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Diversity, Phylogenetics And Life Cycles Of Diplostomoidean (Digenea: Diplostomoidea) Of The Upper Midwest

Jakson Martens

DIVERSITY, PHYLOGENETICS AND LIFE CYCLES OF DIPLOSTOMOIDEAN (DIGENEA: DIPLOSTOMOIDEA) OF THE UPPER MIDWEST

by

Jakson Martens

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This document, submitted in partial fulfillment of the requirements for the degree from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

Dr. Vasyl Tkach_____

Dr. Jefferson Vaughan_____

Dr. Jeffrey Bell_____

This document is being submitted by the appointed advisory committee as having met all the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

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Department Biology

Degree Master of Science

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Jakson Martens

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ABSTRACT

The superfamily Diplostomoidea Poirier, 1886 consists of a diverse, globally distributed group of parasitic flatworms parasitic as adults in reptiles, birds, mammals, and very rarely, fish. The superfamily Diplostomoidea has a tumultuous history full of genus synonymizations and resurrections. Currently, the superfamily consists of 39 genera parasitizing primarily piscivorous animals as adults to include birds, reptiles, and mammals. This group of parasitic worms remains a highly active area of research and has recently undergone several major systematic changes including the abandonment of a subfamily-based system. Additionally, the influx of data entries in GenBank has lead to confusing nomenclature and misidentifications at the genus level. We have clarified the identity of species belonging to *Posthodiplostomum, Diplostmum, Austrodiplostomum, Tylodelphys*, *Neodiplostomum, Crassiphiala,* and *Neofibricola* through molecular tools and the description of new species.

CHAPTER 1:

Introduction

The superfamily Diplostomoidea Poirier, 1886 consists of a diverse, globally distributed group of parasitic flatworms. (e.g., Dubois, 1936a; Niewiadomska, 2002a–g; Blasco-Costa & Locke, 2017) parasitic as adults in reptiles, birds, mammals, and very rarely, fish. Diplostomoideans feature a unique holdfast organ thought to play a role in digestion and attachment to the host gastrointestinal tract. Diplostomoideans have complex life cycles and typically utilize two intermediate hosts before completing their lifecycle in the definitive host (Fig. 1). The first intermediate host is always a mollusk, usually a snail. Snails are infected by motile larvae called miracidia which may hatch from the egg in water. Accordingly, the miracidium penetrates the snail body. Inside the snail, miracidium transforms into a mother sporocyst capable of asexual reproduction. In the Diplostomoidea, the mother sporocyst produces daughter sporocysts which, in turn, produce a very large number of motile, free-swimming cercariae. Cercariae present a wide range of morphology across the Diplostomoidea but usually they consist of a body and a tail used for propulsion. These motile cercaria leave the snail and seek out the second intermediate host to encyst upon or within. Once encysted in the second intermediate host the parasite develops into a dormant stage referred to as the metacercaria which is infective to the definitive host. A variety of invertebrate and vertebrate animals may play a role of the second intermediate host of diplostomoideans, including mollusks, annelids, teleost fishes (most commonly) and amphibians. Reptiles (e.g., Uhrig et al., 2015) and small mammals can serve as paratenic hosts. Several diseases are associated with the infection of larval diplostomoideans on or within their second intermediate host. Species of

Crassiphiala Van Haitsma, 1925, *Diplostomum* von Nordmann, 1832, *Posthodiplostomum* Dubois, 1936, *Tylodelphys* Diesing, 1850 and *Uvulifer* Yamaguti, 1934 are recognized as common agents of black spot disease and ocular diplostomiasis (e.g., Hunter, 1933; Lemly & Esch, 1984; Chappell et al., 1994; Overstreet & Curran, 2004; Bullard et al., 2008; Matisz et al., 2010; McAllister et al., 2013). The definitive host is typically a piscivorous bird; however, reptiles, mammals and non-piscivorous birds, may also be parasitized (Dubois, 1936a, b;

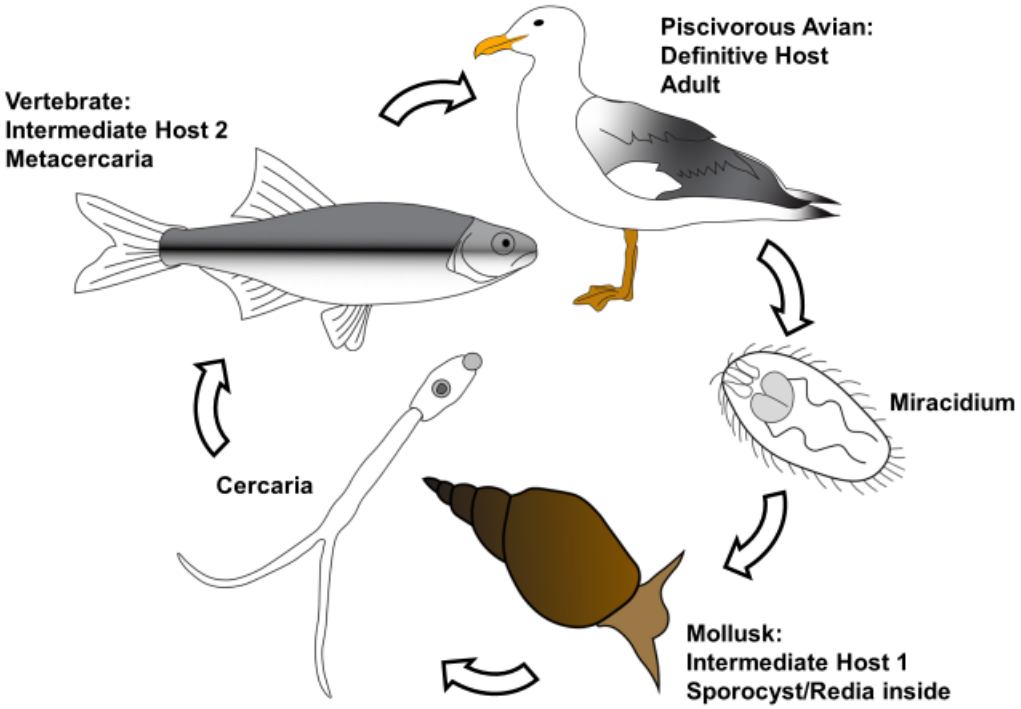


Figure 1. Normal life cycle of a diplostomid parasite. Arrows indicate transition to next stage of development. Eggs and sporocyst/redia not shown.

Niewiadomska, 2002a–g; Blasco-Costa & Locke, 2017).

The superfamily Diplostomoidea has a tumultuous history full of genus synonymizations and resurrections (Achatz et al., 2019a, c, 2020a). Currently, the superfamily consists of 39 genera parasitizing primarily piscivorous animals as adults to include birds, reptiles, and mammals. However, it is important to note that non-piscivorous animals or animals that are not

normally piscivorous can be definitive hosts for diplostomoideans as well. (Achatz et al. 2022b, 2023). Historically, there has been numerous attempts to propose a system of the Diplostomoidea using various sets of characters, however, none of them was universally accepted (e.g., Dubois, 1938, 1953, 1968, 1970a, b, 1982, 1987, 1989; La Rue, 1926a, b, 1957; Niewiadomska 2002; Sudarikov, 1959, 1960a, b, 1961, 1997; Yamaguti, 1958, 1971).This group of parasitic worms remains a highly active area of research (Locke et al. 2018, Gallozo et al. 2022, Gordy & Hanington 2019, Achatz et al. 2019a,b,c,d, 2021b,c, 2022b, López-Jiménez et al. 2018, 2019) and has recently undergone several major systematic changes (Achatz et al. 2021c, 2022b, 2022c, 2023), including the abandonment of a subfamily-based system. Thanks in part to technological advancements which allow for cost-effective sequencing, the biodiversity of the Diplostomoidea is still actively expanding which impacts our understanding of the systematics on a near annual basis. The focus on diplostomoidean research can be explained in part by their global distribution, impact on hosts and frequent use in ecological studies.

CHAPTER 2:

General Methods

Generalized methods are provided here to avoid repeating the same details in each chapter. Diplostomoideans collected in each study, alignment information and parameters of analyses are provided in the 'Methods' section of individual chapters, deviations from the general methods are discussed within the data chapter to which it pertains.

General Morphological Methods

Adult specimens belonging to Diplostomoidea were collected from a variety of mammalian and avian definitive hosts as well as teleost intermediate hosts in North and South

America, Europe, Asia, and Africa. Live digeneans removed from hosts were briefly rinsed in saline, killed with hot water and preserved in 80% ethanol. Dead digeneans were immediately preserved in 80% ethanol. Specimens for light microscopy were stained with aqueous alum carmine and mounted permanently according to Lutz et al. (2017). Specimens were measured using an Olympus® BX53 microscope (Olympus America, Center Valley, Pennsylvania, USA) equipped with a digital imaging system. Voucher specimens were deposited in various collections with the majority of specimens deposited in the Harold W. Manter Laboratory (HWML), University of Nebraska State Museum, Lincoln, NE, USA. Exceptions are noted in text where applicable. If collection was deemed impossible within the time constraints of the program, type species and voucher specimens were borrowed for our study. We use the terms prosoma and opisthosoma as explained by Achatz et al. (2019a) and Tkach et al. (2020).

General Molecular Methods

Genomic DNA was extracted from either fragments (in the case of larger specimens) or whole individuals of each species according to the methods described by Tkach and Pawlowski (1999). Different regions of the nuclear ribosomal RNA operon (18S, ITS1, 5.8S, ITS2, 28S) as well as mitochondrial *Cox1* gene were used in ths study for species differentiation and phylogenetic inference. A complete list of all PCR and sequencing primers can be found in table 1. Primers used to amplify DNA from specific taxa are mentioned below in corresponding chapters. The final length of each gene fragment used for analysis is described where applicable (i.e. in the figure captions for each tree figure). DNA was amplified by polymerase chain reactions (PCRs) using Onetaq (New England Biolabs, Ipswich, MA) in a T100™ thermal cycler (Bio-Rad, Hercules, CA, USA). PCR protocols generally follow manufacturer protocols with the exception of annealing temperature and time as well as extension time. For nuclear DNA,

annealing temperature was set to 53°C for 45 seconds with an extension time of 1.5 minutes. For mitochondrial mtDNA, annealing temperature was set to 45°C for 45 seconds and with an extension time of 1 minute. An ExoSAP-IT PCR clean-up enzymatic kit from Affymetrix (Santa Clara, California, USA) was used to clean-up the PCR products following the manufacturer's protocol. PCR products were cycle-sequenced directly using BrightDye® Terminator Cycle Sequencing Kit chemistry (MCLAB, San Francisco, California, USA)and run on an ABI 3130 automated capillary sequencer (Life Technologies, Grand Island, New York, USA). PCR primers were used for sequencing of 18S, 28S and *cox1* genes as well as the ribosomal ITS region. Contiguous sequences were assembled using Sequencher version 4.2 software (GeneCodes Corp., Ann Arbor, Michigan, USA). Phylogenetic interrelationships among members of the Diplostomoidea Poirier, 1886 were analysed using the 18S, 28S and *cox1* sequence data, in part, to match previously published data. Data gathered from GenBank are denoted and accession numbers are provided along with the corresponding citations within each chapter of the results. Sequences were aligned with the assistance of ClustalW as implemented in MEGA7 (Kumar et al., 2016); prior to analyses, the alignments were trimmed to the length of the shortest respective sequence. In the instances where GenBank data were utilized, efforts were made to include all relevant taxa when possible. However, short or questionable sequences were excluded from our analyses. Outgroup selection will be justified separately for each analysis. Phylogenetic analyses were conducted using Bayesian inference (BI) as implemented in MrBayes Ver. 3.2.6 software (Ronquist and Huelsenbeck, 2003). The model section utilized will be discussed for each analysis. MEGA7 (Kumar et al., 2017) was used to determine the best-fitting nucleotide substitution model for the datasets. BI analyses of 18S, 28S and *cox1* of the Diplostomoidea were carried out with the following settings:

Table 1: PCR and Sequencing primers used for amplification and sequencing of the respective gene fragment. PCR primers were used in a 10pm concentration while Sequencing primers were used at a 2pm concentration, there is no nucleotide difference between PCR and sequencing primers. The primers of interest for each chapter are noted in the methods section and dictates which primers were utilized for PCR vs sequencing or both.

Gene Fragment	Primer Name	Nucleotide Sequence
Cox 1		
	acox650R	5'-CCA AAA AAC CAA AAC ATA TGC TG-3'
	BS_CO1_INT_F	5'-ATT AAC CCT CAC TAA ATG ATT TTT TTY TTT YTR ATG CC-3'
	BS_CO1_INT_R	5'-TAA TAC GAC TCA CTA TAA AAA AAA MAM AGA AGA RAA MAC MGT AGT AAT-3'
	Cox1_Schist_5'	5'-TCT TTR GAT CAT AAG CG-3'
	Dipl_Cox_3′	5′ -WAR TGC ATN GGA AAA AAA CA–3′
	Dipl_Cox_5′	5′ -ACK TTR GAW CAT AAG CG-3′
	Dipl650R	5′-CCA AAR AAY CAR AAY AWR TGY TG-3′
	JB5	5'-AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG-3'
	Plat-diploCOX1F	5'-CGT TTR AAT TAT ACG GAT CC-3'
	Plat-diploCOX1R	5'-AGC ATA GTA ATM GCA GCA GC-3'
	Dice1F	5'-ATT AAC CCT CAC TAA ATT WCN TTR GAT CAT AAG-3'
	Dice14R	5'-TAA TAC GAC TCA CTA TAC CHA CMR TAA ACA TAT GAT G-3'
18S		
	18S-8	5′ -GCA GCC GCG GTA ATT CCA GC-3′
	WB1	5′ -CTT GTT ACG ACT TTT ACT TCC-3′
	WormA	5′ -GCG AAT GGC TCA TTA AAT CAG-3′
	WormB	5′ -ACG GAA ACC TTG TTA CGA CT-3′
28S		
	1500R	5′ –GCT ATC CTG AGG GAA ACT TCG–3′
	d58F	5′ -GCG GTG GAT CAC TCG GCT CGT G-3′
	digL2	5′ –AAG CAT ATC ACT AAG CGG–3′
	DPL1300R	5' – GCC TTT GGG TTT CGT AAC GCC – 3'
	DPL1450R	5' – GAC GGG CCG GTG ATG CGC C – 3'
	DPL250F	5' – GGG TTG TTT GTG AAT GCA GCC C – 3'
	DPL350R	5' – GTT TAC CTC TGA GCG GTT TCA CG – 3'
	DPL600F	5′ -CGG AGT GGT CAC CAC GAC CG-3′
	DPL700R	5′ -CAG CTG ATT ACA CCC AAA G-3′
	ECD2	5' – CTT GGT CCG TGT TTC AAG ACG GG – 3'
ITS1 + 5.8S + ITS2		
	300R	5′ –CAA CTT TCC CTC ACG GTA CTT G–3′
	ITSf	5′ –CGC CCG TCG CTA CTA CCG ATT G–3′
	D1	5' – AGG AAT TCC TGG TAA GTG CAA G – 3'
	300F	5' – CAA GTA CCG TGA GGG AAA GTT G – 3'
	D2	5' – CGT TAC TGA GGG AAT CCT GGT – 3'

Markov chain Monte Carlo chains run for 3,000,000 generations with a sample frequency of 1000, log-likelihood scores were plotted and only the final 75% of trees were used to produce the consensus trees. The number of generations was considered sufficient when the s.d. value reduced well below 0.01.

Any deviations from the above-mentioned standard will be noted where appropriate.

CHAPTER 3:

Unravelling the diversity of the Crassiphialinae (Digenea: Diplostomidae) with molecular phylogeny and descriptions of five new species

3.1 Introduction

Crassiphialinae Sudarikov, 1960 is a relatively large subfamily of the Diplostomidae Poirier, 1886. Its members parasitize, as adults, a variety of avian and mammalian definitive hosts worldwide. Despite the large number of studies on the Crassiphialinae, the systematics of the subfamily is complex and has always been unstable (Dubois, 1970; Shoop, 1989; Niewiadomska, 2002). Previous molecular phylogenetic studies have cast doubt on the validity of the Crassiphialinae based on the position of *Crassiphiala* Van Haitsma, 1925 and *Uvulifer* Yamaguti, 1934 being separate from *Bolbophorus* Dubois, 1934, *Ornithodiplostomum* Dubois, 1936 and *Posthodiplostomum* Dubois, 1936 (e.g. Achatz et al., 2019c).

Posthodiplostomum is a large, widely distributed and often reported crassiphialine genus whose members as adults parasitize piscivorous birds throughout the world (Dubois, 1968; Niewiadomska, 2002). This genus is well-known to fisheries biologists and wildlife disease ecologists due to its association with fish diseases and a common use of these parasites as models

in ecological studies (e.g. Lane et al., 2015; Boone et al., 2018). The metacercariae of *Posthodiplostomum* are associated with 'black spot' disease when encysted on the skin or fins of their fish second intermediate hosts (Horák et al., 2014); these metacercariae are also commonly referred to as 'white grub' when encysting within fish tissues and organs (Boone et al., 2018). These 'white grub' are commonly associated with a variety of pathologies in fishes and may cause death (Hoffman, 1958; Spall & Summerfelt, 1969; Lane & Morris, 2000).

