98% supported clade of (*Tylodelphys* sp. VVT1 + an 98% supported clade of (*Tylodelphys immer* Dubois, 1961 + *Tylodelphys aztecae* García-Varela et al., 2016)). The second clade of *Tylodelphys* spp. (which included *T.* cf. *americana*) formed a weakly supported cluster with *Austrodiplostomum* spp. *Tylodelphys* cf. *americana* and an unidentified diplostomid cercaria (*Tylodelphys* sp. 4 (L) (=Diplostomidae sp. 1 Type 1 (R))) formed an 89% supported clade. The *Austrodiplostomum* clade was strongly supported (100%). *Austrodiplostomum mordax* Szidat & Nani, 1951 formed a sister branch to a 100% supported clade containing the remaining *Austrodiplostomum* spp., including *Austrodiplostomum* sp. VVT1 from *Pelecanus onocrotalus* Linnaeus. *Austrodiplostomum compactum* (Lutz, 1928) formed a sister group to a 98% supported clade containing two previously published sequences of *Austrodiplostomum* sp. 1 and 2 (L) + *Austrodiplostomum* sp. VVT1 (Fig. 3).

The *cox*1 alignments were 362 bp long. We provide the phylogenetic tree based on *cox*1 data from *Diplostomum* spp. as a supplement (Supplementary Fig. S1) due to the large size of the tree. Due to the large number of taxa and the presence of basal polytomies in both *Diplostomum* and *Tylodelphys/Austrodiplostomum* trees, we have numbered the main clades for the convenience of presenting results and following the discussion (Fig. 4; Supplementary Fig. S1).

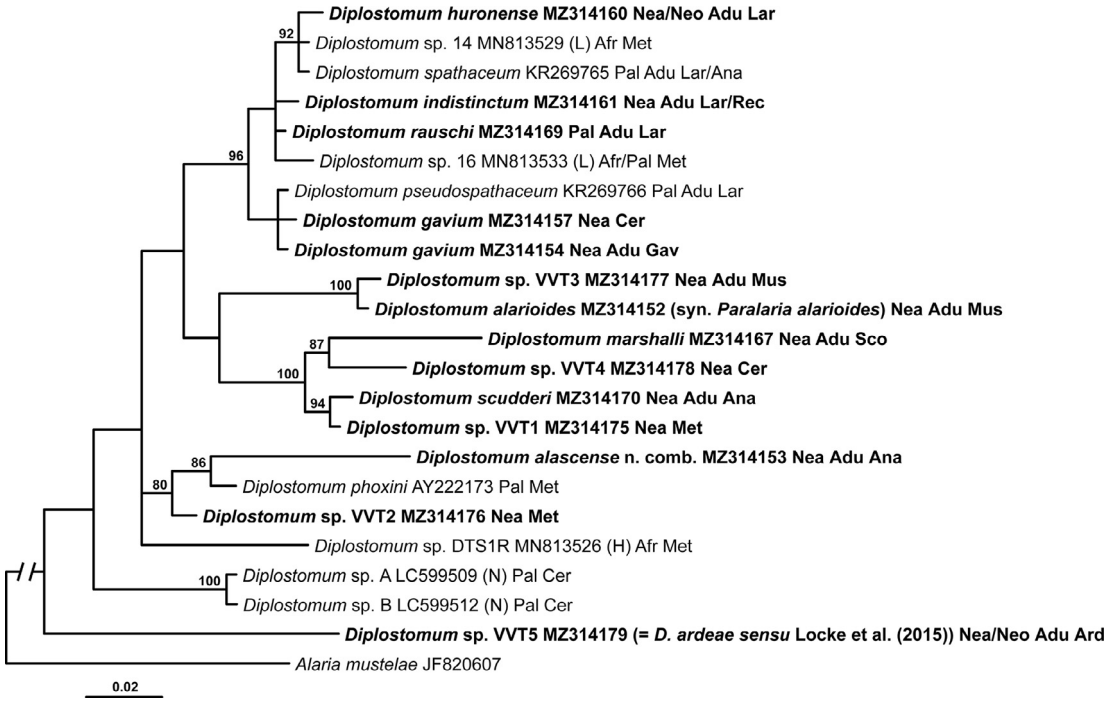

Fig. 2. Phylogenetic interrelationships among 21 taxa of *Diplostomum* (including a former *Paralaria* sp.) based on Bayesian inference analysis of partial 28S rRNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale bar indicates the number of substitutions per site. GenBank accession numbers are provided after the names of species. References to origins of species numbering/naming systems are provided in parentheses after GenBank accession numbers followed by the biogeographical realms where specimens were collected, life stages of isolates and families of definitive hosts (for adult isolates and larvae molecularly matched to adult forms). Abbreviations for references to the original designations of species-level lineages: H, Hoogendoorn et al. (2020); L, Locke et al. (2010a, 2010b, 2015); N, Nakao and Sasaki, (2021). The previously accepted/published names are provided in parentheses after GenBank accession numbers. Abbreviations for biogeographical realms: Afr, Afrotropical realm; Nea, Nearctic realm; Neo, Neotropical realm; Pal, Palaearctic realm. Abbreviations for life stage: Adu, adult; Cer, cercaria; Met, metacercaria. Abbreviations for family of definitive host: Ana, Anatidae; Ard, Ardeidae; Gav, Gaviidae; Lar, Laridae; Mus, Mustelidae; Rec, Recurvirostridae; Sco, Scolopacidae.

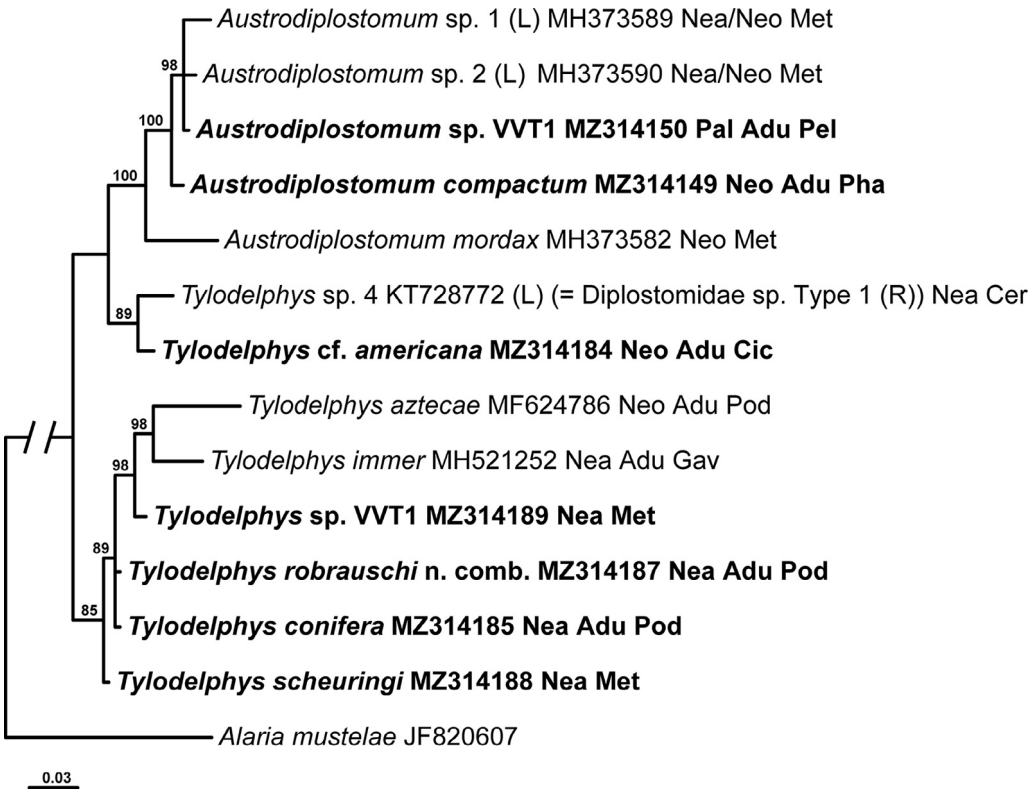

Fig. 3. Phylogenetic interrelationships among 13 taxa of *Tylodelphys* and *Austrodiplostomum* spp. based on Bayesian inference analysis of partial 28S rRNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale bar indicates the number of substitutions per site. GenBank accession numbers are provided after the names of species. References to origins of species numbering/naming systems are provided in parentheses after GenBank accession numbers followed by the biogeographical realms where specimens were collected, life stages of isolate and families of definitive hosts (for adult isolates and larvae molecularly matched to adult forms). Abbreviations for references to the original designations of species-level lineages: L, Locke et al. (2010a, 2010b, 2015); R, Rosser et al. (2016a). The previously accepted/published names are provided in parentheses after GenBank accession numbers. Abbreviations for biogeographical realms: Nea, Nearctic realm; Neo, Neotropical realm; Pal, Palaearctic realm. Abbreviations for life stage: Adu, adult; Cer, cercaria; Met, metacercaria. Abbreviations for families of definitive hosts: Cic, Ciconiidae; Gav, Gaviidae; Pel, Pelecanidae; Pha, Phalacrocoracidae; Pod, Podicipedidae.