Members of the genus *Ornithodiplostomum* have attracted significant attention from researchers due to their association with disease in fishes; their metacercariae are known to encyst on the brain of their fish second intermediate hosts (e.g. Matisz et al., 2010). Another crassiphialine genus, *Mesoophorodiplostomum* Dubois, 1936, has been only reported from the Nearctic and is much less studied than some of the larger and more broadly distributed genera. A close relationship among *Posthodiplostomum, Ornithodiplostomum* and *Mesoophorodiplostomum* has been recently demonstrated using sequences of the ITS1 + 5.8S + ITS2 as well as *cox*1 (Blasco-Costa and Locke, 2017; López-Hernández et al., 2018).

Despite the fact that larval specimens of *Posthodiplostomum* spp. are commonly collected and studied using molecular tools, few molecular studies have provided species identifications based on adult morphology (e.g. Locke et al., 2018). At present, only *Posthodiplostomum centrarchi* Hoffman, 1958, *Posthodiplostomum nanum* Dubois, 1937 and *Mesoophorodiplostomum pricei* (Krull, 1934) have DNA sequence data from adult specimens (Locke et al., 2010, 2018; López-Hernández et al., 2018).

We generated partial 28S rDNA and *cox*1 gene sequences from 28 species/species-level lineages belonging to seven genera of crassiphialines from Africa, Europe and the New World.

Table 2
Hosts, geographical origin, GenBank IDs and Harold W. Manter Laboratory (HWML) and Museum of Southwestern Biology (MSB) accession numbers of digeneans collected in this study.

Taxa	Host species	Geographical origin	Museum accession number	GenBank ID	
				28S	*cox*1
Bolbophorus cf. *confusus*	*Pelecanus onocrotalus*	Ukraine	–	MZ710936	MZ707162
Cercocotyla rhodesiensis	*Halcyon malimbica*	Uganda	HWML 216634; MSB:Para:3 2014	MZ710937	MZ707163
Cercocotyla sp.	*Ceryle maxima*	Uganda	–	MZ710938	MZ707164
Posthodiplostomoides kinsellae n. sp.	*Halcyon malimbica*	Uganda	HWML 216635, 216636	MZ710939	MZ707165
Posthodiplostomum cf. *anterovarium* n. comb.[a]	*Lepomis cyanellus* (liver)	Minnesota, USA	HWML 216637	MZ710940, MZ710941	MZ707166
	Lepomis gibbosus (liver)	Minnesota, USA	–	MZ710942	MZ707167
Posthodiplostomum anterovarium n. comb.[a]	*Pelecanus erythrorhynchos*[c]	New Mexico, USA	MSB:Para:3 2011	MZ710943, MZ710944	MZ707168
Posthodiplostomum centrarchi	*Ambloplites rupestris*	Minnesota, USA	–	MZ710945	MZ707169
	Anhinga anhinga	Mississippi, USA	HWML 216638	MZ710946, MZ710947	MZ707170, MZ707171
	Anhinga anhinga	Louisiana, USA	HWML 216639; MSB:Para:3 2016	MZ710948	MZ707172
	Ardea alba	Mississippi, USA	–	–	MZ707173, MZ707174
	Ardea herodias	Georgia, USA	HWML 216641; MSB:Para:3 2018	MZ710949, MZ710950	MZ707175, MZ707176
	Lepomis cyanellus (liver)	Minnesota, USA	HWML 216642	MZ710951, MZ710952	MZ707177, MZ707178
	Lepomis cyanellus (skin)	Minnesota, USA	HWML 216643	MZ710953	MZ707179

	Lepomis macrochirus (heart)	Minnesota, USA	–	–	MZ707180
	Lepomis macrochirus (liver)	Minnesota, USA	–	–	MZ707181
	Lepomis macrochirus (mesentery)	Minnesota, USA	–	–	MZ707182
	Lepomis macrochirus (spleen)	Minnesota, USA	–	–	MZ707183
	Megaceryle alcyon	Mississippi, USA	–	MZ710954	MZ707184
Posthodiplostomum cuticola	*Nycticorax nycticorax*	Ukraine	HWML 216644; MSB:Para:3 2012	MZ710955	MZ707185
Posthodiplostomum erickgreenei n. sp.	*Pandion haliaetus*[d]	Montana, USA	HWML 216645, 216646	MZ710956	MZ707186
Posthodiplostomum eurypygae n. sp.	*Eurypyga helias*[e]	Pantanal, Brazil	HWML 216647, 216648	MZ710957	MZ707187
Posthodiplostomum macrocotyle	*Busarellus nigricollis*	Pantanal, Brazil	HWML 216649	MZ710958, MZ710959	MZ707188, MZ707189
Posthodiplostomum microsicya	*Tigrisoma lineatum*	Pantanal, Brazil	HWML 216650	MZ710960	–
Posthodiplostomum minimum	*Ardea herodias*	North Dakota, USA	HWML 216651; MSB:Para:3 2017	MZ710961	MZ707190
	Nycticorax nycticorax	Mississippi, USA	HWML 216653	MZ710962	MZ707191
Posthodiplostomum nanum	*Ardea alba*	Mississippi, USA	HWML 216654	MZ710963	MZ707192
Posthodiplostomum orchilongum	*Ardea alba*	Mississippi, USA	HWML 216655	MZ710964	–
	Egretta caerulea	Mississippi, USA	HWML 216656; MSB:Para:3 2015	MZ710965, MZ710966	MZ707193
Posthodiplostomum pacificus n. sp.	*Larus californicus*	California, USA	HWML 216657	MZ710967	MZ707194

Posthodiplostomum cf. *podicipitis* n. comb.[b]	*Catostomus commersonii* (skin)	Minnesota, USA	–	MZ710968	MZ707195
	Lophodytes cucullatus	North Dakota, USA	HWML 216658	MZ710969, MZ710970	MZ707196, MZ707197
	Pimephales promelas (brain)	Minnesota, USA	–	MZ710971	MZ707198
Posthodiplostomum pricei n. comb.[a]	*Larus delawarensis*	North Dakota, USA	HWML 216659; MSB:Para:3 2013	MZ710972, MZ710973	MZ707199, MZ707200
Posthodiplostomum ptychocheilus n. comb.[a]	*Mergus merganser*	Minnesota, USA	HWML 216660; MSB:Para:3 2019	MZ710974	MZ707201
Posthodiplostomum recurvirostrae n. sp.	*Recurvirostra americana*	North Dakota, USA	HWML 216661	MZ710975	MZ707202
Posthodiplostomum sp. 11[b]	*Chrosomus eos*	Minnesota, USA	–	MZ710976	MZ707203
	Unidentified fish (eyes)	North Dakota, USA	–	MZ710977	MZ707204
Posthodiplostomum sp. 17	*Lophodytes cucullatus*	North Dakota, USA	HWML 216662	MZ710978	MZ707205
Posthodiplostomum sp. 18	*Physa gyrina*	Oregon, USA	–	MZ710979, MZ710980	MZ707206, MZ707207
Posthodiplostomum sp. 18	*Pelecanus erythrorhynchos*	Oregon, USA	HWML 216663	MZ710981	MZ707208
Posthodiplostomum sp. 19	*Physa* sp.	Minnesota, USA	–	MZ710982, MZ710983	MZ707209
Posthodiplostomum sp. 20	*Physa gyrina*	Oregon, USA	–	MZ710984	MZ707210
Posthodiplostomum sp. 20	*Physa gyrina*	Oregon, USA	–	MZ710985-MZ710988	MZ707211
Posthodiplostomum sp. 21	*Tigrisoma lineatum*	Pantanal, Brazil	–	MZ710989	MZ707212
Posthodiplostomum sp. 21	*Jabiru mycteria*	Pantanal, Brazil	–	MZ710990	MZ707213
Posthodiplostomum sp. 22	*Ardea alba*	Pantanal, Brazil	HWML 216664	MZ710991	MZ707214

Posthodiplostomum sp. 22	*Ardea cocoi*	Pantanal, Brazil	–	MZ710992	MZ707215
Posthodiplostomum sp. 22	*Tigrisoma lineatum*	Pantanal, Brazil	HWML 216665	MZ710993	MZ707216
Posthodiplostomum sp. 23	*Ardea herodias*	Georgia, USA	HWML 216666	MZ710994, MZ710995	MZ707217, MZ707218
Pulvinifer macrostomum	*Gallinago gallinago*	Minnesota, USA	HWML 216667; MSB:Para:3 2020	MZ710996	MZ707219

[a] Previously included in *Mesoophorodiplostomum.*
[b] Previously included in *Ornithodplostomum.*
[c] Host deposited in the Museum of Southwestern Biology (NK250053; MSB:Para:19549).
[d] Host deposited in the Philip L. Wright Zoological Museum (UMZM:Bird:22149).
[e] Host deposited in the Museum of the Universidade Federal de Mato Grosso (UFMT 4865).
Note: The localization of metacercariae in the second intermediate host is provided, when possible, in parentheses.

The new 28S sequences were used to explore the phylogenetic position of crassiphialine taxa among other major lineages of diplostomoideans, re-evaluate their systematics and aid ecological studies and disease diagnostics. Detailed phylogenetic analyses of 28S and *cox*1 sequences were conducted for closely related *Posthodiplostomum*, *Ornithodiplostomum* and *Mesoophorodiplostomum*. When possible, type-species of corresponding genera were used in our analyses. Furthermore, four new species of *Posthodiplostomum* are described from the New World as well as one new species of another crassiphialine genus, *Posthodiplostomoides* Williams, 1969, from Africa.

3.2 Materials & Methods

Adult diplostomid digeneans were obtained from a variety of avian hosts, while larval diplostomids were collected from snail and fish species in the New World, Africa and Europe (Table 2). Worms fixed and stained according to our standard methods. Type-series and

morphological vouchers were deposited in the collection of the H. W. Manter Laboratory, University of Nebraska, Lincoln, Nebraska, USA and the Museum of Southwestern Biology (MSB), University of New Mexico, Albuquerque, New Mexico, USA (Table 2). Host specimens were deposited in the Philip L. Wright Zoological Museum (UMZM), University of Montana, Missoula, Montana, USA, the MSB, and the Museum of the Universidade Federal de Mato Grosso (UFMT), Brazil.

Genomic DNA was extracted and amplified using our standard methods and the following primers: digL2, 1500R, Plat-diploCOX1F, Cox1_Schist_5', Dipl_Cox_5', BS_CO1_INT_F, Plat-diploCOX1R, acox650R, JB5, Dipl650R, Dipl_Cox_3', and BS_CO1_INT_R. Sequencing was performed using our standard methods with PCR primers and the additional internal primers DPL600F and DPL700R. Alignments were done using our standard methods.

Phylogenetic analyses

The phylogenetic positions of *Bolbophorus*, *Cercocotyla* Yamaguti, 1939, *Mesoophorodiplostomum*, *Ornithodiplostomum*, *Posthodiplostomoides*, *Posthodiplostomum* and *Pulvinifer* Yamaguti, 1933 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 topology presented by Achatz et al. (2019d). This alignment included newly generated sequences of *Bolbophorus* cf. *confusus* (Krause, 1914) (type-species; *n* = 1), *Cercocotyla* spp. (*n* = 2), *M. pricei* (type-species; *n* = 1), *Ornithodiplostomum ptychocheilus ptychocheilus* (Faust, 1917) (type-species; *n* = 1), *Posthodiplostomoides kinsellae* n. sp. (*n* = 1), *Posthodiplostomum* spp. (including the type-species; *n* = 6) and *Pulvinifer*

macrostomum (Jägerskiöld, 1900) (type-species; *n* = 1) and previously published sequences of other crassiphialines including *Bolbophorus* spp. (*n* = 4), *Crassiphiala* (*n* = 2), *Ornithodiplostomum* (*n* = 1), *Posthodiplostomum* (*n* = 4) and *Uvulifer* (*n* = 2). This alignment also included non-crassiphialine diplostomids (*n* = 11) as well as members of the Proterodiplostomidae Dubois, 1936 (*n* = 2) and the Strigeidae Railliet, 1919 (*n* = 12).

Based on the results of the initial, broader analysis of 28S data, two subsequent analyses based on 28S and *cox*1 of *Posthodiplostomum* + *Ornithodiplostomum* + *Mesoophorodiplostomum* were conducted. Both analyses used the unidentified genus of diplostomid sequenced by Hoogendoorn et al. (2019) as the outgroup based on the results of the initial 28S analysis. The second alignment of 28S included newly generated sequences of *Posthodiplostomum* (*n* = 21) including the type-species *Posthodiplostomum cuticola* (von Nordmann, 1832), *Ornithodiplostomum* (*n* = 1) including the type-species *O. p. ptychocheilus*, *Mesoophorodiplostomum* (*n* = 3) including the type-species *M. pricei*, and previously published sequences of *Posthodiplostomum* (*n* = 8), *Ornithodiplostomum* (*n* = 1) and previously unidentified diplostomids (*n* = 4).

The alignment of *cox*1 sequences included new sequences of *Posthodiplostomum* (*n* = 25) including the type-species *Po. cuticola*, *Ornithodiplostomum* (*n* = 4) including the type-species *O. p. ptychocheilus*, *Mesoophorodiplostomum* (*n* = 5) including the type-species *M. pricei*, and previously published sequences of *Posthodiplostomum* (*n* = 15), *Ornithodiplostomum* (*n* = 11), *Mesoophorodiplostomum* (*n* = 3) and an unidentified diplostomid (*n* =1).

BI for 28S was performed using our standard methods. The BI analysis for the *cox*1 dataset used similar conditions, however, the dataset was analyzed as codons and ran for 6,000,000 generations. Pairwise comparisons for each locus were carried out using MEGA7.

Several genera referred to in text begin with the letter 'P'. To avoid confusion and redundancy, we refer to *Pandion* as *Pa*., *Pelecanus* as *Pe*., *Posthodiplostomum* as *Po*., *Posthodiplostomoides* as *Ps*. and *Pulvinifer* as *Pu*.

3.3 Results and discussion

Molecular phylogenies

The initial 28S alignment was 1,092 bp long; 60 bases were excluded from the analysis due to ambiguous homology. The phylogenetic tree resulting from the BI analysis of 28S clearly demonstrated non-monophyly of the Diplostomidae and Strigeidae (Fig. 2). Overall, the phylogeny consisted of a large basal polytomy with multiple independent clades. Importantly, members of the subfamilies of the Diplostomidae (i.e. Crassiphialinae and Diplostominae Poirier, 1886) were non-monophyletic.

Bolbophorus spp. formed two distinct clades. The first clade (unsupported) included a larval specimen of *Bolbophorus* as a sister group to a 100% supported clade of *B.* cf. *confusus* + two other unidentified *Bolbophorus* species-level lineages (Fig. 2). *Bolbophorus damnificus* Overstreet & Curran, 2002 was positioned in a separate clade in the basal polytomy from the other members of *Bolbophorus. Cercocotyla* spp. formed an independent 100% supported clade in the basal polytomy. *Uvulifer* + *Crassiphiala* + *Posthodiplostomoides* formed a 100% supported clade in the basal polytomy of the Diplostomoidea. Within this clade, *Crassiphiala* + *Posthodiplostomoides* formed a weakly supported cluster (Fig. 2). Interestingly, *Pu.*

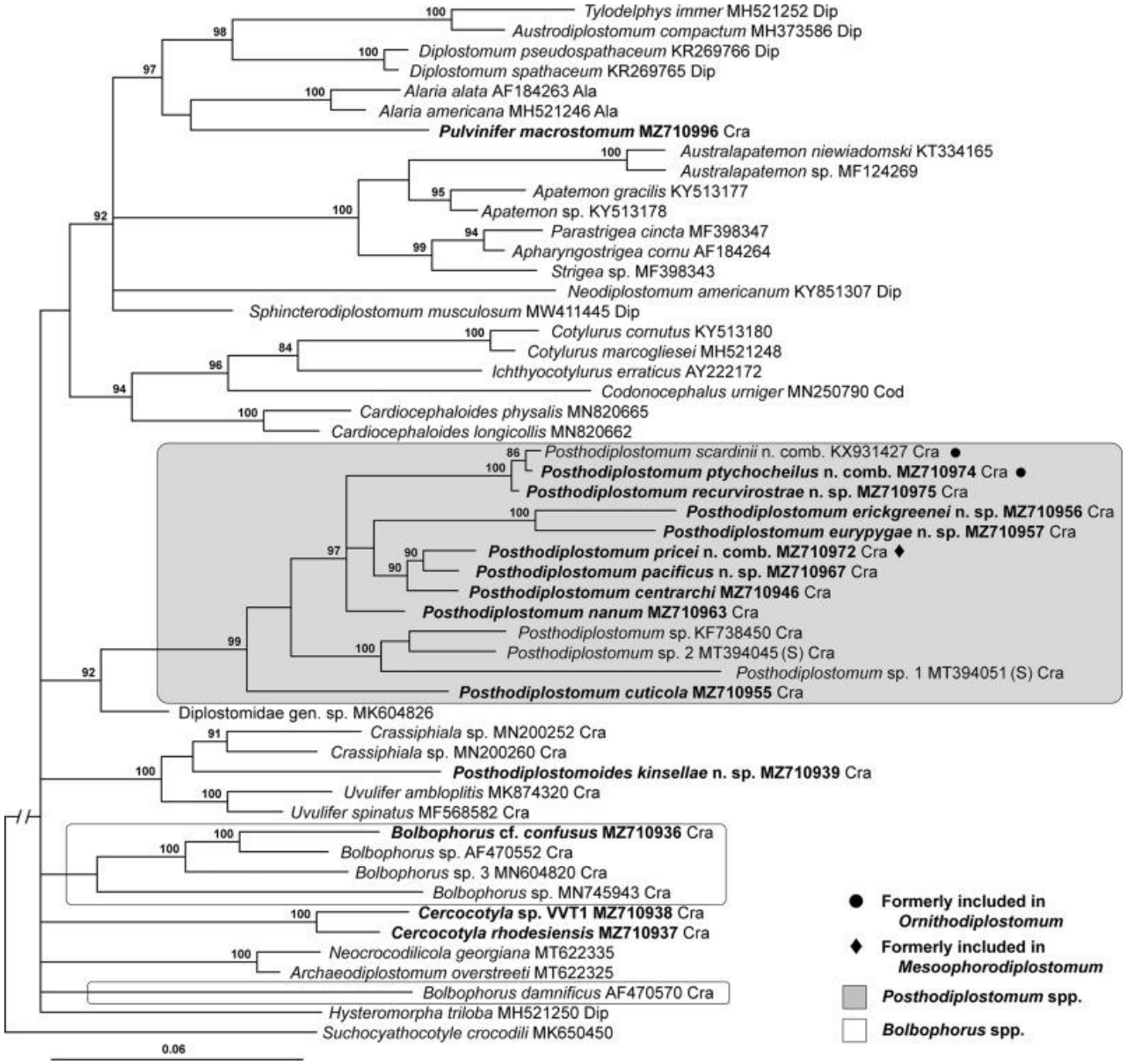


Figure 2. Phylogenetic interrelationships among 51 diplostomoidean taxa based on Bayesian Inference (BI) analysis of partial 28S rDNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale-bar indicates the number of substitutions per site. Reference to the origin of species numbering/naming system of *Posthodiplostomum* spp. in the analysis is provided in parentheses after GenBank accession numbers followed by subfamilies of members of the Diplostomidae included in the analysis. Abbreviation for reference to the original designations of species-level lineages: S, Sokolov and Gordeev (2020). Abbreviations for subfamilies: Ala, Alariinae; Cod, Codonocephalinae; Cra, Crassiphialinae; Dip, Diplostominae.

macrostomum was positioned in a 97% supported clade with non-crassiphialine diplostomids. This clade contained subclades of *Alaria* Schrank, 1788 + *Pulvinifer* and *Diplostomum* + a clade of [*Austrodiplostomum* Szidat & Nani, 1951 + *Tylodelphys* Diesing, 1850].

The unidentified diplostomid of Hoogendoorn et al. (2019) (GenBank: MK604826) + cluster of *Posthodiplostomum* + *Ornithodiplostomum* + *Mesoophorodiplostomum* formed a fairly well-supported monophyletic clade (92%) within the basal polytomy of the Diplostomoidea (Fig. 2). This clade of the three genera was 99% supported with *Po. cuticola* positioned as a sister group to the weakly supported clade containing the remaining taxa (Fig. 2). Phylogenetic relationships among taxa within the *Posthodiplostomum* + *Ornithodiplostomum* + *Mesoophorodiplostomum* clade are discussed in detail below.

The second 28S alignment that included only members of *Posthodiplostomum*, *Ornithodiplostomum* and *Mesoophorodiplostomum* was 1,093 bp long; 28 bases were excluded from the analysis due to ambiguous homology. The tree topology was overall well-resolved (Figs. 3 and 4). *Posthodiplostomum cuticola* (type-species of the genus) was positioned as a sister group to a 100% supported clade which contained the remaining taxa. The four sequences from larval *Posthodiplostomum* specimens collected in Eastern Asia (Palearctic and Indomalayan realms) formed a 100% supported clade, which was separated from the 100% supported cluster containing the remaining *Posthodiplostomum*, *Mesoophorodiplostomum* and *Ornithodiplostomum* and *Mesoophorodiplostomum* sequences. This cluster contained 7 clades. Clades I–VI formed a weakly supported clade separated from clade VII (polytomy of *Po. nanum* + *Posthodiplostomum* sp. 23 + *Posthodiplostomum* sp. of Hernández-Mena et al. (2017); 100% supported). Clades I–VI were overall positioned in a polytomy (Fig. 3).

Clades I and II clustered in a weakly supported clade within the weakly supported polytomy. Clade I (100% support) included several unidentified species-level lineages of *Posthodiplostomum* and *Ornithodiplostomum* larvae without matching sequences from adults. *Posthodiplostomum* sp. 17 appeared as a sister group to a 100% supported cluster containing the remaining members of Clade I (Fig. 3). This 100% supported cluster was mostly a polytomy that included *Posthodiplostomum* sp. 19, *Ornithodiplostomum* cf. *podicipitis* Yamaguti, 1939, *O. p. ptychocheilus* (type-species of *Ornithodiplostomum*), *Posthodiplostomum recurvirostrae* n. sp., *Ornithodiplostomum scardinii* (Shulman, 1952) and a 100% supported clade of *Posthodiplostomum* sp. 18 + (*Posthodiplostomum* sp. 20 + *Posthodiplostomum* sp. 11).

Clade II (100% support) consisted primarily of *Posthodiplostomum* taxa with morphologically identified adults (Fig. 3) and was well-resolved. *Posthodiplostomum eurypygae* n. sp. was positioned as a sister group to a 100% supported clade which contained all other members of the clade. Within this clade, *Posthodiplostomum orchilongum* Noble, 1936 formed a sister branch to a weakly supported clade containing *Posthodiplostomum erickgreenei* n. sp. + a 100% supported clade of [*Posthodiplostomum macrocotyle* Dubois, 1937 + a 99% supported clade with four other species-level lineages]. That 99% supported clade positioned *Posthodiplostomum* sp. 9 of Hoogendoorn et al. (2019) as a sister group to a 98% supported clade of [*Posthodiplostomum* sp. 21 + an 82% supported cluster of (*Posthodiplostomum* sp. 22 + *Posthodiplostomum microsicya* Dubois, 1936)].

Clades III, IV and V formed a poorly supported cluster (Fig. 3). Clade III (99% support) contained *Posthodiplostomum pacificus* n. sp. as a sister group to an unsupported polytomy of *M. pricei*, *Mesoophorodiplostomum anterovarium* Dronen, 1985 and an unidentified diplostomid (GenBank: KU221112). Clade IV (100% supported) consisted of a polytomy with *Po. centrarchi*

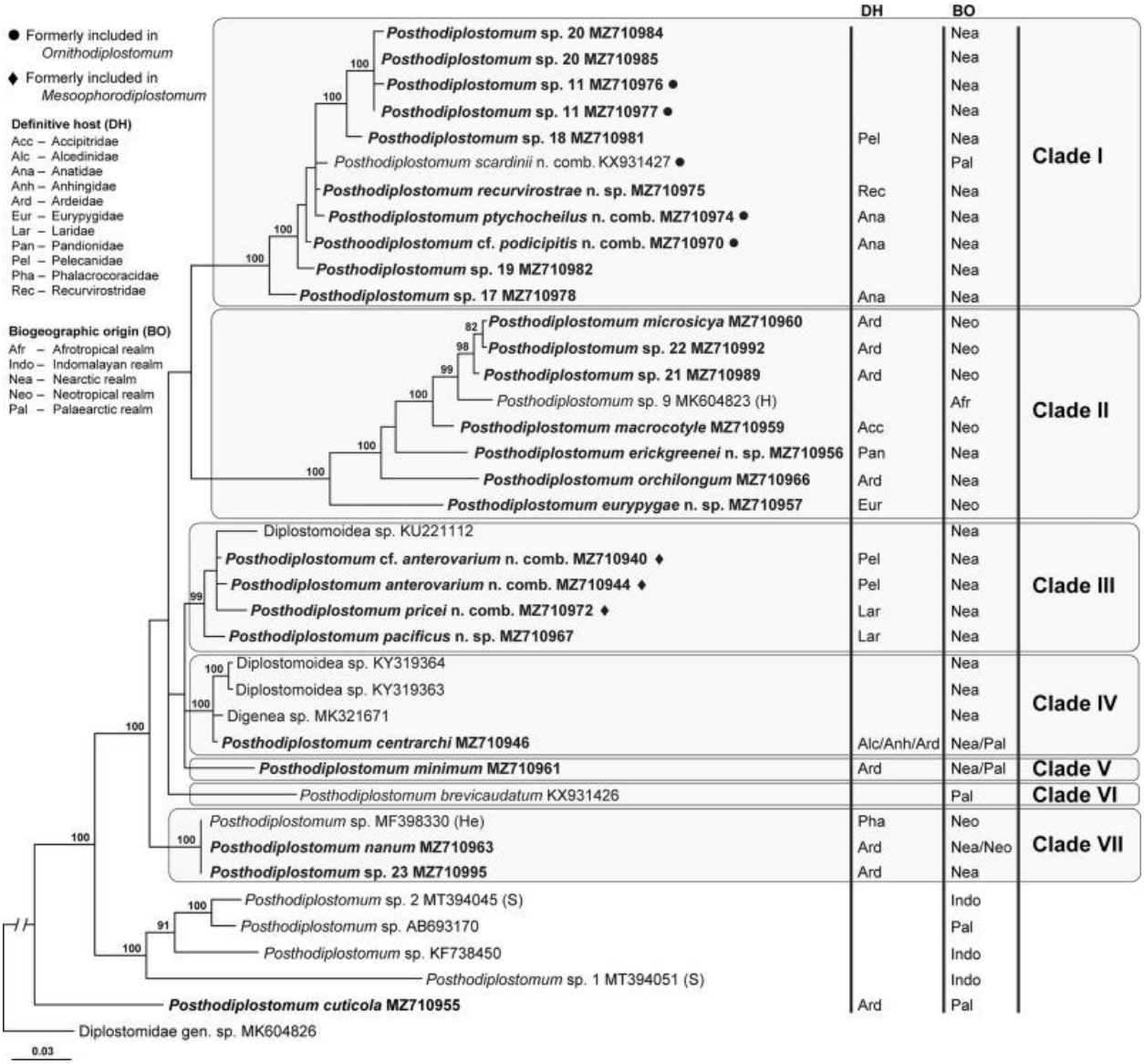


Figure 3. Phylogenetic interrelationships among 38 taxa of *Posthodiplostomum* (syns. *Ornithodiplostomum* and *Mesoophorodiplostomum*) based on Bayesian Inference (BI) analysis of partial 28S rDNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale-bar indicates the number of substitutions per site. Reference to origin of species numbering/naming systems of are provided in parentheses after GenBank accession numbers. Biogeographical realm where specimens were collected and family of definitive host (for adult isolates and larvae molecularly matched to adult forms) are provided when possible. Abbreviations for references to the original designations of species-level lineages: He, Hernández-Mena et al. (2017); Ho, Hoogendoorn et al. (2019); S, Sokolov and Gordeev (2020).

+ an unidentified diplostomid (GenBank: MK321671) + a 100% supported cluster of two unidentified diplostomids (GenBank: KY319363, KY319364). Clade V only contained *Posthodiplostomum minimum* (MacCallum, 1921). Clade VI appeared as an independent branch and only contained *Posthodiplostomum brevicaudatum* (von Nordmann, 1832) (Fig. 3).

The *cox*1 alignment was 363 bp long; the resulting phylogenetic tree was characterized by an overall weakly supported branch topology. Other recent molecular phylogenetic studies (e.g. Hernández-Mena et al., 2017; López-Hernández et al., 2018; Hoogendoorn et al., 2019; Achatz et al., 2019a, c, 2020a; Tkach et al., 2020) have repeatedly demonstrated that analyses of faster mutating genes often produce topologies which are much less resolved than those based on slower mutating genes such as 28S (e.g. Hernández-Mena et al., 2017; Hoogendoorn et al., 2019; Achatz et al., 2019a, c, 2020a; Tkach et al., 2020). Therefore, we opt to not discuss the results of this analysis in detail, although we provide the resulting tree (Supplementary Figure S1) to allow for comparison of some of the better resolved clades.

Non-monophyly of the Crassiphialinae

At present, the Diplostomidae contains four subfamilies: the Crassiphialinae, Diplostominae, Alariinae Hall et Wigdor, 1918 and Codonocephalinae Sudarikov, 1959. According to Niewiadomska (2002), members of the Crassiphialinae are united based on vitellarium that is typically confined to the opisthosoma (= hindbody), a copulatory bursa that may be protrusible and ‘Neascus’ type metacercariae; whereas members of the Diplostominae are united based on vitellarium located in both parts of the body, a copulatory bursa that is not protrusible and ‘diplostomulum’ type metacercariae. Furthermore, Niewiadomska (2002) stated that members of these two subfamilies only parasitize birds as adults. Members of the Alariinae

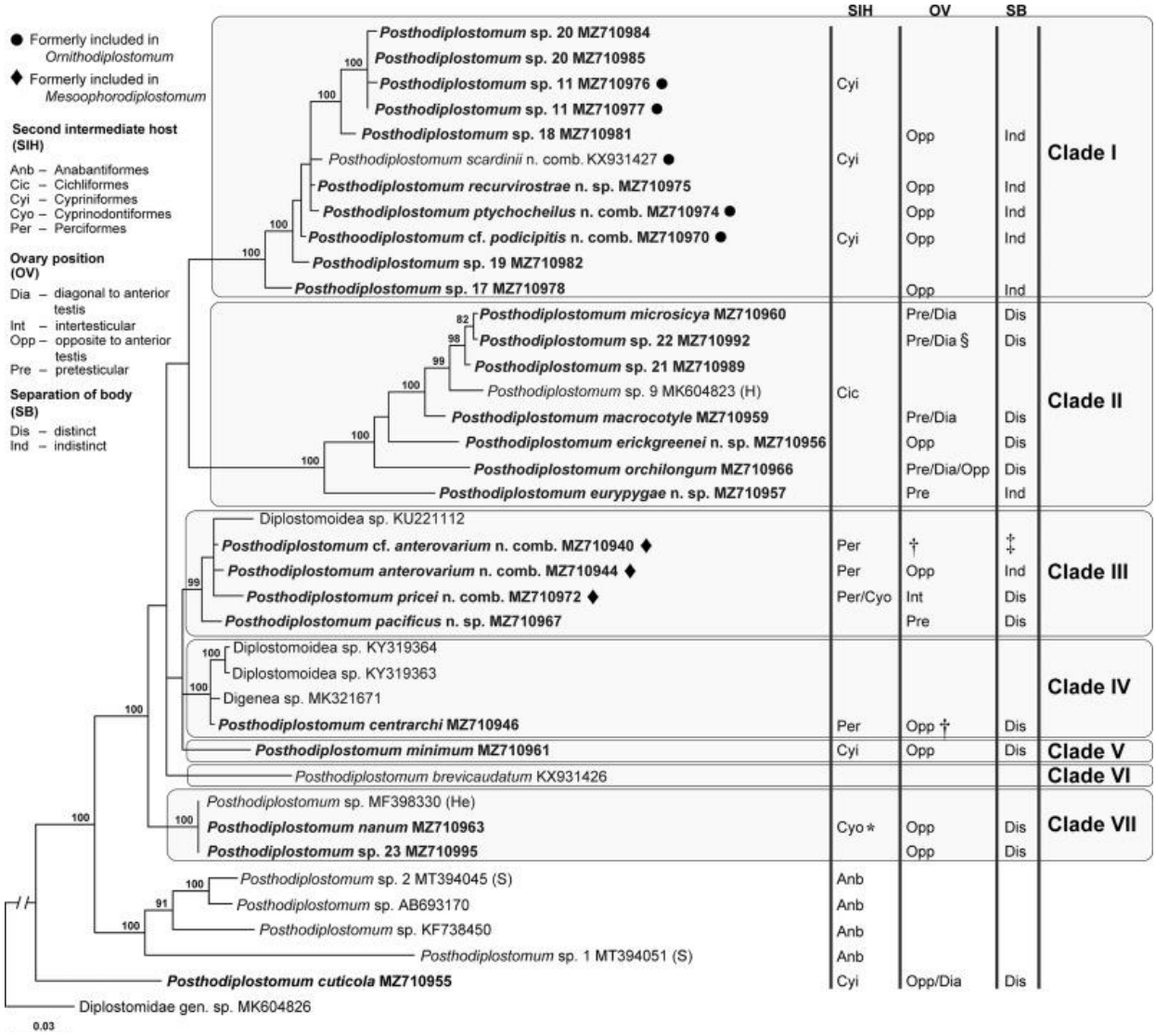


Figure 4. Phylogenetic interrelationships among 38 taxa of *Posthodiplostomum* (syns. *Ornithodiplostomum* and *Mesoophorodiplostomum*) based on Bayesian Inference (BI) analysis of partial 28S rDNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale-bar indicates the number of substitutions per site. Reference to origin of species numbering/naming systems of are provided in parentheses after GenBank accession numbers. Order of second intermediate hosts (for larvae and adults molecularly matched to larval forms), position of ovary and level of distinction between prosoma and opisthosoma in adult stages provided when possible. Abbreviations for references to the original designations of species-level lineages: He, Hernández-Mena et al. (2017); Ho, Hoogendoorn et al. (2019); S, Sokolov and Gordeev (2020). * Collected from experimental infection by López-Hernández et al. (2018). § Ovary intertesticular or opposite to anterior testis in immature specimens. † Ovary intertesticular in immature specimens. ‡ Prosoma and opisthosoma distinct in immature

also possess ‘diplostomulum’ type metacercariae, but often have mesocercarial stages as well. In addition, alariines parasitize mammals as adults. The only member of the Codonocephalinae, *Codonocephalus urniger* (Rudolphi, 1819), has progenetic metacercariae, an infundibular prosoma and several other unique morphological characters (Achatz et al., 2019b). Our broader analysis of 28S (Fig. 2) included multiple genera representing two out of the three diplostomid subfamilies (i.e. the Crassiphialinae and Diplostominae) which contain more than a single genus.