The majority of *Diplostomum* spp. formed a 100% supported polytomous cluster with multiple well-supported internal clades, some of them well-resolved (Supplementary Fig. S1; clades D-I-D-XVI). Only a single clade (clade D-XVII) comprising *D. ardeae sensu* Locke et al. (2015), *Diplostomum lunaschiae* Locke, Drago, Núñez, Rangel e Souza & Takemoto, 2020 and *Diplostomum* sp. VVT5, was positioned separately from the larger polytomy. We only focus on the 10 clades containing species with newly generated DNA sequence data.

Clade D-I consisted of two strongly supported, larger sub-clades. The first major sub-clade (100% support) contained a large group of species-level lineages and two named species, *D. baeri* and *D. phoxini*. Notably, sequences of lineages belonging to the *D. baeri* complex appeared in three different strongly supported (100%, 92% and 100%) clusters. Within this clade, *Diplostomum* sp. VVT2 formed a 100% supported clade with a sequence of *D. baeri sensu* Galazzo et al. (2002) (MF142196; Ubels et al., 2018) (Supplementary Fig. S1). The second major sub-clade also included several species-level lineages and only a single named species, *D. alascense* n. comb. Within this clade, *D. alascense* n. comb. was clustered with a sequence of a metacercaria previously identified as *Diplostomum* sp. 2 (M) in a 100% supported clade.

Strongly supported (97%) clade D-II contained a polytomy with several species-level lineages, including our *Diplostomum* VVT1 and VVT4, as well as one named species, *D. scudderi*. Within this clade, *Diplostomum* sp. VVT4 + *Diplostomum* sp. 17 (L) formed a 100% supported clade.

Another weakly supported clade in the basal polytomy consisted of two strongly supported clades (D-III and D-IV). Clade D-III was split into two sub-clades (Supplementary Fig. S1) that comprised sequences of *D. gavium* (=*Diplostomum* sp. 3 (M)) and *D. pseudospathaceum*. The first sub-clade (99% support) contained sequences of *D. gavium,* while the second sub-clade (95% support) contained *D. pseudospathaceum*. Clade D-IV (100% support) only contained newly generated sequences of *D. indistinctum* + *Diplostomum* sp. 4 (M). Clade D-VI (100% support) only consisted of newly generated sequences of *D. huronense* and *Diplostomum* sp. 1 (M). Notably, *D. indistinctum sensu* Galazzo et al. (2002) and *D. huronense sensu* Galazzo et al. (2002) were positioned in the tree separately from our isolates of *D. huronense* and *D. indistinctum*.

Diplostomum rauschi + *Diplostomum* sp. Lineage 2 (B) formed a 100% supported cluster within clade D-VIII (84% support). Clade D-VIII also included a 100% supported group of *Diplostomum* sp. 16 (L) sequences.

The isolates of *D. spathaceum* formed a 100% supported cluster (clade D-IX) that appeared in the *cox*1 tree (Supplementary Fig. S1). In the 100% supported Clade D-X, *D. alarioides* was basal to the strongly supported clade of *Diplostomum* sp. VVT3 + *Diplostomum* sp. 10 (L). Clade D-XIII (100% support) consisted of *D. marshalli* and *Diplostomum* sp. A (Go).

The second major well-supported (94%) clade of *Diplostomum* (clade D-XVII), which was separate from the largest polytomy described above, contained *D. lunaschiae* + *Diplostomum* sp. VVT5 + *D. ardeae sensu* Locke et al. (2015).

This *cox*1 based phylogeny of *Tylodelphys* + *Austrodiplostomum* (Fig. 4) consisted of a polytomy with nine well-supported clades (clades A-I, T-I-T-VIII) and a sister clade (T-IX) which only con-

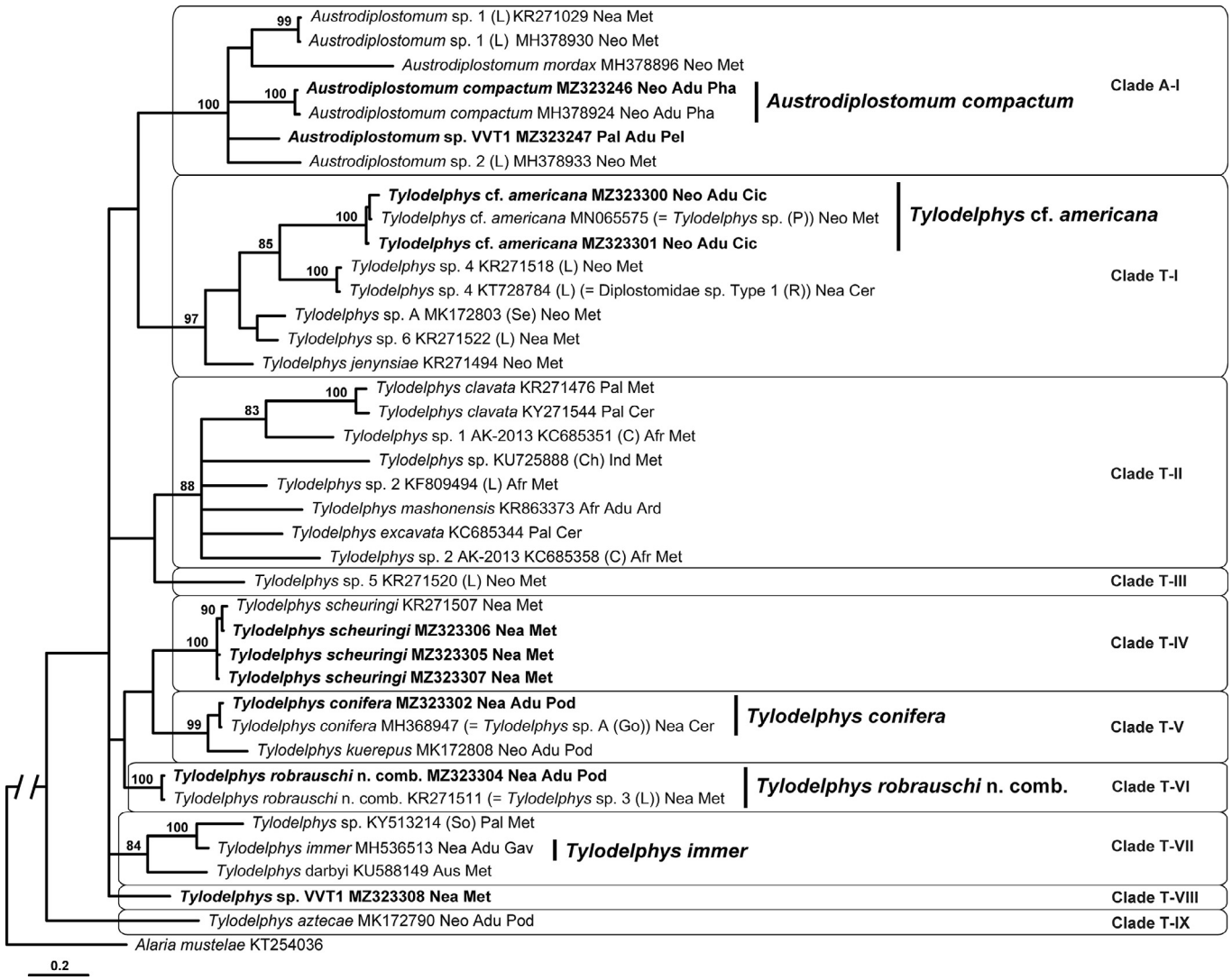

Fig. 4. Phylogenetic interrelationships among 27 taxa of *Tylodelphys* and *Austrodiplostomum* spp. based on Bayesian inference analysis of partial *cox*1 mtDNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale bar indicates the number of substitutions per site. GenBank accession numbers are provided after the names of species. References to origins of species numbering/naming systems are provided in parentheses after GenBank accession numbers followed by the biogeographical realms where specimens were collected, life stages of isolate and families of definitive hosts (for adult isolates and larvae molecularly matched to adult forms). Abbreviations for references to the original designations of species-level lineages: C, Chibwana et al. (2013); Ch, Chaudhary et al. (unpublished); Go, Gordy and Hanington (2019); Locke et al. (2010a, 2010b, 2015; P, Pelegrini et al. (2019); R, Rosser et al. (2016a); Se, Sereno-Uribe et al. (2019a); So, Soldánová et al. (2017). Abbreviations for biogeographical realms: Afr, Afrotropical realm; Aus, Australasian realm; Ind, Indomalayan realm; Nea, Nearctic realm; Neo, Neotropical realm; Pal, Palaearctic realm. Abbreviations for life stages: Adu, adult; Cer, cercaria; Met, metacercaria. Abbreviations for families of definitive hosts: Ard, Ardeidae; Cic, Ciconiidae; Gav, Gaviidae; Pel, Pelecanidae; Pha, Phalacrocoracidae; Pod, Podicipedidae.

tained *T. aztecae*. We opt to only discuss the six clades containing isolates with newly generated DNA sequences.