Our broader analysis based on 28S sequences (Fig. 2) clearly demonstrates the non-monophyly of the Diplostomidae as well as two of its subfamilies (i.e. the Diplostominae and Crassiphialinae). Likewise, several recent molecular phylogenetic studies have demonstrated non-monophyly of these currently accepted taxa (e.g. Blasco-Costa and Locke, 2017; Hernández-Mena et al., 2017; Achatz et al., 2019b, c, d, 2020b, 2021a; Queiroz et al., 2020; Tkach et al., 2020). Prior to our study, only five genera of crassiphialines had available 28S sequence data (*Bolbophorus*, *Crassiphiala*, *Ornithodiplostomum*, *Posthodiplostomum* and *Uvulifer*). Previous studies demonstrated *Crassiphiala* and *Uvulifer* form a clade independent from *Bolbophorus*, *Ornithodiplostomum* and *Posthodiplostomum* (e.g. Achatz et al., 2019c). Our 28S analysis included members of additional crassiphialine genera *Cercocotyla*, *Mesoophorodiplostomum*, *Posthodiplostomoides* and *Pulvinifer*, as well as the type-species of *Bolbophorus* (*B.* cf. *confusus*) (Fig. 2).

The molecular phylogenetic analysis of the Diplostomoidea based on 28S (Fig. 2) did not unite the members of the Crassiphialinae or Diplostominae. Instead, members of both subfamilies formed several independent clades in the basal polytomy of the Diplostomoidea. In fact, *Alaria* (Alariinae), *Diplostomum* (Diplostominae), *Austrodiplostomum* (Diplostominae), *Tylodelphys* (Diplostominae) and *Pulvinifer* (Crassiphialinae) formed a 97% supported clade.

Our analysis failed to provide any support for the currently recognized Crassiphialinae and Diplostominae.

Morphological analysis has demonstrated the lack of any consistent morphological features in adults which could be used to reliably differentiate between taxa forming the clades of the Crassiphialinae or Diplostominae (Fig. 2). The difference in distribution of vitellarium between members of the Crassiphialinae and Diplostominae is very inconsistent. Numerous crassiphialine species have vitellarium in both parts of the body (e.g. *B. confusus* and *Posthodiplostomoides* spp.). The protrusible nature of the copulatory structures should also not be relied on for separation of subfamilies considering that only some, but not all, crassiphialines have a protrusible genital bursa (Niewiadomska, 2002). In addition, some diplostomines also possess protrusible genital bursae/cones (e.g. some *Dolichorchis* Dubois, 1961 and *Tylodelphys*).

Interestingly, *Codonocephalus* Diesing, 1850 was positioned within a strongly supported clade (94%) of *Cardiocephaloides* Sudarikov, 1959 and *Cotylurus* Szidat, 1928 + *Ichthyocotylurus* Odening, 1969 (Fig. 2). It is possible that familial placement of *Codoncephalus* should be re-evaluated. *Codonocephalus* shares some morphological features with both the Diplostomidae and Strigeidae (Niewiadomska, 2002; Achatz et al., 2019b).

Recently, Tkach et al. (2020) proposed discontinuing the use of subfamilies within the diplostomoidean family Proterodiplostomidae based on the non-monophyletic nature of its constituent subfamilies. The abandonment of subfamilies has also been relatively recently proposed for other large digenean families such as the Cryptogonimidae Ward, 1917, Dicrocoeliidae Looss, 1899 and Echinostomatidae Looss, 1899 (Miller and Cribb, 2008; Tkach et al., 2016, 2018). Based on our molecular phylogenetic analysis (Fig. 2), which is consistent with other recent molecular phylogenetic studies of the Diplostomidae (e.g. Hernández-Mena et

al., 2017; Achatz et al., 2019b, c, d, 2020b, 2021a; Queiroz et al., 2020; Tkach et al., 2020), it is our opinion that the subfamilies of the Diplostomidae should also be abandoned. Therefore, we do not consider the four diplostomid subfamilies to be valid. It is likely that the subfamilies of the Strigeidae should also be considered invalid due to their non-monophyletic nature. However, detailed morphological study of independent clades of strigeids is necessary to determine if any morphological features may be used to erect new subfamilies (or families). Undoubtedly, a detailed re-evaluation of the system of the diplostomoidean families is required. However, such a re-evaluation is well beyond the scope of the present study.

Status of *Bolbophorus*

Bolbophorus spp. are associated with diseases in fishes (Markle et al., 2014, 2020). Interestingly, members of *Bolbophorus*, as currently recognized formed two independent clades in our analysis of 28S (Fig. 2). The first clade was composed of four species/species-level lineages (two of which are only currently known from larvae), including the specimen tentatively identified as the type-species of the genus. The second clade only contained *B. damnificus*; the separate position of *B. damnificus* demonstrates that the species belongs to a separate genus. However, detailed morphological re-evaluation of *Bolbophorus* spp. is necessary to properly address the generic placement of *B. damnificus*.

Unfortunately, the single specimen of *B.* cf. *confusus* available in our collection was entirely used for DNA extraction. *Bolbophorus confusus* was originally described from specimens collected from Dalmatian pelican *Pelecanus crispus* Brunch from Europe by Krause (1914) and later redescribed by Dubois (1934, 1938) based on the original material and additional specimens collected from the great white pelican *Pelecanus onocrotalus* Linnaeus

from Europe and American white pelican *Pelecanus erythrorhynchos* Gmelin from Minnesota, USA. Our specimen was collected from *Pe. onocrotalus* in Ukraine. No other species of *Bolbophorus* is currently known to be distributed in Europe.

Currently there are 11 unique 28S sequences from *B. damnificus* and four unique 28S sequences of *Bolbophorus* sp. of Overstreet et al. (2002) available in GenBank. We suspect that at least some of these sequences contain errors or represent additional species, in part, due to the presence of indels limited to individual sequences (e.g. GenBank: AF470546 compared to AF470538). Furthermore, the intraspecific variation among 28S sequences of *B. damnificus* reaches 1.6% and the intraspecific variation among 28S sequences *Bolbophorus* sp. from Overstreet et al. (2002) is up to 0.4%. These levels of intraspecific variation are substantially greater than within the *Bolbophorus* sp. of Hoogendoorn et al. (2019) (0% intraspecific variation) and *Posthodiplostomum* spp. (up to 0.1% intraspecific variation) in the present study. Moreover, some *cox*1 sequences (e.g. GenBank: AF470578 compared to AF470614) generated by Overstreet et al. (2002) from isolates of these species have single nucleotide indel sites, which is not possible in a coding gene. Sequencing of freshly collected adult specimens of *B. damnificus* and *Bolbophorus* sp. of Overstreet et al. (2002) is necessary to evaluate the status of these taxa and clarify the systematic position of *B. damnificus*.

Validity of *Ornithodiplostomum* and *Mesoophorodiplostomum*

Ornithodiplostomum and *Posthodiplostomum* are differentiated based on the presence/absence of an ejaculatory pouch (present in *Ornithodiplostomum* spp. *vs* absent in *Posthodiplostomum* spp.) as well as the level of separation between prosoma and opisthosoma (indistinct in *Ornithodiplostomum* spp. *vs* more or less distinct in *Posthodiplostomum* spp.; Fig.

5) (Dubois, 1968; Niewiadomska, 2002). *Ornithodiplostomum p. ptychocheilus*, the type-species of *Ornithodiplostomum*, was originally described as having an ejaculatory pouch; however, it was not shown on the illustrations of the adult provided by Van Haitsma (1930) and Dubois (1936, 1968). It appears that the pouch-like terminal portion of the seminal vesicle was considered an ejaculatory pouch. In our opinion, this terminal portion of the seminal vesicle is not an 'ejaculatory pouch' based on the original illustrations provided by Van Haitsma (1930) and our well-fixed adult specimens of *O. p. ptychocheilus*. Based on the original descriptions, the only *Ornithodiplostomum* species that appears to have a well-developed ejaculatory pouch is *Ornithodiplostomum garambense* (Baer, 1959), which was originally placed into the genus *Prolobodiplostomum* Baer, 1959 (Baer, 1959; Dubois, 1968). Furthermore, in our 28S analyses (Figs. 2–4) the sequence of *Po. recurvirostrae* (which clearly lacks an ejaculatory pouch) was positioned in a strongly supported clade with *O. p. ptychocheilus*.

Dubois (1944) transferred *O. podicipitis* into *Posthodiplostomum* based on the lack of an ejaculatory pouch. Later, Dubois (1968) returned it to *Ornithodiplostomum* based on the lack of clear differentiation between the prosoma and opisthosoma as well as the fact that it was not described from a member of *Ardea* Linnaeus. Our specimens of *O.* cf. *podicipitis* also clearly lack an ejaculatory pouch. Similar to *Po. recurvirostrae*, this species was positioned within a clade with *O. p. ptychocheilus* (Figs. 3 and 4). The terminal portion of the seminal vesicle of some *Posthodiplostomum* spp. (e.g. *Po. minimum*, *Po. macrocotyle* also appears pouch-like) (Dubois, 1968; present material). Hence, the presence/absence of an ejaculatory pouch does not appear to be a valid feature enabling differentiation among these genera based on well-fixed adult specimens.

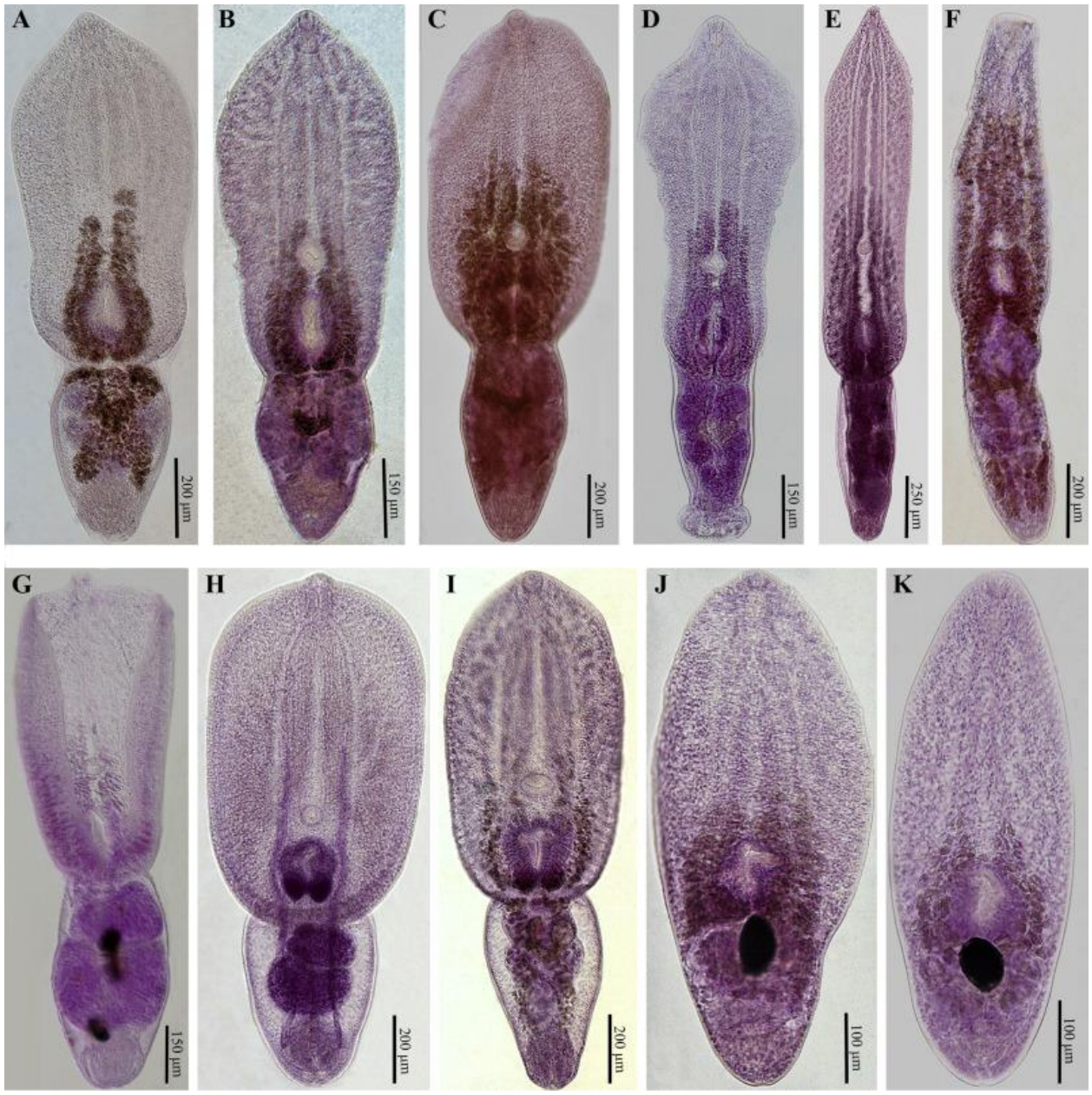


Figure 5. Photographs of *Posthodiplostomum* spp. **A** *Po. cuticola*. **B** *Po. minimum*. **C** *Po. orchilongum*. **D** *Po. centrarchi*. **E** *Po. pricei*. **F** *Po. eurypygae* n. sp. **G** *Po. erickgreenei* n. sp. **H** *Posthodiplostomum* sp. 22. **I** *Po. macrocotyle*. **J** *Po. ptychocheilus*. **K** *Po. recurvirostrae* n. sp.

The adult specimens of taxa from Clade I (including *Ornithodiplostomum* spp.) in our second 28S analysis (Fig. 4) lacked a clear distinction between prosoma and opisthosoma. However, *Po. eurypygae*, which was positioned as the basal branch in Clade II, also lacks a clear distinction between the prosoma and opisthosoma (Figs. 4, 5). Other taxa with corresponding adults included in Clade II have a distinct prosoma and opisthosoma. Furthermore, *M. anterovarium*, which was positioned in Clade IV, also has a weakly separated prosoma and opisthosoma as an adult. However, *Po. pacificus* and *M. pricei*, members of Clade IV, both have a distinct prosoma and opisthosoma. Thus, the combination of molecular phylogenetic data and morphological analysis convincingly demonstrates that the lack of clear separation between prosoma and opisthosoma are not suitable for differentiation of *Ornithodiplostomum* and *Posthodiplostomum*.

The flame-cell formulae provided by Niewiadomska (2002) differs between *Ornithodiplostomum* and *Posthodiplostomum*. However, Dubois (1968) already cast doubt on the reported flame-cell formula in *O. p. ptychocheilus* (type-species of *Ornithodiplostomum*). Furthermore, a dissertation on the larvae of *O. ptychocheilus* by Hendrickson (1978) (likely *O. p. ptychocheilus*) demonstrated that the flame-cells of larval *O. ptychocheilus* are difficult to observe and the author was unable to confirm the number of flame-cells. It remains unclear if the flame-cell formula actually differs between *Ornithodiplostomum* and *Posthodiplostomum*. The flame-cell formula of *Mesoophorodiplostomum* spp. is currently unknown.

Mesoophorodiplostomum is differentiated from *Posthodiplostomum* and *Ornithodiplostomum* based on the position of the ovary (intertesticular in the type-species of *Mesoophorodiplostomum vs* pretesticular or at level of anterior testis in *Posthodiplostomum* and *Ornithodiplostomum* spp.) (Niewiadomska, 2002; López-Hernández et al., 2018; present data).

However, some authors have noted that the ovary can be intertesticular in some immature specimens of *Po. centrarchi* and *Po. brevicaudatum* (Palmieri, 1977; Stoyanov et al., 2017). The ovary of many species of *Posthodiplostomum* (e.g. *Po. recurvirostrae*, *Po. minimum*, *Posthodiplostomum obesum* (Lutz, 1928) is positioned opposite to the anterior testis. In fact, the second known member of *Mesoophorodiplostomum* (*M. anterovarium*) has an ovary which is opposite to the anterior testis (Dronen, 1985). Dronen (1985) remarked that his new species fit characteristics of both *Mesoophorodiplostomum* and *Posthodiplostomum* and only tentatively assigned its genus.

Molecular phylogenies based on 28S (Figs. 2–4) consistently positioned *Mesoophorodiplostomum* (including the type-species *M. pricei*) within clades of *Posthodiplostomum*. Interestingly, *M. pricei* and *M. anterovarium* formed a strongly supported clade with *Po. pacificus* (Figs. 3 and 4), a species with a pretesticular ovary. These results make it clear that the position of ovary is not suitable to distinguish between these three genera.

Our analyses of 28S (Figs. 2 and 3) positioned *Po. cuticola* (type-species of *Posthodiplostomum*) as a sister group to several other clades of *Posthodiplostomum*, *Ornithodiplostomum* and *Mesoophorodiplostomum*. If *Ornithodiplostomum* and *Mesoophorodiplostomum* were to be maintained as separate genera, then the several other clades of *Posthodiplostomum* would require the erection of at least four additional genera. However, morphological features in adult stages do not support the erection of these new genera. For instance, *Po. centrarchi* was originally considered a subspecies of *Po. minimum* due to its extremely similar morphology. However, the 28S phylogeny (Fig. 3) placed these taxa in only a weakly supported clade together with a clade of *Po. pacificus* + *Mesoophorodiplostomum* spp. Clade II contained another previous synonym of *Po. minimum*, namely *Po. orchilongum* as well

as several other species which closely conform to the morphological diagnosis of *Posthodiplostomum* (e.g. *Po. macrocotyle*, *Po. microsicya*). Based on the phylogenetic position of the type-species, *Po. cuticola*, and lack of consistent morphological differences in the adult stages, we consider *Ornithodiplostomum* and *Mesoophorodiplostomum* to be junior synonyms of *Posthodiplostomum*; we transfer all members of these two genera into *Posthodiplostomum*.

Considering the new synonymy, we provide updated species-level lineage numbers for the previously published *Posthodiplostomum* species-level lineages (Table 3). This increases the number of recognized *Posthodiplostomum* species-level lineages in GenBank to 23, including our data (Supplementary Table S1).

López-Hernández et al. (2018) suggested that *Posthodiplostomum* clades may potentially be separated based on the localisation of metacercariae in fishes. *Posthodiplostomum cuticola* (von Nordman, 1832) are known to encyst on the skin of fishes; it formed a sister branch to all other *Posthodiplostomum* spp. in our 28S phylogenies (Figs. 2–4). However, *Posthodiplostomum centrarchi* Hoffman, 1958 and *Posthodiplostomum* cf. *podicipitis* (Yamaguti, 1939) n. comb. were also found on the skin of fishes in the present study (Table 2), although *Po. centrarchi* was more commonly found in visceral organs (e.g. liver and spleen). Based on the currently available data, the site of infection in fishes does not seem to be suitable for separating *Posthodiplostomum* clades.

An amended description of *Posthodiplostomum* is provided below.

Posthodiplostomum Dubois, 1936

Diagnosis (after Niewiadomska, 2002, amended): Digenea: Diplostomidae. Body bipartite, distinctly or indistinctly; prosoma flat or concave, oval, sometimes elongate, linguiform

Table 3 New and updated *Posthodiplostomum* species-level lineage numbers and their corresponding previously-accepted species-level lineage numbers

Updated species-level lineage number	Previously-accepted species-level lineage number	Representative GenBank accession number	Reference
Posthodiplostomum sp. 10	*Ornithodiplostomum* sp. 1	HM064737	Moszczynska et al. (2009)
Posthodiplostomum sp. 11	*Ornithodiplostomum* sp. 2	KT831368	Moszczynska et al. (2009)
Posthodiplostomum sp. 12	*Ornithodiplostomum* sp. 3	HM064780	Moszczynska et al. (2009)
Posthodiplostomum sp. 13	*Ornithodiplostomum* sp. 4	HM064788	Moszczynska et al. (2009)
Posthodiplostomum sp. 14	*Ornithodiplostomum* sp. 8	MH368943	Locke et al. (2010)
Posthodiplostomum sp. 15	Diplostomidae gen. sp. X	MH368849	Gordy and Hanington (2019)
Posthodiplostomum sp. 16	*Posthodiplostomum* sp. 4	MH368945	Gordy and Hanington (2019)
	Posthodiplostomum sp. UG2	LC511187	Komatsu et al. (2020)
	Posthodiplostomum sp. UG3	LC511188	Komatsu et al. (2020)
Posthodiplostomum sp. 17	–	MZ707205	Present study
Posthodiplostomum sp. 18	–	MZ707206	Present study
Posthodiplostomum sp. 19	–	MZ707209	Present study
Posthodiplostomum sp. 20	–	MZ707210	Present study
Posthodiplostomum sp. 21	–	MZ707212	Present study
Posthodiplostomum sp. 22	–	MZ707214	Present study
Posthodiplostomum sp. 23	–	MZ707217	Present study

Note: A single representative GenBank accession number is provided for each new or updated species-level lineage as well as the reference to the origin of the corresponding previously accepted species-level lineage number

or lanceolate; opisthosoma short or long, oval or claviform to subcylindrical. Pseudosuckers absent; holdfast organ subspherical or oval, with cavity opening *via* median slit. Oral and ventral sucker present; oral sucker often weakly developed; pharynx small. Testes two, tandem, different in size and shape; anterior testis asymmetrical or transversely-oval; posterior testis larger,

bilobed, reniform or cordiform, sometimes twisted, often with indentation anteriorly. Ovary ellipsoidal or oval, pretesticular, opposite to anterior testis or intertesticular, median, lateral or diagonal to anterior testis. Vitellarium typically in prosoma and opisthosoma. Copulatory bursa eversible, with terminal or subterminal opening. Genital cone present in most species, surrounded by prepuce, encloses hermaphroditic duct, which is formed at its base by union of uterus and ejaculatory duct; ejaculatory pouch typically absent, terminal portion of seminal vesicle may appear sac-like. Typically in piscivorous birds. Cosmopolitan. Metacercariae in fishes.

Type-species: Po. cuticola (von Nordmann, 1832).

Other species: Po. anterovarium (Dronen, 1985) n. comb., *Po. australe* Dubois, 1937, *Po. bi-ellipticum* Dubois, 1958, *Po. botauri* Vidyarthi, 1938, *Po. boydae* Dubois, 1969, *Po. brevicaudatum* (von Nordmann, 1832), *Po. centrarchi* Hoffman, 1958, *Po. erickgreenei* n. sp., *Po. eurypygae* n. sp., *Po. garambense* (Baer, 1959) n. comb., *Po. giganteum* Dubois, 1988, *Po. grande* (Diesing, 1850), *Po. grayii* (Verma, 1936), *Po. ixobrychi* (Lung Tsu-pei, 1966), *Po. linguaeforme* Pearson & Dubois, 1985, *Po. macrocotyle* Dubois, 1937, *Po. mehtai* Gupta & Mishra, 1974, *Po. microsicya* Dubois, 1936, *Po. mignum* Boero, Led & Brandetti 1972, *Po. milvi* Fotedar & Bambroo, 1965, *Po. minimum* (MacCallum, 1921), *Po. nanum* Dubois, 1937, *Po. obesum* (Lutz, 1928), *Po. oblongum* Dubois, 1937, *Po. opisthosicya* Dubois, 1969, *Po. orchilongum* Noble, 1936, *Po. pacificus* n. sp., *Po. podicipitis* (Yamaguti, 1939) n. comb., *Po. pricei* (Krull, 1934) n. comb., *Po. prosostomum* Dubois & Rausch, 1948, *Po. ptychocheilus ptychocheilus* (Faust, 1917) n. comb., *Po. ptychocheilus palaearcticum* (Odening, 1963) n. comb., *Po. recurvirostrae* n. sp., *Po. scardinii* (Shulman, 1952) n. comb.

Descriptions of new taxa

Posthodiplostomoides kinsellae Achatz, Chermak, Martens, Pulis & Tkach n. sp.

Taxonomic summary

Type-host: *Halcyon malimbica* Shaw (Aves: Alcedinidae).

Type-locality: Kibale National Park (0°21'31.4"N, 30°22'50.2"E), Manairo, Uganda.

Type-material: The type-series consists of four fully mature specimens deposited in the HWML. Holotype: HWML 216635, labeled ex *Halcyon malimbica*, small intestine, Uganda, 20 March 2013, coll. E. Pulis. Paratypes: HWML 216636 (lot of 2 slides), labels identical to the holotype.

Site in host: Small intestine.

Representative DNA sequences: GenBank: MZ710939 (28S), MZ707165 (*cox*1).

ZooBank registration: The Life Science Identifier (LSID) for *Postodiplostomoides kinsellae* n. sp. is urn:lsid:zoobank.org:act:554358B0-8853-4FC4-95F3-FAF877E8DE20.

Etymology: The species is named after J. M. Kinsella for his outstanding contributions to the field of parasitology and being an incredible colleague.

Description

[Based on 4 adult specimens; measurements of holotype given in text; measurements of entire series given in Table 4; Fig. 6] Body 1,171 long, consisting of distinct prosoma and opisthosoma. Prosoma oval, widest at level of ventral sucker, 571 × 339, posterior portion somewhat concave; opisthosoma cylindrical, 580 × 206, somewhat narrower than prosoma. Prosoma:opisthosoma length ratio 1. Forebody 26% of body length. Tegument of prosoma armed with fine spines. Oral sucker subterminal, 58 × 55. Pseudosuckers present, 56–66 × 42. Ventral

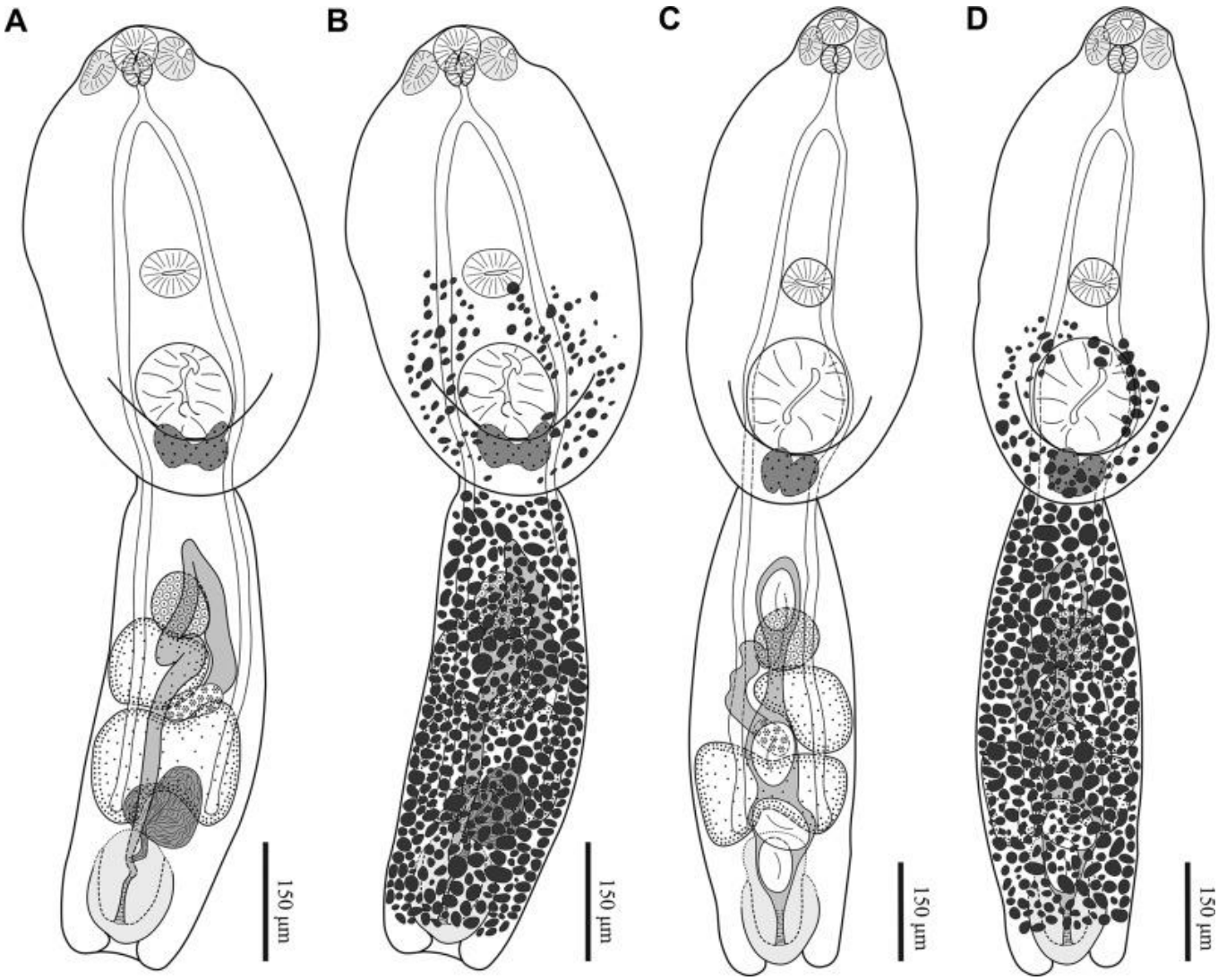


Figure 6. *Posthodiplostomoides kinsellae* n. sp. **A** Ventral view of the holotype, vitellarium omitted. **B** Ventral view of the holotype, vitellarium shown. **C** Ventral view of a paratype, vitellarium omitted. **D** Ventral view of a paratype, vitellarium shown.

sucker somewhat larger than oral sucker, 59 × 73, located near mid-length of prosoma; oral:ventral sucker width ratio 0.8. Holdfast organ 151 × 127, subspherical with ventral muscular portion, posterior to ventral sucker, typically positioned in posterior-most quarter of prosoma. Proteolytic gland dorsal to posterior part of holdfast organ. Prepharynx not observed. Pharynx

oval, 43 × 34. Oesophagus 29 long. Caecal bifurcation in anterior-most 25% of prosoma length. Caeca slender, extending to near posterior margin of posterior testis.

Testes 2, tandem, occupying about half of opisthosoma; anterior testis entire, subspherical or reniform, 111 × 125, posterior testis somewhat bi-lobed, saddle-like, 134 × 183. Seminal vesicle primarily post-testicular, portions ventral to posterior part of posterior testis, compact, was well-observed only in holotype, continues as short ejaculatory duct. Ejaculatory duct joins metraterm dorsally to form hermaphroditic duct near proximal part of genital cone. Hermaphroditic duct opens at tip of genital cone; genital cone with ventral prepuce within genital atrium. Genital cone and prepuce occupy majority of genital atrium. Genital pore terminal.

Ovary pretesticular, subspherical, 75 × 76. Oötype and Mehlis' gland not well-observed. Laurer's canal not observed. Vitellarium extending posteriorly from level of or slightly posterior to the level of the ventral sucker to about the posterior margin of the opisthosoma; vitellarium sparsely distributed in prosoma. Vitelline reservoir intertesticular. Uterus ventral to gonads, contains no egg in holotype, up to five eggs in paratypes (88–105 × 56–67).

Excretory vesicle and pore not observed.

Remarks

Posthodiplostomoides kinsellae n. sp. belongs to the genus based on the presence of pseudosuckers and a genital cone with genital prepuce. The new species differs from the two

Table 4
Ranges of morphometric characters of *Posthodiplostomoides* spp.