Clade A-I (100% support) contained all *Austrodiplostomum* taxa included in our analysis with unresolved internal topology. Within this clade, *A. mordax* n. comb. appeared as a sister group to a weakly supported cluster containing *Austrodiplostomum* sp. 1 (L).

The strongly supported (97%) clade T-I contained two nominal species, *T.* cf. *americana* and *Tylodelphys jenynsiae* Szidat, 1969, and a few not yet identified species-level lineages (Fig. 4). *Tylodelphys jenynsiae* was positioned as a sister group to a weakly supported clade consisting of a cluster of an 85% supported clade containing (a 100% supported clade of *Tylodelphys* sp. 4 (L) (=*Diplostomidae* sp. 1 Type 1 (R)) + a 100% supported clade of *T.* cf. *americana* (=*Tylodelphys* sp. MN065575 (P))) + an unsupported clade of (*Tylodelphys* sp. A (Se) + *Tylodelphys* sp. 6 (L)).

The clades T-IV (100% support), T-V (99% support) and T-VI (100% support) formed a weakly supported cluster. Clade T-IV contained only isolates of *T. scheuringi*, while clade T-V contained *Tylodelphys kuerepus* Sereno-Uribe, Andrade-Gómez, Ponce de León & García-Varela, 2019 + a cluster of (*T. conifera* + *Tylodelphys* sp. A (Go)). Clade T-VI only contained *T. robrauschi* n. comb. + *Tylodelphys* sp. 3 (L).

Clade T-VII (84% support) included *Tylodelphys darbyi* Presswell & Blasco-Costa, 2019 and a 100% supported cluster of *T. immer* + *Tylodelphys* sp. (KY513214) (So). *Tylodelphys* sp. VVT1 formed another independent clade (T-VIII) in the polytomy of *Tylodelphys* spp.

3.2. Pairwise comparisons

Due to the relatively limited amount of available 28S sequence data, it was possible to provide pairwise comparisons of all available named species or species-level lineages regardless of parasite life stage (*Diplostomum*: Supplementary Table S1; *Tylodelphys* and

Austrodiplostomum: Supplementary Table S2). Due to the greater number of *cox*1 sequences available in GenBank, we provide pairwise comparisons only for *cox*1 sequences included in our phylogenetic analyses (*Diplostomum*: Supplementary Table S3; *Tylodelphys* and *Austrodiplostomum*: Supplementary Table S4). However, we discuss below only pairwise comparisons of *Diplostomum*, *Tylodelphys* and *Austrodiplostomum cox*1 sequences from morphologically identified adults or larvae matched genetically with adult specimens included in our analysis (Supplementary Table S5). Intraspecific variations of *cox*1 sequences were studied using two closely related representative species of *Diplostomum* (Supplementary Table S6) and a species of *Tylodelphys* (Supplementary Table S7); multiple identical sequences of species and species-level lineages were not included in analyses.

The interspecific divergence in 28S sequences of *Diplostomum* spp. was generally low (0–3.7%; Supplementary Table S1). *Diplostomum* sp. A (N) *versus Diplostomum* sp. B (N), *D. gavium versus D. pseudospathaceum* and *Diplostomum* sp. 14 (L) *versus D. spathaceum* showed the lowest levels of divergence. In contrast, *D. marshalli versus Diplostomum* sp. VVT5 demonstrated the greatest divergence. Similarly, the interspecific divergence in 28S sequences of *Tylodelphys* and *Austrodiplostomum* spp. was also generally low (0–1.9% and 0.1–1.3%, respectively; Supplementary Table S2). Among *Tylodelphys* taxa, the pair *T. conifera versus T. robrauschi* n. comb. were the least divergent, whereas *T. aztecae versus Tylodelphys* sp. 4 (L) showed the greatest difference. Among *Austrodiplostomum*, the pair *Austrodiplostomum* sp. VVT1 *versus Austrodiplostomum* sp. 2 (L) were the least divergent, while *A. mordax versus Austrodiplostomum* sp. 1 (L) and *A. mordax versus Austrodiplostomum* sp. 2 were the most divergent. The 28S sequences of the two *Dolichorchis* species-level lineages differed by 1%.

No intraspecific variation was detected among the newly obtained 28S sequences of *D. huronense* ($n = 2$), *D. indistinctum* ($n = 6$), *D. spathaceum* ($n = 2$), *T.* cf. *americana* ($n = 2$) and *Dolichorchis lacombeensis* Lunaschi & Drago, 2006 ($n = 2$), whereas isolates of *D. gavium* ($n = 4$) differed by up to 0.1% (1 nucleotide).

Diplostomum spp. showed interspecific divergence levels of 2.3–16.3% in the sequenced fragment of *cox*1 gene (Supplementary Tables S5, S6). *Diplostomum pseudospathaceum versus D. gavium* had the lowest level of interspecific divergence, whereas *Diplostomum* sp. VVT5 *versus D. rauschi* (KJ726449) (=*Diplostomum* sp. Lineage 2 (B)) were the most divergent. The interspecific divergence levels of *cox*1 sequences among *Tylodelphys* spp. (4.4–14.6%) and *Austrodiplostomum* spp. (9.1–13%) were similar to the interspecific divergence of *cox*1 sequences among *Diplostomum* species. *Tylodelphys conifera* (MH368947) (=*Tylodelphys* sp. A (Go)) *versus T. kuerepus* had the highest level of similarity among *Tylodelphys* species/species-level lineages, whereas *T.* cf. *americana versus Tylodelphys mashonensis* Beverley-Burton, 1963 were most divergent. Among *Austrodiplostomum*, *A. compactum versus Austrodiplostomum* sp. VVT1 were least divergent, while *A. mordax versus Austrodiplostomum* sp. VVT1 were the most divergent. The two *Dolichorchis* species-level lineages differed by 12.9–13.6% in *cox*1 sequences.

As expected, intraspecific variation of the representative species of *Diplostomum* and *Tylodelphys* was lower than their respective interspecific divergences. Multiple sequences of *D. pseudospathaceum* ($n = 14$) from the Palaearctic showed up to 1.8% difference in *cox*1 sequences (Supplementary Table S6), while *D. gavium* ($n = 17$) from the Nearctic differed only by up to 0.8%. At the same time, these two species differed by 2.3–4.1%. Isolates of *T. conifera* ($n = 3$) varied up to 0.5% (Supplementary Table S7), while isolates of *Do. lacombeensis* ($n = 2$) had 0.7% intraspecific variation.

4. Discussion

4.1. Generation of new molecular data

Although members of *Diplostomum* are widely distributed and often included in ecological and evolutionary studies, few sequences of adult specimens were available (e.g., Georgieva et al., 2013; Blasco-Costa et al., 2014; Pérez-del-Olmo et al., 2014; Brabec et al., 2015; Locke et al., 2015; Hoogendoorn et al., 2020). Prior to our study, sequences of 28S from morphologically identified adults were only available for three species of *Diplostomum*, two species of *Tylodelphys* and two species of *Austrodiplostomum*. No DNA sequence data were previously available for a member of *Paralaria* or *Dolichorchis*. Here, we provide 28S DNA sequence data from morphologically identified adults of 10 nominal species of *Diplostomum*, five nominal species of *Tylodelphys*, one nominal species of *Austrodiplostomum* and one nominal species of *Dolichorchis*. In total, we provided new ribosomal and mitochondrial DNA sequence data of 15 species/species-level lineages of *Diplostomum*, six species/species-level lineages of *Tylodelphys*, two species/species-level lineages of *Austrodiplostomum*, two species/species-level lineages of *Dolichorchis* and an unknown diplostomid lineage. To the best of our knowledge, this is the first study to generate DNA sequence data of a *Diplostomum* species collected in Chile and the first to report an *Austrodiplostomum* species in the Palaearctic.