Species	*Ps. kinsellae* n. sp.		*Ps. opisthadenicus*	*Ps. leonensis*[b]
Host	*Halcyon malimbica*		*Scopus umbretta*	*Bubulcus ibis*
Locality	Uganda		Zimbabwe	Sierra Leone
Reference	Present study		Dubois and Beverly-Burton (1971)	Williams (1967)
	Holotype and paratypes (n = 3)[a]	Hologenophore	n = 9	n = not provided
Body length	1,171–1,389 (1,252)	–	Up to 1,800	950–1,100
Prosoma length	569–721 (620)	–	630–770	490–580
Prosoma width	334–360 (344)	–	250–280	320–380
Opisthosoma length	580–686 (625)	–	670–1,050	460–520
Opisthosoma width	206–246 (232)	182	200–290	240–270
Prosoma:opisthosoma length ratio	0.9–1.1 (1.0)	–	0.7[c]	1.2[c]
Forebody (% of body length)	54–58 (56)	–	66%[c]	59[c]
Oral sucker length	56–58 (57)	–	47–60	50–60
Oral sucker width	55–56 (55)	–	57–68	50–80
Pseudosucker length	54–66 (59)	–	–	–
Pseudosucker width	28–43 (39)	–	–	–
Ventral sucker length	55–59 (58)	–	60–73	40–55
Ventral sucker width	67–73 (69)	–	65–78	57–75
Oral sucker:ventral sucker width ratio	0.8 (0.8)	–	0.9[c]	0.9[c]
Holdfast organ length	132–175 (153)	–	90–125	80–100
Holdfast organ width	127–167 (142)	–	90–120	80–100
Pharynx length	36–45 (41)	–	37–42	30–50
Pharynx width	34–37 (35)	–	30–37	20–30
Oral sucker:pharynx length ratio	1.2–1.6 (1.4)	–	1.23[c]	1.2[c]
Oesophagus length	29–60 (40)	–	–	–
Anterior testis length	111–127 (119)	–	85–175	80–120

Anterior testis width	125–144 (140)	–	195–270	190–260
Posterior testis length	123–141 (133)	–	160–250	120–160
Posterior testis width	183–227 (210)	–	200–270	180–240
Ovary length	75–85 (80)	72	50–68	60–100
Ovary width	76–95 (84)	85	90–105	50–70
Number of eggs	0–5	4	1	0–2
Egg length	88–97 (91)	63–67	–	73
Egg width	56–66 (61)	89–105	–	52
Anterior vitellarium free zone (% of prosoma length)	52–59 (55)	–	80[c]	46[c]
Posterior vitellarium free zone (% of opisthosoma length)	5–6 (5)	–	6[c]	16[c]

[a] Mean provided for *Posthodiplostomoides kinsellae* n. sp. in parentheses after range.
[b] Obtained from experimental infection by Williams (1967).
[c] Calculated based measurements of line drawing in original description.

other known *Posthodiplostomoides* species, *Posthodiplostomoides leonensis* (Williams, 1967) and *Posthodiplostomoides opisthadenicus* Dubois & Beverly-Burton, 1971, based on the distribution of the vitellarium (sparsely distributed in the prosoma and extending anteriorly to about the level of the ventral sucker or somewhat more posterior to it in the new species *vs* densely distributed in prosoma extending anterior to the level of the ventral sucker in *Ps. leonensis* and vitellarium in prosoma restricted to the area around holdfast organ in *Ps. opisthadenicus*), and the distinction between prosoma and opisthosoma (clearly distinct in the new species *vs* much less distinct in the two other species). This new species of *Posthodiplostomoides* can be further distinguished from the other two species in the possession of a larger holdfast organ (132–175 × 132– 167 µm in *Ps. kinsellae vs* 80–100 × 80–100 µm in *Ps. leonensis* and 90–125 × 90–120 µm in *Ps. opisthadenicus*).

Pairwise comparisons of *Posthodiplostomum* spp.

Many of the sequences of *Posthodiplostomum* spp. available in GenBank were obtained from larval stages; these larval stages typically cannot be reliably identified to the species based on morphology alone. Unfortunately, comparisons with the previously published sequences suggest that at least some sequences contain errors as they include numerous ambiguous sites and indels of lengths that cannot be divided by three (e.g. 1–2 nucleotides long) in the protein-coding gene *cox*1. Comparisons of DNA sequences must only utilize accurate sequences.

The interspecific divergence of 28S sequences among *Posthodiplostomum* spp. was generally low (0–9.6%). *Posthodiplostomum* sp. 20 *vs Posthodiplostomum* sp. 11 were the least divergent at 0%, whereas *Po. orchilongum vs Posthodiplostomum* sp. 1 of Sokolov and Gordeev (2020) (GenBank: MT394051) were the most divergent at 9.6%.

Intraspecific variation was only detected within four *Posthodiplostomum* spp. with multiple 28S sequences: *Po. anterovarium*, *Po. centrarchi*, *Posthodiplostomum* sp. 11 and *Posthodiplostomum* sp. 20. Interestingly, three out of 11 partial 28S sequences of *Po. centrarchi* contained an ambiguous site (cytosine or thymine), while the remaining eight had a thymine at the same position. *Posthodiplostomum anterovarium*, *Posthodiplostomum* sp. 11 and *Posthodiplostomum* sp. 20 each had a single ambiguous base.

The interspecific divergence of *cox*1 sequences among *Posthodiplostomum* spp. was much greater than among 28S sequences (4.1–22.3%) and overall similar to the interspecific divergence of *cox*1 sequences demonstrated within other diplostomoidean genera (3.4–19.8%) (e.g. Hernández-Mena et al., 2014; Gordy et al., 2017; Locke et al., 2018; López-Hernández et al., 2018; Achatz et al., 2020b and references therein; Tkach et al., 2020). *Posthodiplostomum minimum* (MacCallum 1921) and *Posthodiplostomum* sp. 16 were the least divergent at 4.1%;

Posthodiplostomum cuticola and *Posthodiplostomum brevicaudatum* were the most divergent at 22.3%. Despite only 0–0.1% difference between 28S sequences of *Posthodiplostomum* sp. 11 and *Posthodiplostomum* sp. 20, these two species-level lineages differed by 9.6–10.2% in *cox*1 sequences.

Due to the similarity of *cox*1 sequences among *Po. minimum* and *Posthodiplostomum* sp. 16 in the pairwise comparisons of all *Posthodiplostomum* spp., an additional alignment limited to *cox*1 sequences of *Po. minimum* and *Posthodiplostomum* sp. 16 was analyzed; this additional alignment was 72 nucleotides longer than the alignment used for general pairwise comparisons of *Posthodiplostomum* spp. The pairwise comparisons based on this longer alignment demonstrated *Po. minimum vs Posthodiplostomum* sp. 16 to be 5.3–6.0% different.

An additional alignment was analyzed to explore the intraspecific variation of *Po. anterovarium* (= *Posthodiplostomum* sp. 1 and sp. 2 of Moszczynska et al. (2009)). The additional alignment was 25 nucleotides longer than the alignment used for general pairwise comparisons of *Posthodiplostomum* spp. The *cox*1 sequence of the adult specimen of *Po. anterovarium* (GenBank: MZ707168) was 3.0–3.5% different from the data of the larval specimens previously referred to as *Posthodiplostomum* sp. 1 and *Posthodiplostomum* sp. 2 of Moszczynska et al. (2009) as well as the sequences from our larval specimens; the larval specimens of the previously accepted *Posthodiplostomum* sp. 1 and sp. 2 of Moszczynska et al. (2009) differed by 2.8–3.8%. Our *cox*1 sequences from larvae and *Posthodiplostomum* sp. 2 of Moszczynska et al. (2009) varied by up to 2.5%. Importantly, the level of variation among *cox*1 sequences of the adult *Po. anterovarium* and genetically similar larvae are gradual (). In our opinion, the differences detected among the *cox*1 sequences of these isolates do not provide enough support to consider these separate species/species-level lineages without clear

morphological differences in adult specimens. As such, we consider these larvae (e.g., *Posthodiplostomum* spp. 1 and 2 of Moszczynska et al. (2009)) to be *Po.* 'cf.' *anterovarium* until matching sequences from adults will become available.

Remarks on *Posthodiplostomum* diversity

In the present study, we have generated new ribosomal and mitochondrial DNA sequences of the type species of *Bolbophorus* Dubois, 1934, two species of *Cercocotyla* Yamaguti, 1939, one new species of *Posthodiplostomoides*, 23 species/species-level lineages of *Posthodiplostomum* (syns. *Mesoophorodiplostomum* and *Ornithodiplostomum*) and the type-species of *Pulvinifer*. We provided DNA sequence data from adults of 19 species/species-level lineages, 14 of which were identified to species based on adult morphology. In addition, our DNA sequences represent 14 species/species-level lineages of *Posthodiplostomum*, which lacked previously published DNA sequence data.

Our results show that the currently known diversity of *Posthodiplostomum* is underestimated. The genus, as recognized in this study, was represented in the Nearctic by 12 nominal species. Our data, combined with previous studies, demonstrated the presence of at least 17 species-level lineages in the Nearctic. Furthermore, the morphology of our specimens of *Posthodiplostomum* sp. 21 and 22 suggests the presence of at least two additional species in the Neotropics; however, our adult specimens of these species-level lineages are not sufficient for description. We hypothesize that the diversity of *Posthodiplostomum* in other biogeographic realms has been similarly underestimated.

Our specimens of *Po. minimum* from the great blue heron *Ardea herodias* L. and black-crowned night heron *Nycticorax nycticorax* (L.) closely conform to the original description of

Po. minimum collected from *A. herodias* in a zoo in New York, USA by MacCallum (1921) and the subsequent description of *Po. minimum* provided by Dubois and Rausch (1948) based on specimens collected from *A. herodias* and *N. nycticorax* in the Midwestern United States (e.g. Wisconsin, Michigan and Ohio). *Posthodiplostomum* sp. UG1 of Komatsu et al. (2020) (GenBank: LC511186) is clearly conspecific with our *Po. minimum* based on comparison of *cox*1 data (0–0.7% divergence in partial *cox*1 sequences). At the same time, *Posthodiplostomum* sp. 16 (= *Posthodiplostomum* sp. 4 of Gordy and Hanington (2019); e.g. GenBank: MH368945) and *Posthodiplostomum* sp. UG2 and UG3 of Komatsu et al. (2020) (GenBank: LC511187 and LC511188) appear to be conspecific based on comparison of *cox*1 sequences (0–1.8% divergence in partial *cox*1 sequences). The *cox*1 sequences of *Po. minimum* (= *Posthodiplostomum* sp. 4 of Moszczynska et al. (2009)) and *Posthodiplostomum* sp. 16 (= *Posthodiplostomum* sp. 4 of Gordy & Hanington (2019) and UG2 and UG3 of Komatsu et al. (2020)) also differ by 5.3–6%. In our opinion, this range of divergence exceeds what can be reasonably expected for intraspecific variation based on currently available data for the diplostomoideans. It is critical that adults which correspond to the genotype of *Posthodiplostomum* sp. 16 are collected for proper morphological comparison with *Po. minimum*. The presently available data demonstrate that at least three species of *Posthodiplostomum*, *Po. centrachi*, *Po. minimum* and *Posthodiplostomum* sp. 16, have Holarctic distributions.

Posthodiplostomum orchilongum is currently considered a synonym of *Po. minimum* (Dubois, 1938, 1968). Our phylogenetic analyses (Figs. 3 and 4) clearly demonstrate that these taxa represent distinct species-level lineages. These two species are most easily distinguished based on differences in the holdfast organ (typically subspherical or transversely-oval in *Po.*

orchilongum vs longitudinally-oval in *Po. minimum*) as well as the anterior extent of vitellarium (extending more anteriorly to the level of the ventral sucker in *Po. orchilongum vs* typically only reaching to the level of or slightly anterior to the level of the ventral sucker in *Po. minimum*). Based on the results of our molecular phylogenetic analyses as well as morphological differences, we restore *Po. orchilongum* as an independent species. We expect that additional differences may be found in other stages of the life cycle.

Prior to this study, *Posthodiplostomum nanum* was known to be distributed only in the Neotropics (Dubois, 1937; López-Hernández et al., 2018). This is the first report of *Po. nanum* in the Nearctic region. However, it is important to note that *Po. nanum* studied by López-Hernández et al. (2018) has vitellarium in both the prosoma and opisthosoma, whereas the material originally described by Dubois (1937) has vitellarium only in the prosoma. Our specimens are conspecific with *Po. nanum* studied by López-Hernández et al. (2018) based on morphology as well as the comparison of *cox*1 sequences (1.4% difference). The distribution of the vitellarium has been demonstrated to be rather stable within a *Posthodiplostomum* species (Pérez-Ponce de León, 1995; present study). It is likely that the specimens currently identified as *Po. nanum* represents a novel species. Similar to the situation regarding *Po. minimum*, DNA sequences from specimens that conform to the original description of *Po. nanum* by Dubois (1937) are needed to test if the two morphotypes are conspecific.

Our specimens of *Po.* cf. *podicipitis* from a hooded merganser *Lophodytes cucullatus* (L.) are morphologically similar to the original description of specimens from the little grebe *Tachybaptus ruficollis* (Pallas) (*Podiceps ruficollis*) collected in Japan by Yamaguti (1939). It is possible that our material represents a novel species based on the difference in the order of definitive host (Anseriformes *vs* Podicipediformes) as well as the fact that the distribution range

of *Ta. ruficollis* does not extend into the Nearctic, nor does the geographical range of *L. cucullatus* extend into the Palaearctic. Unfortunately, data on snail intermediate hosts of these taxa are not available. However, at this point we consider the description of our material as a novel species premature until comparable data of *Po. podicipitis* from *Ta. ruficollis* in Japan become available.

Mesoophorodiplostomum was previously considered a separate genus (Dubois, 1936; Niewiadomska, 2002), in part, based on the position of the ovary (intertesticular in *Postodiplostomum pricei* (Krull, 1934) n. comb., the former type-species of *Mesoophorodiplostomum*). Our examination of ovary position of *Posthodiplostomum* spp. included in our 28S analysis (Fig. 4) demonstrated some clades to have relatively stable position of ovary (e.g. the ovary of members of Clade I was opposite to the anterior testis). However, other clades that include multiple species/species-level lineages (i.e. Clades II and III) had a variable position of the ovary. Importantly, previous authors have demonstrated that the position of the ovary may change during development (e.g. Stoyanov et al., 2017) or in adults (e.g. Palmieri, 1977). Our specimens of *Po. anterovarium*, *Po. centrachi* and *Posthodiplostomum* sp. 22 demonstrate variation in ovary position between the more immature and mature adult specimens (e.g. intertesticular in immature forms that transitions to pretesticular in adults of *Po. centrarchi*) (Fig. 4). Therefore, the exact position of the ovary should not be heavily relied upon for differentiation of *Posthodiplostomum* spp. except in fully mature adult specimens.

Most *Posthodiplostomum* spp. have a relatively distinct prosoma and opisthosoma. However, members of the former *Ornithodiplostomum* (Clade I; Fig. 4) as well as *Po. anterovarium* (Clade III; Fig. 4) and *Po. eurypygae* (Clade II; Fig. 4) have relatively indistinct separation between prosoma and opisthosoma. While this feature is suitable for assisting with

differentiation of many *Posthodiplostomum* spp., it is clearly not suitable for supra-specific systematics. It is worth noting that among *Posthodiplostomoides* spp., only the new species described here have a clearly distinct prosoma and opisthosoma. At the same time, all other morphological features support its generic placement.

Our analyses demonstrate that Diplostomoidea sp. (GenBank: KU221112, KY319363 and KY319364), Digenean sp. (GenBank: MK321671) and Diplostomidae gen. sp. X (GenBank: MH368849) belong to *Posthodiplostomum* (Figs. 2–4). Identity of these forms will need to be established in the future by matching their sequences to sequences of properly fixed and identified adult digeneans.

Biogeography and host associations of *Posthodiplostomum*

Considering the ecological relevance of members of *Posthodiplostomum*, notably as major causative agents of white grub and black spot disease in fishes, it is critical to understand the diversity of *Posthodiplostomum* spp. worldwide as well as their host-associations throughout their life cycles.

The 28S analysis of *Posthodiplostomum* spp. positioned *Po. cuticola* from the Palaearctic (Ukraine) as a strongly supported sister group to all other *Posthodiplostomum* spp. (Fig. 3). Likewise, four isolates of *Posthodiplostomum* spp. larvae from the Indomalayan (India and Vietnam) and Palaearctic (Japan) realms were positioned in a 100% supported clade separate from the 100% supported clade containing the remaining *Posthodiplostomum* spp. The position of *Po. cuticola* and the clade from the Indomalayan and Palaearctic realms strongly suggest an Old World origin of the genus. The strong support and branch lengths of the cluster of the four *Posthodiplostomum* spp. larvae from the Indomalayan (India and Vietnam) and Palaearctic

(Japan) realms suggest that members of the cluster may be endemic to Southeastern Asia and nearby regions (i.e. Japan).

Only two of the seven clades within the larger internal cluster of *Posthodiplostomum* spp. (Fig. 3) contained species from a single biogeographic realm, Nearctic in case of Clade III and Palaearctic in case of Clade VI. The remaining five clades contained representatives from more than one biogeographic realm. The branch topology within Clade II suggests a dispersal from the Neotropics into the Nearctic and Afrotropics (Fig. 3) while the branch topology in Clade I clearly suggests the dispersal of *Po. scardinii* from Nearctic to Palaearctic. Clades IV, V and VII failed to demonstrate any clear patterns of biogeography. *Posthodiplostomum centrarchi* (Clade IV; Nearctic and Palaearctic), *Po. minimum* (Clade V; Nearctic and Palaearctic) and *Po. nanum* (Clade VII; Nearctic and Neotropics) were collected in two biogeographic realms. Distribution of diplostomoideans (e.g. *Diplostomum ardeae* Dubois, 1969 and *Diplostomum huronense* (La Rue, 1927)) across multiple biogeographic realms has been previously demonstrated with DNA sequence data (e.g. Locke et al., 2020; Achatz et al., 2021c). In part, the extremely broad distribution of some *Posthodiplostomum* spp. may be facilitated by the broad geographical distribution and migratory nature of many of the avian definitive hosts; for instance, *A. alba* and *N. nycticorax* both have essentially worldwide distributions and are semi-migratory. The wide geographical distribution of *Posthodiplostomum* spp. is also possible due to the ubiquity of their potential snail intermediate hosts.