4.2. The status of Paralaria

Kraus (1914) established the genus *Paralaria* for *P. clathrata* (type-species) and the newly described *P. pseudoclathrata*, both from otters. In addition, Kraus (1914) noted the presence of a genital cone which could be inverted. Dubois (1937) described *D. alarioides* from the giant river otter *Pteronura brasiliensis* (Gmelin) (syn. *Lutra brasiliensis* Gmelin) collected in Brazil. Later, Dubois (1944) established the genus *Enhydridiplostomum* for *Diplostomum fosteri* McIntosh, 1939 and transferred both *D. fosteri* and *D. alarioides* into *Enhydridiplostomum*. Members of *Enhydridiplostomum* also parasitize otters but lack a genital cone. Although Dubois (1963) maintained *Enhydridiplostomum* as a valid genus, he later changed his opinion and considered *Enhydridiplostomum* a synonym of the subgenus *Paralaria* (see Dubois, 1968). Other authors (e.g., Yamaguti, 1971; Schoop, 1989) viewed *Enhydridiplostomum* as a valid genus. In the most recent systematic revision of the Diplostomidae by Niewiadomska (2002), *Paralaria* is considered a valid genus with *Enhydridiplostomum* as its synonym.

Interestingly, the generic diagnosis of *Paralaria* by Niewiadomska (2002) reflected features characteristic of the former *Enhydridiplostomum*, but not other species of *Paralaria*. Notably, the lack of a genital cone is typical of *P. fosteri* and *P. alarioides*, whereas the type-species *P. clathrata* as well as *P. pseudoclathrata* were originally described and clearly illustrated with a genital cone. The results of our phylogenetic analyses clearly demonstrate *P. alarioides* along with an unidentified species-level lineage, both from otters, as members of *Diplostomum*. The nested position of both species from otters among species from birds in all our analyses likely reflects a secondary evolutionary host-switching event from birds into mammalian hosts (Figs. 1, 2; Supplementary Fig. S1). Based on our molecular phylogenies along with morphological evidence, we return *P. alarioides* to *Diplostomum* as *D. alarioides*. We expect that *P. fosteri* may also belong to *Diplostomum*; however, DNA sequence data are needed for a well-grounded conclusion and nomenclatural action. Considering the current inaccurate generic diagnosis of *Paralaria* provided by

Niewiadomska (2002), we provide an amended diagnosis of the genus.

Paralaria Kraus 1914: Diagnosis (after Niewiadomska, 2002, amended): Body distinctly bipartite; prosoma elongate, spatulate, shorter to equal, rarely longer than claviform opisthosoma. Pseudosuckers present. Ventral sucker smaller than, or similar in size to oral sucker; pharynx large. Holdfast organ oval, elongate, with median slit; its anterior margin extends beyond middle of prosoma. Testes two, oval, trilobed posteriorly; lateral lobes may be subdivided into dorsal and ventral lobes; anterior testis median, asymmetrical, cuneate; posterior larger, may be symmetrical or massive. Ovary oval or reniform, median, in middle or at anterior margin of opisthosoma. Vitelline follicles densely distributed in prosoma, extend from level just posterior to ventral sucker to level of ovary. Copulatory bursa with dorso-subterminal opening. Genital cone present or absent. Hermaphroditic duct opens ventrally in dorsal wall of copulatory bursa. In otters. North and South America. Mesocercariae in anurans. Cercariae with four paracetabular penetration gland-cells; flame-cell formula 2[(1 + 1 + 1) + (1 + 1 + [2])] = 14. Metacercariae of 'diplostomulum' type. Type-species: *P. clathrata* (Diesing, 1850). Other species: *P. pseudoclathrata* (Krause, 1914); *P. fosteri* (McIntosh, 1939).

Diplostomum alarioides was previously reported from the North American river otter *L. canadensis* in Georgia and North Carolina, U.S.A. (Sawyer, 1961; Miller and Harkema, 1968) and Ontario, Canada (Pearson, unpublished) as well as American mink *Neovison vison* (Schreber) from North Carolina (Miller and Harkema, 1964). Our specimens of *D. alarioides* from *L. canadensis* collected in Mississippi closely conform to the original description from specimens collected in Brazil (Dubois, 1937, 1968). *Lontra canadensis* and *Pt. brasiliensis* (type-host) do not overlap in their geographical distributions. However, both species share some overlap in geographical distributions with the Neotropical otter *Lontra longicaudis* (Olfers) (Polechla & Carrillo-Rubio, 2009; Rheingantz et al., 2014; Bouley et al., 2015). We hypothesise that *D. alarioides* from the Nearctic and Neotropics will prove to be separate species once DNA sequence data from the Neotropical forms are available. However, without a genetic comparison description of our specimens as a separate species is premature. Until now, only *D. alarioides* has been reported from the Nearctic. Clustering of the species-level lineage *Diplostomum* sp. VVT3 with *D. alarioides*, along with their 8.8% difference in *cox*1 sequences, indicates the presence of a second species of *Diplostomum* in Nearctic otters. Collection of well-fixed adult specimens are needed for description. It is worth noting that *Diplostomum* sp. 10 (L) is likely conspecific with *Diplostomum* sp. VVT3 (Supplementary Fig. S1; Supplementary Table S3). *Diplostomum* sp. 10 (L) was previously found in the eyes (non-lens) of the rock bass *Ambloplites rupestris* (Rafinesque) and the fathead minnow *Pimephales promelas* (Rafinesque) (Locke et al., 2015).

4.3. Remarks on other Diplostomum species

Diplostomum ardeae sensu Locke et al. (2015) from the great blue heron *Ardea herodias* (Linnaeus) in Canada and *Diplostomum* sp. VVT5 from the little blue heron *Egretta caerulea* (Linnaeus) collected in Mississippi have identical 28S partial sequences and only 0.6% different partial *cox*1 sequences. Our specimens of *Diplostomum* sp. VVT5 do not fit the original morphological description of *D. ardeae* (Dubois, 1969b; Supplementary Fig. S2). The differences include the opisthosoma:prosoma length ratio which is 0.65 in our specimens and 0.47–0.51 in *D. ardeae*, together with the ventral sucker:oral sucker width ratio that is approximately 1.5 in our specimens, whereas suckers are of approximately the same size in *D. ardeae*. Additionally, our specimens lack a strongly defined separation between prosoma and opisthosoma as opposed to the well-defined separation between prosoma and opisthosoma in *D. ardeae* (see Dubois, 1969b; Supplementary Fig. S2). *Diplostomum* sp. VVT5 sequenced here is morphologically closest to *D. scudderi* (syn. *Diplostomum baeri eucaliae* Hoffman & Hundley, 1957); however, the two species have several morphological differences (see Hoffman & Hundley, 1957 and Supplementary Fig. S2) and differ by 2.9% between partial 28S sequences and by 11% between partial *cox*1 sequences (Supplementary Tables S1, S3, S5). Therefore, we believe that *Diplostomum* sp. VVT5 and *D. ardeae sensu* Locke et al. (2015) represent a currently undescribed species. Detailed descriptions of these materials will be published elsewhere.

Diplostomum sp. VVT5 formed a sister branch to all other *Diplostomum* species (Figs. 1, 2; Supplementary Fig. S1), as previously demonstrated in other recent molecular phylogenetic studies (e.g., Locke et al., 2015; Hernández-Mena et al., 2017; Pelegrini et al., 2019; Locke et al., 2020). Some previous studies (e.g., Pelegrini et al., 2019) have suggested that this form may belong to a separate genus; however, Locke et al. (2020) considered it to be a species of *Diplostomum* based on its morphology. Our morphological study of adult *Diplostomum* sp. VVT5 does not provide any evidence supporting its placement in a separate genus. However, it is worth noting that *Diplostomum* sp. VVT5 and *D. lunaschiae* have a weakly bipartite body, similar to *Tylodelphys* spp., whereas many other *Diplostomum* spp. have a distinctly bipartite body (e.g., Shigin, 1993; Dubois, 1968, 1969a, b; Niewiadomska, 2002; Locke et al., 2020; present study).