Based on the positions of *Po. cuticola* as well as *Po. centrarchi*, *Po. nanum* and *Posthodiplostomum* sp. 23 (Fig. 3), it would not be unreasonable to hypothesize that the ancestors of these diplostomoideans parasitized ardeid definitive hosts (e.g. herons). Additional 28S sequence data from other species of *Posthodiplostomum*, many of which parasitize ardeids,

are necessary to further test this hypothesis. In addition, our phylogenetic analysis of *Posthodiplostomum* spp. based on 28S sequences (Fig. 3) revealed several secondary definitive host-switching events in the evolutionary history of *Posthodiplostomum*.

Clades I, II, III and VII (Fig. 3) included species which originate from a variety of definitive hosts. The members of Clade I included adults collected from anatids (common merganser Mergus merganser L. and *L. cucullatus*; three *Posthodiplostomum* species/species-level lineages), a recurvirostrid (American avocet *Recurvirostra americana* Gmelin; *Po. recurvirostrae*) and a pelecanid (*Pe. erythrorhynchos*; *Posthodiplostomum* sp. 18). The position of *Posthodiplostomum* sp. 17 from *L. cucullatus* as a sister branch to the 100% supported clade which contained other members of Clade I, as well as the positions of *Po.* cf. *podicipitis* (collected from *L. cucullatus*) and *Po. ptychocheilus* (collected from a *M. merganser*) within the 100% supported clade suggest a possible host switch from merganser ducks to avocets and pelicans (Fig. 3; Table 2). However, the adult specimens of the other five species-level lineages within this clade remain to be collected and sequenced, which should clarify the picture of their host associations. Clade II demonstrates multiple transitions among lineages of avian definitive hosts (Fig. 3). For instance, *Po. eurypygae* from a eurypygid (sun bittern *Eurypyga helias* (Pallas)) was positioned as a sister group to species collected from ardeids (great egret *Ardea alba* L., cocoi heron *Ardea cocoi* L., little blue heron *Egretta caerulea* (L.) and rufescent tiger heron *Tigrisoma lineatum* (Boddaert); four *Posthodiplostomum* species/species-level lineages), accipitrids (black-collared hawk *Busarellus nigricollis* (Latham); *Po. macrocotyle*), a ciconiid (jabiru *Jabiru mycteria* (Lichtenstein)) and a pandionid (western osprey *Pandion haliaetus* (L.); *Po. erickgreenei*). Interestingly, three species/species-level lineages (*Po. microsicya*, *Posthodiplostomum* sp. 21 and 22) from *T. lineatum* formed a strongly supported clade (99%)

which indicates a single transition to *T. lineatum*. Clade III included species collected from larids (California gull *Larus californicus* (Lawrence) and ring-billed gull *Larus delawarensis* Ord; two *Posthodiplostomum* species/species-level lineages) and a pelecanid (*Pe. erythrorhynchos* ; *Po. anterovarium*). Clade VII included two species/species-level lineages from ardeids (*A. alba* and *A. herodias*) and a single species-level lineage from a phalacrocoracid (neotropic cormorant *Nannopterum brasilianum* (Gmelin)). More data on definitive and intermediate hosts are necessary to address the directionality of host-switching within these two clades.

Our 28S tree of *Posthodiplostomum* spp. (Fig. 4) revealed some associations between the strongly supported clusters/clades and the order of their fish second intermediate hosts. For instance, four species-level lineages from the Indomalayan and Palaearctic realms (GenBank: AB693170, KF738450, MT394045 and MT394051) were collected from fishes in the order Anabantiformes Britz, whereas three species-level lineages from Clade I (Fig. 4) were collected from fishes in the order Cypriniformes Bleeker. Although all former members of *Mesoophorodiplostomum* (Clade III; Fig. 4) were collected from perciform fishes, one species (*Po. pricei*) was found in fishes from the order Cyprinodontiformes Berg. The fish second intermediate hosts of many *Posthodiplostomum* species-level lineages are currently unknown, thus, it can be anticipated that some of these relationships may change once more data regarding the second intermediate hosts become available.

To the best of our knowledge, this is the first report of *Posthodiplostomum* spp. (or its new synonyms) from sunbitterns (Eurypygidae Selby), anhingas (Anhingidae Reichenbach) and avocets (Recurvirostridae Bonaparte). Based on our newly collected and sequenced specimens (Table 2) it is clear that *Posthodiplostomum* spp. and its new synonyms parasitize at least members of the orders Accipitriformes Vieillot (e.g. hawks and osprey), Charadriiformes Huxley

(e.g. gulls, avocets), Eurypygiformes Hackett, Kimball, Reddy, Bowie, Braun, Braun, Chojnowski, Cox, Han, Harshman, Huddleston, Marks, Miglia, Moore, Sheldon, Steadman, Witt & Yuri (sunbitterns), Pelecaniformes Sharpe (e.g. pelicans, herons) and Suliformes Sharpe (e.g. anhingas, cormorants). It is worth noting that literature data (e.g. Dubois, 1968) claim that *Posthodiplostomum* spp. parasitize other orders of avian definitive hosts (e.g. Podicipediformes). It will be interesting to see how taxa collected from members of other avian orders, such as Podicipediformes (grebes), will impact the topologies of the *Posthodiplostomum* phylogenies.

Management strategies focused on the definitive hosts of *Posthodiplostomum* spp. must target a wide diversity of fish-eating birds, besides the most commonly reported ardeid hosts, as previously suggested by some authors (e.g. Lane and Morris, 2000). Our data from adult specimens expands the reference set of *Posthodiplostomum* spp. sequences which is critical for future ecological and systematic studies on agents of white grub and 'black spot' disease worldwide. Our results further demonstrate that management strategies should also consider other birds that may not be commonly viewed as piscivorous, such as avocets. However, snail controlling measures may be the more realistic and efficient avenue as opposed to limiting access of avian definitive hosts to water bodies.

CHAPTER 4:

Phylogenetic relationships and further unknown diversity of diplostomids (Diplostomida: Diplostomidae) parasitic in kingfishers

4.1 Introduction

Kingfishers (Alecedinidae Rafinesque) are known to be definitive hosts of a wide range of digeneans that use fishes as second intermediate hosts (Van Haitsma, 1925; Hoffman, 1956; Boyd & Fry, 1971; Merino *et al.,* 2003; Muzzall *et al.,* 2011). Among these digeneans, members of the Diplostomidae Poirier, 1886 are most common worldwide, including the New World (Hoffman, 1956; Muzzall *et al.,* 2011; López-Jiménez *et al.,* 2018; Achatz *et al.,* 2019a,b, 2021a,b). Members of nine diplostomid genera are known to parasitize kingfishers as adult parasites (Dubois, 1968; Niewiadomska, 2002; Achatz *et al.,* 2021a,b. The genera *Crassiphiala* Van Haitsma, 1925 and *Uvulifer* Yamaguti, 1934 (López-Jiménez *et al.,* 2018; Achatz *et al.,* 2019a,b, 2021a,b) are known to encyst on the skin/fins of fishes and often cause 'black spot disease' in their fish second intermediate hosts (Van Haitsma, 1925; Hunter, 1933; Hoffman, 1956); their association with potential health concerns of fishes has led to interest in revealing the identities of these digeneans.

Herein, we obtained DNA sequences from seven diplostomid taxa infecting kingfishers, including *Crassiphiala* and *Uvulifer* spp., collected in North and South America as well as the Philippines. Partial sequences of 28S gene were used to study the interrelationships of these diplostomids and *cox*1 data were utilized for differentiation among closely related species. We provide descriptions of a new diplostomid genus and three new species of diplostomids. Additionally, we provide an amended diagnosis of *Crassiphiala* and a description of *Crassiphiala bulboglossa* Van Haitsma, 1925, the type-species of the genus, using newly

collected high quality specimens. We have generated the first DNA sequence data of a member of *Subuvulifer* Dubois, 1952.

4.2 Materials and methods

Adult diplostomids were collected from the intestines of a belted kingfisher Megaceryle alcyon (Linnaeus) in North Dakota, USA, ringed kingfisher Megaceryle torquata (Linnaeus) and green kingfisher *Chloroceryle americana* (Gmelin) in Pantanal, Mato Grosso State, Brazil, M. torquata in Lábrea, State of Amazonas, Brazil and white-throated kingfisher *Halcyon smyrnensis* (Linnaeus) from the Mindoro Island, Philippines. A single *M. alcyon* from North Dakota was collected using the federal collecting permit MB072162-0. A single *M. torquata* from Lábrea was collected as a part of the biodiversity survey funded by the National Science Foundation, USA, based on the collecting permit 37740-4 from the Ministry of the Environment (Ministério do Meio Ambiente), Brazil. Birds in Pantanal were collected based on the collecting permit 10698-1 and birds in the Philippines were obtained for parasitological examination from Dr. Carl Oliveros as a part of the biodiversity survey funded by the National Science Foundation. Type series and morphological vouchers of adult specimens are deposited in the Museu Paraense Emílio Goeldi (MPEG), Belém, Brazil and the collection of the H. W. Manter Laboratory, University of Nebraska, Lincoln, Nebraska, USA (table 5). Previously deposited vouchers identified as *Crassiphiala* spp. and sequenced by Achatz et al. (2019b) were re-examined. Metacercariae of Uvulifer semicircumcisus Dubois et Rausch, 1950 were collected from the skin and fins of the Northern redbelly dace Chrosomus eos Cope in Minnesota, USA. Fixation and staining of all specimens follow our general methods.

The DNA extraction, amplification, sequencing, and alignment also follow our general methods. We used the the following primers for amplification and sequencing; digL2, 1500R for 28S and Plat-diploCOX1F, Cox1_Schist_5', Dipl_Cox_5', BS_CO1_INT_F, Plat-diploCOX1R, JB5, Dipl650R, Dipl_Cox_3' and BS_CO1_INT_R for cox1 (table 1).

Table 5. Hosts, GenBank accession numbers and museum accession numbers assigned by the Museu Paraense Emílio Goeldi (MPEG) and Harold W. Manter Laboratory (HWML) for diplostomids studied in present work. Abbreviations for life stage: A, adult; C, cercaria; M, metacercaria. GenBank accession numbers for new sequences generated in the present study are in bold.

Taxa	**Life stage**	**Host species**	**Geographic origin**	**Museum No.**	**GenBank Accession numbers**	
					28S	***cox*1**
Crassiphiala bulboglossa	A	*Megaceryle alcyon*	North Dakota, USA	HWML 216888–216893	–	**OP688075–OP688082**
*C. bulboglossa**	A	*Megaceryle alcyon*	Minnesota, USA	–	MN200254	MN193952
*C. bulboglossa**	M	*Chrosomus eos*	Minnesota, USA	–	MN200255	MN193953
*C. bulboglossa**	M	*Umbra limi*	Minnesota, USA	–	MN200256	MN193954, MN193955
Crassiphiala jeffreybelli n. sp.	A	*Megaceryle torquata*	Pantanal, Brazil	MPEG 000335–000339 HWML 216896	–	**OP688085**
C. jeffreybelli n. sp.	A	*Chloroceryle americana*	Pantanal, Brazil	–	**OP687981**	**OP688086**
Crassiphiala wecksteini n. sp.	A	*Megaceryle torquata*	Pantanal, Brazil	MPEG 000349–000354 HWML 216014, 216894, 216895	**OP687979, OP687980**	**OP688083, OP688084**
C. wecksteini n. sp.‡	A	*Megaceryle torquata*	Pantanal, Brazil	HWML 216014	MN200261	MN193959, MN193960

Pseudocrassiphiala tulipifera n. sp.	A	*Megaceryle torquata*	Lábrea, Brazil	–	**OP687982**	**OP688087**
P. tulipifera n. sp. †	A	*Megaceryle torquata*	Pantanal, Brazil	MPEG 000340–000348 HWML 216013, 216897, 216898	MN200258–MN200260	MN193957, MN193958
Pseudocrassiphiala sp. VVT1	A	*Chloroceryle americana*	Pantanal, Brazil	–	**OP687983**	**OP688088**
Subuvulifer glandulaxiculus	A	*Halcyon smyrnensis*	Philippines	HWML 216918–216925	**OP687984–OP687986**	**OP688089–OP688092**
Uvulifer semicircumcisus	M	*Chrosomus eos*	Minnesota, USA	–	**OP687987**	**OP688093**
U. semicircumcisus	A	*Megaceryle alcyon*	North Dakota, USA	HWML 216926–216927	–	**OP688094–OP688096**

* Previously published as *Crassiphiala* sp. lineage 2 of Achatz et al. (2019b).
‡ Previously published as *Crassiphiala* sp. lineage 5 of Achatz et al. (2019b).
† Previously published as *Crassiphiala* sp. lineage 4 of Achatz et al. (2019b).

Phylogenetic relationships of the diplostomid taxa studied in the present work were estimated based on an alignment of partial 28S sequences. The alignment included newly generated sequences from members of *Crassiphiala*, *Uvulifer*, *Subuvulifer* and the new genus (table 5) and previously published sequences of 37 diplostomids and 14 strigeids. The alignment included representatives from all currently sequenced genera of the Diplostomidae and Strigeidae Railliet, 1919; we only included sequences that were at least 1,100 base pairs (bp) long to avoid significant loss of data. *Suchocyathocotyle crocodili* (Yamaguti, 1954) was used as the outgroup for the analyses based on the study by Achatz *et al.* (2019c).

The best-fitting nucleotide substitution models for the alignment was determined using MEGA7. The analysis used the general time-reversible model with estimates of invariant sites and gamma-distributed among-site variation (GTR + G + I) model. The standard BI was

performed in addition to ML analysis. Nodal support of ML analysis was estimated by performing 1,000 bootstrap pseudoreplicates. Pairwise comparisons were performed using MEGA7.

4.3 Results

Molecular phylogenies

Upon trimming to the length of the shortest sequence, the alignment was 1,115 bp long; 33 nucleotide sites were excluded due to ambiguous homology. The strongly supported topologies were identical between the BI and ML analyses. The Diplostomidae and Strigeidae were clearly non-monophyletic throughout the basal polytomy (Fig. 7); at the same time, the representatives of the Proterodiplostomidae formed a strongly supported clade (100% BI; 99% ML). These results are generally similar to those published and discussed in previous molecular phylogenetic studies (e.g., Blasco-Costa & Locke, 2017; Hernández-Mena *et al.*, 2017; Achatz *et al.*, 2019b,c, 2021a,b, 2022a,b; Tkach *et al.*, 2020; Locke *et al.*, 2021). Therefore, below we focus on details related to the clades that contain newly generated data.

Members of *Subuvulifer*, *Crassiphiala*, *Uvulifer, Posthodiplostomoides* Williams, 1969 and the new genus formed a strongly supported clade (100% BI; 97% ML) in the basal polytomy of the Diplostomoidea consisting of five clades/branches with unresolved relationships, each representing a single genus (Fig. 7). *Subuvulifer* and *Posthodiplostomoides* were represented in the tree by a single species each, *Subuvulifer glandulaxiculus* Pearson et Dubois, 1985 and *Posthodiplostomoides kinsellae* Achatz, Chermak, Martens, Pulis et Tkach, 2021. The clade containing two species of *Pseudocrassiphiala* n. gen. was strongly supported (100% BI; 99% ML).

The strongly supported (100 BI; 99% ML) clade containing *Crassiphiala* spp. (weak support in BI and ML) included two clusters: *Crassiphiala jeffreybelli* n. sp. + *Crassiphiala* p. lineage 1 of Achatz et al. (2019b) (99% BI; 100% ML) and C. bulboglossa + Crassiphiala wecksteini n. sp. + Crassiphiala sp. lineage 3 of Achatz et al. (2019b) (100% BI; 99% ML).

Lastly, the clade of *Uvulifer* spp. included a well-supported (99% BI; 92% ML) grouping of *Uvulifer pequenae* + *Uvulifer spinatus* and a weakly supported clade of *Uvulifer elongatus* + a strongly supported cluster (100% BI; 99% ML) of *Uvulifer ambloplitis* + *U. semicircumcisus*.

Descriptions

At the time of publication of the previous diagnosis of *Crassiphiala* (Niewiadomska 2002), the genus was considered monotypic. We provide a new diagnosis of the genus to accommodate the features of the new species described herein as well as *Crassiphiala ceryliformis* Vidyarthi, 1938. The latter species was originally placed in *Crassiphiala* and subsequently transferred into *Uvulifer*; we return it to *Crassiphiala* based on morphological evidence (see detailed discussion below).

Family Diplostomidae Poirier, 1886

Diagnosis. Body distinctly bipartite; prosoma generally flattened or with slight concavity, much shorter than cylindrical opisthosoma. Tegument unarmed. Oral sucker present; ventral sucker and pseudosuckers absent. Holdfast organ elliptical or bulbous, with median opening, may occupy entire width of prosoma. Pharynx present; ceca reach level of seminal vesicle. Testes 2, tandem. Seminal vesicle compact, winding, with pouch-like expansion at proximal end.