The morphology of our specimens of *D. huronense* from the kelp gull *Larus dominicanus* Lichtenstein collected in Chile and from the ring-billed gull *Larus delawarensis* Ord collected in Illinois, U.S.A. closely conforms to the original description by La Rue (1927) of specimens from the European herring gull *Larus argentatus* Pontoppidan collected at Lake Huron (Supplementary Table S8; Supplementary Fig. S2). Similarly, the morphology of our specimens of *D. indistinctum* from *La. delawarensis* collected in North Dakota, U.S.A. closely conforms to the original description by Guberlet (1922) of specimens from *La. delawarensis* collected in Oklahoma, U.S.A. (Supplementary Table S8; Supplementary Fig. S2). Our *cox*1 sequences of *D. huronense* differ from the previously published sequences of *D. huronense sensu* Galazzo et al. (2002) by 9.9–11% (Supplementary Tables S3 and S5). Locke et al. (2010a) published *cox*1 sequences of adult specimens of *Diplostomum* sp. 1 (M); *Diplostomum* sp. 1 (M) is conspecific with our isolates of *D. huronense* based on the similarity of partial *cox*1 sequences (0.3–1.9%; Supplementary Tables S3 and S5).

Similarly, our *cox*1 sequences from adult specimens of *D. indistinctum* differ by 12.2–12.7% from previously published sequences of *D. indistinctum sensu* Galazzo et al. (2002) (Supplementary Tables S3 and S5). *Diplostomum* sp. 4 (M), another form sequenced from adult specimens (e.g., HM064700), turned out to be conspecific with our isolates of *D. indistinctum* based on the high similarity of *cox*1 sequences (0.6–1.4% divergence; Supplementary Tables S3 and S5).

Galazzo et al. (2002) sequenced the ITS region of *D. huronense* and *D. indistinctum* and studied morphology of the adult forms from experimentally infected *La. delawarensis*. Subsequently Locke et al. (2010a, 2010b) generated *cox*1 data from *D. indistinctum* studied by Galazzo et al. (2002) and additional specimens of *D. huronense* and *D. indistinctum* identified, in part, based on comparison of ITS region sequences, which matched sequences from Galazzo et al. (2002). Galazzo et al. (2002) stated that their specimens were nearly morphologically identical to the original descriptions. Most measurements provided by Galazzo et al. (2002) seem to be consistent with *D. huronense* as described by La Rue (1927). Unfortunately, neither La Rue (1927) nor Galazzo provided ratios of many characters often used for species differentiations (e.g., oral sucker:ventral sucker width ratio). Based on the

line drawings, *D. huronense* described by La Rue has an oral sucker: ventral sucker width ratio of 0.64; in contrast, *D. huronense* illustrated by Galazzo et al. (2002) has oral sucker:ventral sucker width ratio of 0.9 (Supplementary Table S8). Our specimens of *D. huronense* have an oral sucker:ventral sucker width ratio of 0.68–0.80. La Rue (1927) described the vitellarium of *D. huronense* as extending anteriorly to at least the level of the ventral sucker. The vitellarium in the specimen of *D. huronense* illustrated by Galazzo et al. (2002) does not extend beyond the level of the holdfast organ. In contrast, the vitellarium in some of our specimens of *D. huronense* extends anteriorly to the level of the ventral sucker (Supplementary Fig. S2). In our opinion, the sucker ratios, and the anterior extent of vitellarium provide evidence that our specimens fit the original description of *D. huronense* better than those reported by Galazzo et al. (2002).

Guberlet (1922) illustrated *D. indistinctum* with a noticeable narrowing of the anterior part of the opisthosoma immediately posterior to the prosoma (approximately half the width of the widest part of the opisthosoma). The specimen of *D. indistinctum* illustrated by Galazzo et al. (2002) lacked such a narrowing, whereas all our specimens of *D. indistinctum* have a narrowing of the anterior part of the opisthosoma (Supplementary Table S8; Supplementary Fig. S2). In addition, the oral sucker length:pharynx length ratio of *D. indistinctum* based on the illustrations provided by Guberlet (1922) is 0.78–1.06, whereas the oral sucker length: pharynx length ratio of *D. indistinctum* based on the illustration by Galazzo et al. (2002) is 1.66. The oral sucker length:pharynx length ratio of our specimens of *D. indistinctum* is 1.00–1.13, which is much closer to that in the original description than in the material described by Galazzo et al. (2002) (Supplementary Table S8). In our opinion, the presence of a narrowing of the opisthosoma and more similar character ratios compared with the original description support the identification of our specimens as *D. indistinctum*.

Sequences from specimens of *Diplostomum* sp. VVT2 from the yellow perch *Perca flavescens* Mitchill, green sunfish *Lepomis cyanellus* Rafinesque and pumpkinseed *Lepomis gibbosus* (Linnaeus) from Minnesota formed a 100% supported clade with a sequence of *D. baeri* sensu Galazzo et al. (2002) (MF142196) from an isolate collected from *Pe. flavescens* in Michigan (Ubels et al., 2018) (Table 2). The clade that included *Diplostomum* sp. VVT2 + D. baeri sensu Galazzo et al. (2002) from the Nearctic was separate from other clades of the *D. baeri* species complex containing sequences from Palaearctic only (Supplementary Fig. S1). *Diplostomum baeri* was originally described from the long-tailed jaeger *Stercorarius longicaudus* Vieillot collected at Lake Geneva (France and Switzerland) (Dubois, 1937). We find it unlikely that *D. baeri sensu* Galazzo et al. (2002) (and other conspecific lineages identified as *D. baeri* from the Nearctic; Table 2) as well as the *Diplostomum* sp. VVT2 belong to *D. baeri*. We hypothesise that *D. baeri sensu* Galazzo et al. (2002) from Nearctic likely represents a new species. However, sequences of adult specimens of *D. baeri* from the type-host and preferably close to type-locality are needed to define which lineage actually represents *D. baeri*. It is worth noting that specimens of *Diplostomum* sp. VVT2 were found encysted on the skin as well as in the eyes (Table 1). The larvae collected from the skin were encapsulated in melanized cysts.

Diplostomum mergi alascense Dubois, 1969 was originally described from red-breasted merganser *Mergus serrator* (Linnaeus) collected in Alaska (Dubois, 1969a). This taxon can be most easily distinguished from *Diplostomum mergi mergi* Dubois, 1932, described from *M. serrator* collected in Europe, based on the oral: ventral sucker ratio (suckers about the same size in *D. m. alascense* while in *D. m. mergi* the ventral sucker is larger than the oral sucker) and the anterior extent of vitellarium (vitellarium extending to about the level of the ventral sucker in *D. m. alascense versus* vitellarium extending anterior to the level of the ventral sucker in *D. m. mergi*) (Dubois, 1932, 1969a). Our specimens of *D. m. alascense* clearly morphologically conform to the original description and differ by at least 9.1% in sequences of *cox*1 from larval specimens of the *D. mergi* complex collected and sequenced in the Palaearctic (Supplementary Table S9). Furthermore, in our phylogenetic analysis based on *cox*1 gene, Nearctic *D. m. alascense* was positioned separately from the *D. mergi* complex from the Palaearctic (Supplementary Fig. S1). Considering the morphological and genetic differences, we elevate *D. m. alascense* to the level of species as *D. alascense* n. comb.

In total, we have provided species-level identifications for seven species of *Diplostomum* spp. based on adult morphology which were previously published as genetic lineages only (Table 2; Supplementary Table S10).

Table 2
Diplostomum species/species-level lineages sequenced in the present study and the corresponding previously accepted species/species-level lineage names based on BLAST search results of *cox*1 sequences in GenBank. References to the original designations of species-level lineages are provided.

Taxon	Corresponding previously accepted species/species-level lineage	Reference
Diplostomum alarioides[a]	–	Present study
Diplostomum alascense	*Diplostomum* sp. 2	Moszczynska et al. (2009)
Diplostomum gavium	*Diplostomum* sp. 3	Moszczynska et al. (2009)
	Diplostomum baeri	Ubels et al. (2018)
Diplostomum marshali	*Diplostomum* sp. A	Gordy and Hanington (2019)
Diplostomum huronense	*Diplostomum* sp. 1	Moszczynska et al. (2009)
Diplostomum indistinctum	*Diplostomum* sp. 4	Moszczynska et al. (2009)
	D. baeri	Ubels et al. (2018)
Diplostomum pseudospathaceum	*D. pseudospathaceum*	Behrmann-Godel (2013); Georgieva et al. (2013)
Diplostomum rauschi	*Diplostomum* sp. Lineage 2	Blasco-Costa et al. (2014)
Diplostomum scudderi	*Diplostomum* sp. 13	Locke et al. (2015)
	Diplostomum sp. C	Gordy and Hanington (2019)
Diplostomum spathaceum	*D. spathaceum*	Georgieva et al. (2013)
	D. spathaceum LIN1	Blasco-Costa et al. (2014)
	Diplostomum paracaudum	Behrmann-Godel (2013)
Diplostomum sp. VVT1	–	Present study
Diplostomum sp. VVT2	*D. baeri sensu* Galazzo et al. (2002)	Galazzo et al. (2002)
	D. aff. *baeri* LIN2	Gordy et al. (2016)
	D. baeri complex LIN2	Gordy and Hanington (2019)
Diplostomum sp. VVT3	*Diplostomum* sp. 10	Locke et al. (2015)
Diplostomum sp. VVT4	–	Present study
Diplostomum sp. VVT5	*Diplostomum ardeae sensu* Locke et al. (2015)	Locke et al. (2015)

[a] Formerly included in *Paralaria*.

4.4. Non-monophyly of Tylodelphys

Our phylogenetic analyses positioned members of *Austrodiplostomum* nested within *Tylodelphys* (Figs. 1, 3, 4), which indi-

cates the paraphyletic nature of *Tylodelphys*, similar to what has been shown previously (e.g., Locke et al., 2015; Sereno-Uribe et al., 2019b). For instance, the phylogenetic analyses conducted by Sereno-Uribe et al. (2019b), which included only a few *Tylodelphys* spp., demonstrated a non-monophyly of *Tylodelphys* due to the position of *Austrodiplostomum*. *Austrodiplostomum* spp. and *Tylodelphys* spp. have some morphological differences. *Austrodiplostomum* spp. are characterised by a heavily reduced ventral sucker or no ventral sucker at all, and the lack a genital cone. In contrast, *Tylodelphys* spp. typically have a small, but well-developed ventral sucker and a small genital cone (e.g., Dubois, 1938; Szidat and Nani, 1951; Niewiadomska, 2002; Dronen, 2009; Sereno-Uribe et al., 2019a, 2019b). It is worth noting, however, that cercariae of *Austrodiplostomum* spp. are known to possess ventral suckers (e.g., Rosser et al., 2016a; López-Hernández et al., 2019).

Our analysis (Fig. 3) separated *Tylodelphys* spp. into two distinct clades. The first clade (85% support) included majority of *Tylodelphys* (e.g., *T. conifera* and *T. immer*), while the second clade (89% support) only contained *T.* cf. *americana* and *Tylodelphys* sp. 4 (M). *Tylodelphys* cf. *americana* (which has a well-developed ventral sucker and a small genital cone) is characterised by typical *Tylodelphys* morphology and we failed to find morphological features which would warrant its placement into a genus separate from *Tylodelphys*. On the other hand, adult *Austrodiplostomum* spp. have clear morphological differences from adult digeneans from both *Tylodelphys* clades. Based on the results of our phylogenetic analysis, *T.* cf. *americana* and *Tylodelphys* sp. 4, as well as other members of *Tylodelphys* clade T-I in the analysis of *cox*1 (Fig. 4), appear to belong to a separate, genus-level lineage. However, as mentioned above, currently available data are insufficient for a systematic action. Additional morphological and life cycle data on these taxa are necessary to erect a new genus in the future. Therefore, we provisionally maintain *T.* cf. *americana* and *Tylodelphys* sp. 4 (M) within *Tylodelphys*.

The genus *Austrodiplostomum* was originally established for *A. mordax* from the Neotropical cormorant *Phalacrocorax brasilianus* (Gmelin). The genus includes only two species, *A. mordax* and *A. compactum* (syn. *Austrodiplostomum ostrowskiae* Dronen, 2009), parasitic in cormorants of the genus *Phalacrocorax* Brisson (syn. *Nannopterum* (Gmelin)) in the Neotropics (Szidat and Nani, 1951; Sereno-Uribe et al., 2019b). However, larval stages of *Austrodiplostomum* spp. have been identified as far north as the southern United States (Rosser et al., 2016a).

To the best of our knowledge, no member of *Austrodiplostomum* has been previously reported from pelicans. However, two morphologically similar genera *Bursacetabulus* Dronen, Tehrany, & Wardle, 1999 and *Bursatintinnabulus* Tehrany, Dronen & Wardle, 1999 were described based on specimens from the brown pelican *Pelecanus occidentalis* Linnaeus and the northern gannet *Morus bassanus* (Linnaeus), respectively, in the Nearctic (Dronen et al., 1999; Tehrany et al., 1999). Similar to the former species of *Austrodiplostomum*, members of *Bursacetabulus* and *Bursatintinnabulus* lack a ventral sucker. However, members of *Bursacetabulus* and *Bursatintinnabulus* possess a sucker-like copulatory bursa. Our specimens of *Austrodiplostomum* sp. VVT1 from the great white pelican *Pe. onocrotalus* clearly lack a ventral sucker. However, the relatively poor condition of our specimens does not allow us to unequivocally establish whether the copulatory bursa of *Austrodiplostomum* sp. VVT1 is sucker-like. It would not be surprising if *Bursacetabulus* and *Bursatintinnabulus* are found to be synonyms of *Austrodiplostomum*. However, this hypothesis needs to be tested with DNA sequence data from well-fixed adult specimens of the type-species of both genera (i.e., *Bursacetabulus pelecanus* Dronen, Tehrany & Wardle, 1999 and *Bursatintinnabulus macrobursus* (Dronen, Tehrany, & Wardle, 1999)).

4.5. Remarks on Tylodelphys

Tylodelphys podicipina robrauschi Dubois, 1969 was originally described as a subspecies of *Tylodelphys podicipina* (Kozicka & Niewiadomska, 1960) based on specimens collected from the red-necked grebe *Podiceps grisegena* (Boddaert) in Alaska (Dubois, 1969a). The morphology of *T. p. robrauschi* most notably differs from *T. p. podicipina* in the extent of the vitellarium; the vitellarium extends to approximately the level of the ventral sucker in *T. p. robrauschi* (Dubois, 1969a), while in *T. p. podicipina* it extends anteriorly to approximately halfway between the oral and ventral suckers (Kozicka and Niewiadomska, 1960). Heneberg and Sitko (2021) proposed *T. immer* to be a junior synonym of *T. p. podicipina* based on an inaccurate comparison of ribosomal data; while the authors claimed the ITS2 sequences of *T. immer* and *T. p. podicipina* were identicial, the GenBank sequences they refer to, are not identical. Further, Heneberg and Sitko (2021) failed to compare *cox*1 sequences of *T. immer* and *T. p. podicipina*. Our comparison of *cox*1 sequences from *T. p. podicipina*, *T. p. robrauschi* and *T. immer* revealed at least of 8.8% difference between these species (Supplementary Table S11). Therefore, we reject the synonymizaton of *T. immer* with *T. p. podicipina*. Based on morphological differences (e.g., distribution of the vitellarium) and the level of genetic divergence (Supplementary Table S11), we elevate *T. p. robrauschi* to full species rank as *Tylodelphys robrauschi* Dubois, 1969 n. comb.

The 28S DNA sequences are also available from *T. darbyi* from New Zealand (Blasco-Costa et al., 2017); however, inclusion of these sequences would require trimming of our alignment to a much shorter length (777 bp) than used in our 28S analysis (1,116 bp).

To summarise, due to the availability of adult stages, we were able to provide species-level identifications for three genetic lineages of *Tylodelphys* that were previously sequenced only from unidentified larvae (Table 3; Supplementary Table S10).

4.6. Remarks on Dolichorchis and the Diplostominae

As previously demonstrated by other authors (e.g., Achatz et al., 2021a; Blasco-Costa and Locke, 2017; Locke et al., 2018), the

Table 3
Tylodelphys and *Austrodiplostomum* species/species-level lineages sequenced in the present study and the corresponding previously accepted species/species-level lineage names based off BLAST search results of *cox*1 sequences in GenBank. References to the original designations of species-level lineages are provided.

Taxon	Corresponding previously accepted species/species-level lineage	Reference
Austrodiplostomum compactum	*A. compactum*	Sereno-Uribe et al. (2019b)
	Austrodiplostomum ostrowskiae	O'Hear et al. (2014)
	Austrodiplostomum sp.	Farias et al. (unpublished)
Austrodiplostomum sp. VVT1	–	Present study
Tylodelphys cf. *americana*	*Tylodelphys* sp.	Pelegrini et al. (2019)
Tylodelphys conifera	*Tylodelphys* sp. A	Gordy and Hanington (2016)
Tylodelphys immer	*T. immer*	Locke et al. (2018)
Tylodelphys robrauschi n. comb.	*Tylodelphys* sp. 3	Locke et al. (2015)
Tylodelphys scheuringi	*T. scheuringi*	Moszczynska et al. (2009)
Tylodelphys sp. VVT1	–	Present study

Diplostominae was non-monophyletic in our broader analysis of 28S (Fig. 1). Despite the general morphological similarity of *Tylodelphys* and *Dolichorchis*, these two genera were not positioned together in the phylogeny (Fig. 1). It should be noted that the two genera differ in the structure of the anterior testis (asymmetrical in *Dolichorchis* spp. *versus* symmetrical in *Tylodelphys* spp.) and often in the distinction between prosoma and opisthosoma (body distinctly bipartite in *Dolichorchis* spp. *versus* body typically indistinctly bipartite in *Tylodelphys* spp.).

Members of the Diplostominae were positioned in three distinct clades in our analysis: *Diplostomum* + *Tylodelphys*; *Dolichorchis* + *Neodiplostomum* + *Sphincterodiplostomum*; and *Hysteromorpha* Lutz, 1931. Our review of morphology did not demonstrate any obvious morphological features of adult stages which would unite *Dolichorchis*, *Neodiplostomum* and *Sphincterodiplostomum* separately from *Alaria*, *Diplostomum* and *Tylodelphys*.

Only two species of *Dolichorchis* are known from the New World (*Do. lacombeensis* and *Dolichorchis bonariensis* Ostrowski de Núñez, 1970). Our specimens of *Do. lacombeensis* from *Ardea cocoi* (Linnaeus) closely conform to the original description of specimens from *Ar. cocoi* collected in Argentina by Lunaschi and Drago (2006). Our specimen of *Dolichorchis* sp. VVT1 from the bare-faced ibis *Phimosus infuscatus* (Lichtenstein) collected in Brazil was too immature for accurate species identification. However, we suspect that *Dolichorchis* sp. VVT1 represents a novel species-level lineage. *Dolichorchis lacombeensis* and *Dolichorchis* sp. VVT1 are clearly separate lineages based on genetic divergence comparisons; the two species differ by 1% in sequences of 28S and 12.9–13.6% in sequences of cox1. *Dolichorchis bonariensis* has only been reported from cormorants (order Suliformes Sharpe), whereas *Dolichorchis* sp. VVT1 was collected from an ibis (order Pelecaniformes Sharpe). On the other hand, our immature specimens may be the result of accidental infection. This is the first report of a species of *Dolichorchis* outside of Argentina in the New World (Fernandes et al., 2015).

4.7. Pairwise comparisons

Many of the DNA sequences of many *Diplostomum* spp., *Tylodelphys* spp. and *Austrodiplostomum* spp. currently available in GenBank originate from larval stages, which, for the most part, cannot be reliably identified to the species based on morphology. Comparisons of numerous previously published sequences have also suggested poor quality of some sequence data in the GenBank database (i.e., numerous variable sites and indels in the protein-coding gene *cox*1). Adequate comparisons should use only high-quality sequence data, preferably from morphologically identified adults.

The interspecific divergence levels among 28S sequences within *Diplostomum*, *Tylodelphys, Austrodiplostomum* and *Dolichorchis* included in this study (0–3.7%) were similar to that demonstrated within other genera of diplostomoideans (0–4.4%) (e.g., Achatz et al., 2020 and references therein; Tkach et al., 2020).

Interspecific divergence levels in partial *cox*1 sequences of adult *Diplostomum* spp. (2.3–16.3%), *Tylodelphys* spp. (4.4–14.6%), *Austrodiplostomum* spp. (9.1–13%) and *Dolichorchis* (12.9–13.6%) and corresponding larvae (Supplementary Table S5) found in our study were similar to those demonstrated within other diplostomoidean genera (3.4–19.8%) (e.g., Achatz et al., 2020 and references therein; Tkach et al., 2020). The interspecific differences among *cox*1 sequences from adult specimens of *Diplostomum* spp. and corresponding larvae included in our study (2.3–16.3%) were similar to or lower than those reported by Locke et al. (2010a) (9.9–15.1%) and Hoogendoorn et al. (2020) (11.8–14.7%).

However, the interspecific differences in *cox*1 from adult specimens in the present study was slightly higher than those provided by Georgieva et al. (2013) (4.6–14.9%) and Selbach et al. (2015) (4.3–14.7%). It is worth noting, that these studies (Locke et al., 2010a; Georgieva et al., 2013; Selbach et al., 2015; Hoogendoorn et al., 2020) were primarily based on larval specimens. The interspecific differences among *cox*1 sequences from adult specimens of *Tylodelphys* and *Austrodiplostomum* along with the corresponding larvae (4.4–14.6%) obtained in the present study were similar to those previously reported by Blasco-Costa et al. (2017) (8.0–16.5%) and Sereno-Uribe et al. (2019a) (5.0–15%).

Interestingly, Locke et al. (2015) reported 2.10–11.22% interspecific difference among *cox*1 sequences (*Diplostomum* and *Tylodelphys* spp.) between the nearest neighbours in their analysis. Our comparison of partial *cox*1 sequences of morphologically identified adults and corresponding larvae did not show interspecific divergence lower than 2.3% (Supplementary Tables S5, S6). However, it is important to note that almost all comparisons of *cox*1 sequences of species with morphologically identified adults differed by at least 4.4%. The only exception was *D. gavium* and *D. pseudospathaceum* which differ by 2.3–4.1% (Supplementary Table S6) while being very distinct morphologically. It is worth noting that comparisons among members of other diplostomoidean genera identified based on adult morphology typically yield interspecific divergence values much greater than 2.1% (e.g., Hernández-Mena et al., 2014, Achatz et al., 2019a).

4.8. Host associations

Our phylogenetic analyses (Figs. 1, 2; Supplementary Fig. S1) provided evidence of multiple host-switching events among *Diplostomum* spp. The majority of adult *Diplostomum* isolates included in our analyses were collected from the Laridae Rafinesque (gulls). However, our analysis also included *Diplostomum* spp. collected from birds belonging to the Ardeidae Leach (herons), Recurvirostridae Bonaparte (avocets), Gaviidae Forster (loons), Scolopacidae Rafinesque (sandpipers), and Anatidae Leach (ducks), as well as from the Mustelidae Waldheim (otters). Notably, in the 28S trees *Diplostomum* sp. VVT5 (=*D. ardeae sensu* Locke et al., 2015) from *E. caerulea* formed a sister group to the weakly supported clade containing all other *Diplostomum* species (Figs. 1, 2). In the *cox*1 tree, *Diplostomum* sp. VVT5 formed a clade with *D. lunaschiae*, a parasite of the rufescent tiger heron *Tigrisoma lineatum* (Boddaert). Unfortunately, a 28S sequence of *D. lunaschiae* is not available. The phylogenetic position of *Diplostomum* sp. VVT5 in all analyses along with the position of *D. lunaschiae* in the *cox*1 analysis suggests that the ancestral host of *Diplostomum* may have been an ardeid.

Diplostomum spp. from otters and mergansers formed three of the branches within the *Diplostomum* clade, representing separate secondary host-switching events (Fig. 2). However, we did not collect adults of two *Diplostomum* spp. (VVT1, VVT4) clustered in the clade with *D. scudderi*, a parasite of ducks, and *D. marshalli*, a parasite of sandpipers. We posit that these species also parasitize anatids and scolopacids. The diversity of *Diplostomum* spp. in gulls and the presence of more than one clade of species from gulls in our *cox*1 analysis (Supplementary Fig. S1) suggests a long history of radiation within gull hosts. At the same time, in the 28S analysis all *Diplostomum* isolates from gulls formed a single, strongly supported clade (Fig. 2), which suggests that the transition to gulls may have occurred only once. However, this notion might change in the future because several species and species-level lineages included in the *cox*1 analysis lack corresponding 28S data. In addition, nine *Diplostomum* species-level lineages included in the second 28S analysis (Fig. 2) have DNA sequence data available only from larval stages and their definitive hosts remain unknown. It can be anticipated that more comprehensive sequence data will

reveal additional host-switching events in the evolutionary history of this large, cosmopolitan genus.

Our analyses also revealed multiple host-switching events within *Tylodelphys* and *Austrodiplostomum* (Fig. 3). Members of the genus included in our analyses were collected from the Podicipedidae Bonaparte (grebes), Gaviidae Coues (loons), Ciconiidae Grey (storks), Pelecanidae Rafinesque (pelicans) and Phalacrocoracidae Reichenbach (cormorants). In the analysis of 28S (Fig. 3), adult *Tylodelphys* spp. from grebes and loons formed a clade separate from *Tylodelphys* and *Austrodiplostomum* parasitic in storks and cormorants + pelicans. Within this clade, it appears that *Tylodelphys* species likely transitioned from grebes into loons. *Tylodelphys* cf. *americana* from the jabiru *Jabiru mycteria* (Lichtenstein) formed a sister group to *Austrodiplostomum* spp. from cormorants and pelicans. Interestingly, *Austrodiplostomum* sp. VVT1 from pelicans was nested among multiple *Tylodelphys* spp. from cormorants in both 28S and *cox*1 analyses (Figs. 3, 4) suggesting a transition from cormorants to pelicans.

4.9. Biogeography

Previous studies (e.g., Locke et al., 2015, 2020; Hoogendoorn et al., 2020) have demonstrated that some *Diplostomum* spp. are distributed across multiple biogeographic realms (i.e., Palaearctic and Afrotropics; Nearctic and Neotropics) and continents (i.e., Europe and Asia; Africa and Asia). Gibson (1996) proposed that many *Diplostomum* spp. may have a Holarctic distribution based on the mobility and distribution of their avian hosts; however, this has not been previously tested based on molecular data. To date, only Locke et al. (2020) has demonstrated using molecular data that a species of *Diplostomum* (i.e., *D. ardeae sensu* Locke et al. (2015)) is distributed in the Nearctic + Neotropics. In the latter study, the Nearctic samples were collected in Quebec, Canada, and those from the Neotropics were collected in Puerto Rico, near the northern edge of the Neotropics. Our Nearctic samples of *D. huronense* originated from the northern United States and the Neotropic specimens were collected in Chile, substantially farther south than Puerto Rico. This provides a convincing evidence that some *Diplostomum* spp. are broadly distributed throughout the New World.

The broad distribution of *Diplostomum* may be promoted, in part, by the extensive overlapping of bird migration flyways. For instance, the overlap in Atlantic Americas and East Atlantic flyways can facilitate dispersal of species between the New World and Europe (Olsen et al., 2006; Dusek et al., 2014; Ramey et al., 2015, 2016). Blasco-Costa et al. (2014) suggested that the common ancestor of *Diplostomum* spp. may have originated in North America and subsequently dispersed into the Palaearctic. The position of *Diplostomum* sp. VVT5 in our 28S and *cox*1 analyses (Figs. 1, 2; Supplementary Fig. S1), together with *D. lunaschiae* in the *cox*1, provide some support for this hypothesis (Figs. 1, 2). This is further supported by the presence of three other clades of *Diplostomum* spp. from the Nearctic in the broader clade of *Diplostomum* (Fig. 2). Most *Diplostomum* spp. from the Palaearctic formed a single, strongly supported clade in our analysis of 28S (Fig. 2). This clade also contained *Diplostomum* spp. from the Nearctic, Neotropics and Afrotropics. Only *D. phoxini*, from the Palaearctic, appeared on the tree separately from other Palaearctic forms, in a clade with *Diplostomum* sp. VVT2 from the Nearctic. Patterns related to biogeography of *Diplostomum* spp. were less pronounced in the *cox*1 analysis (Supplementary Fig. S1).

The majority of *Tylodelphys* and *Austrodiplostomum* spp. included in our 28S analyses originated from the New World. However, the single species from the Palaearctic (*Austrodiplostomum* sp. VVT1) was deeply nested within a clade of species from the Nearctic + Neotropics (Fig. 3). This provides some evidence that the ancestor of this group also likely originated in the New World. However, our understanding of the biogeographical patterns within these genera may potentially change once ribosomal data (i.e., 28S) from a greater diversity of species from other biogeographical realms become available. Similar to *Diplostomum*, the *cox*1 results did not reveal any well-defined biogeographical patterns for *Tylodelphys* spp. (Fig. 4).

5. Conclusions

We provided new ribosomal and mitochondrial DNA sequence data of 15 species/species-level lineages of *Diplostomum*, six species/species-level lineages of *Tylodelphys*, two species/species-level lineages of *Austrodiplostomum*, two species/species-level lineages of *Dolichorchis* and one species-level lineage of an unidentified diplostomid. Our study has thus significantly expanded the available sequence data from morphologically identified adult stages of *Diplostomum* and *Tylodelphys*.

Our phylogenetic analyses demonstrated some incongruences between the results based on 28S and *cox*1 sequence data (Figs. 2–4; Supplementary Fig. S1). For instance, *Diplostomum* spp. from gulls formed a monophyletic clade in the 28S tree (Fig. 2) but appear in more than one clade in the *cox*1 tree (Supplementary Fig. S1). Similarly, *D. marshalli* and *D. scudderi* appeared within the same 100% supported clade in the 28S tree while being separated in clades D-II and D-XIII within the major polytomy in the *cox*1 tree. Overall, the *cox*1 trees had high support for distal branches and low support for basal branches, while 28S trees were somewhat better resolved despite still containing polytomies close to the base of the trees. A similar discordance and notably lower branch support in *cox*1-based phylogenies have been previously reported in studies of *Diplostomum* spp. (e.g., Brabec et al., 2015, Hoogendoorn et al., 2020) and other diplostomoideans (e.g., Hernández-Mena et al., 2017; Achatz et al., 2019a, 2020; Hoogendoorn et al., 2019, 2020; Heneberg et al., 2020). Although *cox*1 sequences remain an excellent source of data for species differentiation, caution must be taken when *cox*1 sequences are used for phylogenetic inference at taxonomic levels above genus. Unfortunately, the avilability of 28S sequence data for diplostomoideans is lagging far behind the *cox*1 data, which are being generated at a much higher rate.

Our data demonstrated that *P. alarioides* along with an unidentified digenean, both from otters, belong to *Diplostomum* (Fig. 2; Supplementary Fig. S1). Importantly, molecular phylogenetic analyses have demonstrated the non-monophyly of *Tylodelphys* and suggested the need to establish a novel genus which contains *T.* cf. *americana*, despite the lack of clear morphological differences in adult stages (Figs. 3, 4).

Our broader 28S analysis of diplostomoideans (Fig. 1) positioned *Dolichorchis* spp. and the unknown diplostomid separate from *Diplostomum* spp. and *Tylodelphys* spp.; the results of the 28S analysis supported *Dolichorchis* as a distinct genus. Sequences of 10 species of *Diplostomum* and *Tylodelphys* identified based on adult morphology matched previously published sequences of species/species-level lineages identified based on larval morphology only or not identified to species (Tables 2, 3; Supplementary Table S10). In addition, we provided the first DNA sequence data for three species/species-level lineages of *Diplostomum*, one species-level lineage of *Tylodelphys*, one species-level lineage of *Austrodiplostomum*, two species/species-level lineages of *Dolichorchis* and one currently unknown diplostomid.

The results of our phylogenetic analyses revealed multiple host-switching events, notably from avian definitive hosts to otters along with switching between major avian groups. In addition, our results provide evidence for multiple dispersal events between

biogeographical realms in the evolutionary history of the *Diplostomum*, *Tylodelphys* and *Austrodiplostomum* (Figs. 2, 3), together with the molecular evidence that some species are distributed throughout the Nearctic and Neotropics.

Future studies should provide additional DNA sequence data from well-fixed adult specimens to study the interrelationships within the Diplostomidae comprehensively. This approach will help clarify the taxonomy of a large number (at least 30) of yet unidentified species-level lineages of *Diplostomum*, *Tylodelphys* and *Austrodiplostomum* with predominantly Nearctic distribution (Moszczynska et al., 2009; Locke et al., 2010a, 2010b, 2015; Gordy and Hanington, 2019). Furthermore, of particular interest is species identification within the two species complexes: the *D. baeri* species complex as defined by Blasco-Costa et al. (2014) for which the phylogenetic reconstructions have suggested North America as an ancestral area (see Blasco-Costa et al., 2014) and the *D. mergi* species complex as defined by Georgieva et al. (2013) and Selbach et al. (2015) which may appear to be restricted to the Palaearctic. Finally, many lineages of *Tylodelphys* with sequence data reported from larval stages, predominantly metacercariae, still await further taxonomic scrutiny.

Although larval *Diplostomum* spp. and *Tylodelphys* spp. are very commonly reported in ecological studies of fish and mollusk intermediate hosts, limited data from adult *Diplostomum* spp. and *Tylodelphys* spp. were available. Our newly generated DNA sequences from morphologically identified adults significantly expand the reference set of diplostomids at the species level. This knowledge is critical for future ecological studies of larval diplostomids, many of which are agents of fish diseases.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2021.06.002.

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