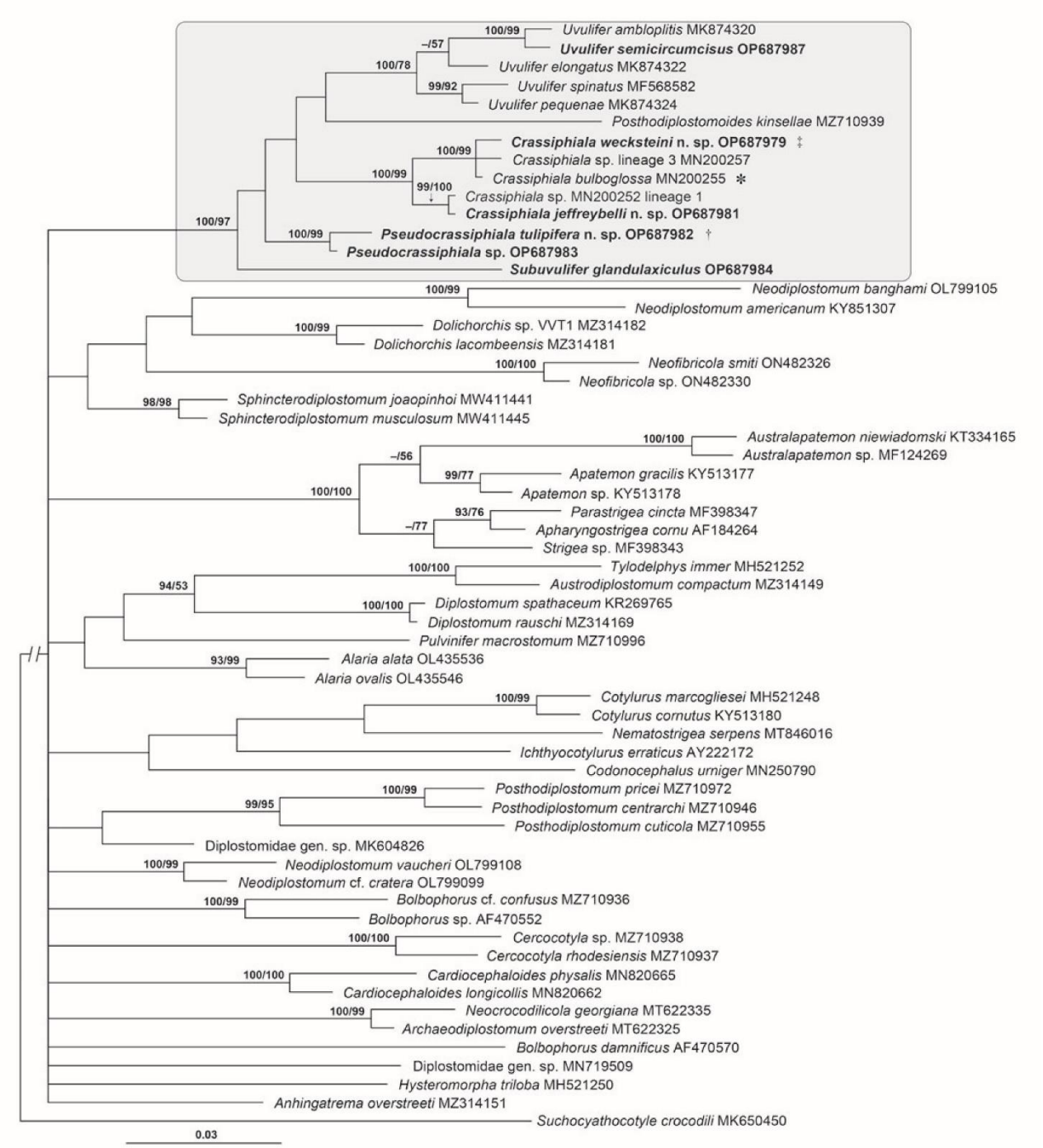


Figure 7. Phylogenetic interrelationships among 59 diplostomoideans based on Bayesian Inference (BI) and Maximum Likelihood analyses of partial 28S rDNA gene sequences. Topology from the BI analysis is provided. Bayesian inference posterior probability/ML bootstrap values are provided above internodes. The BI posterior probability values lower than 90% and ML bootstrap values lower than 50% are not shown. The new sequences generated in this study are in bold. The scale bar indicates the number of substitutions per site. The clade containing digeneans studied in the present work is in the shaded box. GenBank accession numbers are provided after names of taxa. * Previously published as *Crassiphiala* sp. lineage 2 of Achatz *et al.* (2019b). ‡ Previously published as *Crassiphiala* sp. lineage 5 of Achatz *et al.* (2019b). † Previously published as *Crassiphiala* sp. lineage 4 of Achatz *et al.* (2019b).

Ejaculatory pouch absent. Ejaculatory duct, typically short, may be dilated resulting in pouch-like appearance, joins distal part of metraterm to form a short hermaphroditic duct. Hermaphroditic duct opens at apex of genital cone. Genital cone with prepucial (=prepuce-like) fold, opens into genital atrium. Genital atrium with terminal opening. Ovary pretesticular; oötype intertesticular. Vitellarium distributed throughout opisthosoma; vitelline reservoir intertesticular. Excretory pore subterminal on ventral side. In kingfishers. Nearctic, Neotropics, Indomalaya. *Type species: Crassiphiala bulboglossa* Van Haitsma, 1925. *Other species: Crassiphiala ceryliformis* Vidyarthi, 1938, *Crassiphiala jeffreybelli* n. sp. Achatz, Von Holten, Fecchio et Tkach, *Crassiphiala wecksteini* n. sp. Achatz, Von Holten, Fecchio et Tkach.

Morphological description of the type-species of *Crassiphiala*

Crassiphiala bulboglossa Van Haitsma, 1925 (figs 8, 9)

Taxonomic summary:

Type host: *Megaceryle alcyon* (Linnaeus) (Coraciiformes: Alcedinidae).

Site of infection: small intestine.

Type locality: *Douglas Lake Michigan, USA.*

Collection locality in this study: Grand Forks Co., North Dakota, USA

Infection rate: numerous *C. bulboglossa* were found in single *M. alcyon* from North Dakota.

Type material: Slides deposited in the National Museum of Natural History (NMNH), Washington D.C., under accessions USNPC 071491.00.

New specimens deposited: 38 mature specimens deposited in the HWML. Vouchers: HWML 216888, labeled ex. *Megaceryle alcyon*, small intestine, Grand Forks Co., North Dakota, USA, June 07, 2018, coll. T. Achatz. Hologenophores (5 slides): HWML 216889−216893, label identical to the vouchers.

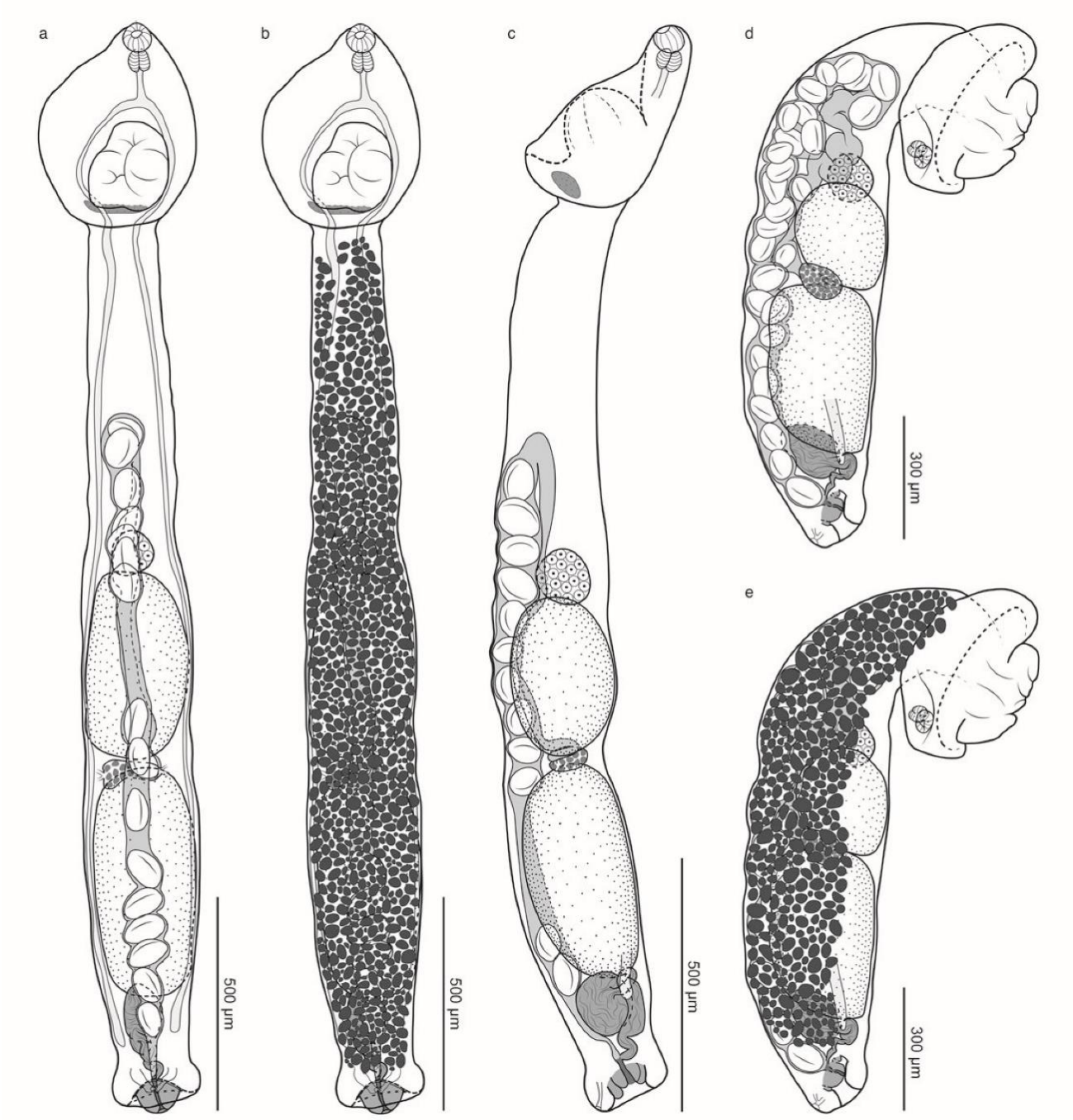


Figure 8. *Crassiphiala bulboglossa.* (a) hologenophore 1, ventral view with vitellarium omitted; (b) hologenophore 2, ventral view with vitellarium shown; (c) voucher 1, relaxed, lateral view; (d) voucher 2, contracted, lateral view with vitellarium omitted; (e) voucher 3, contracted, lateral view with vitellarium shown.

Table 6. Morphometric characters of new diplostomids described in the present study. Ranges provided followed by mean in parentheses.

Species	***Crassiphiala bulboglossa***	***Crassiphiala jeffreybelli,* n. sp.**	***Crassiphiala weckstеini,* n. sp.**	***Pseudocrassiphiala tulipifera,* n. sp.**
Body length	1,694−2,982 (2,649)	1,454−1,728 (1,585)	864−1,317 (1,164)	2,534−4,050 (3,373)
Prosoma length	356−545 (458)	315−374 (339)	230−335 (279)	473−612 (545)
Prosoma width	317−478 (389)	270−291 (281)	198−270 (236)	435−527 (480)
Opisthosoma length	1,252−2,562 (2,187)	1,139−1,511 (1,290)	589−1,020 (892)	2,024−3,502 (2,822)
Opisthosoma width	200−339 (288)	161−212 (187)	154−245 (202)	285−402 (345)
Oral sucker length	45−77 (57)	38−65 (47)	28−45 (38)	19−38 (28)
Oral sucker width	51−75 (63)	38−67 (59)	39−50 (44)	38−49 (45)
Pharynx length	37−59 (47)	40−50 (43)	31−43 (36)	40−50 (45)
Pharynx width	45−52 (48)	49−53 (51)	29−40 (35)	40−54 (49)
Esophagus length	33−72 (52)	21−27 (24)	22−63 (39)	60−88 (73)
Holdfast organ length	194−336 (246)	110−157 (141)	94−191 (150)	205−285 (236)
Holdfast organ width	205−335 (252)	60−93 (72)	132−223 (174)	165−295 (225)
Anterior testis length	206−540 (384)	99−216 (173)	120−207 (175)	323−466 (394)
Anterior testis width	157−284 (221)	65−141 (117)	84−191 (154)	225−327 (271)
Posterior testis length	267−697 (547)	87−211 (164)	154−228 (192)	380−477 (420)
Posterior testis width	160−285 (244)	72−148 (120)	113−175 (151)	225−367 (286)
Ovary length	102−161 (123)	92−107 (99)	55−93 (79)	123−169 (146)
Ovary width	78−134 (107)	72−88 (82)	62−80 (74)	110−138 (129)
Number of eggs	0−32 (17)	2−3 (3)	0−3 (1)	0−20 (4)
Egg length	90−114 (102)	80−93 (88)	103−104 (104)	101−113 (107)
Egg width	48−67 (57)	45−62 (52)	56−63 (60)	46−64 (57)
Prosoma:opisthosoma length ratio	0.2−0.4 (0.2)	0.3 (0.3)	0.2−0.5 (0.3)	0.2−0.3 (0.2)
Opisthosoma length:width ratio	6.2−10.3 (7.8)	5.7−7.7 (7)	3.8−5.2 (4.5)	6.9−10.5 (8.2)
Holdfast organ:prosoma width r	0.5−0.9 (0.6)	0.2−0.3 (0.3)	0.6−1.0 (0.7)	0.3−0.5 (0.4)

Hologenophore DNA sequences: *cox*1: OP688075 (HWML 216889), OP688076 (HWML 216890), OP688077 (HWML 216891), OP688079 (HWML 216892), OP688081 (HWML 216893).

Previously published genetic lineage name: *Crassiphiala* sp. lineage 2 of Achatz *et al.* (2019b)

Description. Based on 38 adult specimens. Measurement ranges given in text and table 6. Body 1,694−2,982 long, consists of distinct prosoma and opisthosoma; prosoma oval, with shallow concavity, 356−545 long, widest at level of holdfast organ, 317−478; opisthosoma elongated, cylindrical, 1,252−2,562 × 200−339; opisthosoma length:width ratio 6.2−10.3. Prosoma:opisthosoma length ratio 0.2−0.4. Tegument unarmed. Oral sucker subterminal, 45−77 × 51−75. Pseudosuckers absent. Holdfast organ oval, with longitudinal aperture, armed with fine spines, proximal portion glandular, 194−336 × 205−335; holdfast organ:prosoma width ratio 0.5−0.9. Proteolytic gland consisting of diffuse gland cells. Prepharynx absent. Pharynx subspherical, 37−59 × 45−52. Esophagus 33−72 long. Cecal bifurcation in anterior 40% of prosoma length. Ceca slender, extend to near level of seminal vesicle.

Testes 2, tandem, rounded, entire, anterior testis 206−540 × 157−284, posterior testis 267−697 × 160−285. Seminal vesicle post-testicular, proximal portion pouch-like, followed by winding distal portion that joins distal part of metraterm to form short hermaphroditic duct. Hermaphroditic duct opens at apex of small muscular genital cone. Genital cone surrounded by small prepucial fold, positioned within genital atrium. Prepucial fold small or not observable when genital cone everted (Fig. 9a vs. Fig. 9b−d). Genital atrium with terminal opening.

Ovary pretesticular, subspherical, 102−161 × 78−134. Oötype and Mehlis' gland intertesticular (not illustrated). Vitelline follicles limited to opisthosoma, distributed from near level of prosoma-opisthosoma junction to near posterior end of opisthosoma. Vitelline reservoir

intertesticular. Uterus ventral to gonads, extends anteriorly beyond level of ovary before turning and extending posteriorly. Uterus in our specimens containing up to 32 eggs. Eggs 90–114 × 48–67. Excretory pore subterminal, ventral.

Remarks

Historically, descriptions of many diplostomoideans were based on laterally oriented specimens, for example see the numerous illustrations in the monograph by Dubois (1968). The same is true in the case of the original description and illustrations of *C. bulboglossa* (Van Haitsma, 1925). Our newly collected adult specimens of *C. bulboglossa* allowed us to study these digeneans in both ventro-dorsal and lateral orientations. Although the description by Van Haitsma (1925) lacked many essential measurements, the description and illustrations closely resemble our contracted, laterally positioned specimens (Fig. 8d,e). Our newly collected digeneans demonstrated substantial morphological variation in appearance. Some of this variation may be the result of different ages of the diplostomids or the crowding effect, since the host studied was infected with many hundreds of these digeneans. The extent of body contraction and eversion of the genital cone are at least partly responsible for the observed morphological variation (figs 7, 8). The partial *cox*1 sequences of *C. bulboglossa* obtained from specimens of different sizes and states of contraction were identical (table 5).

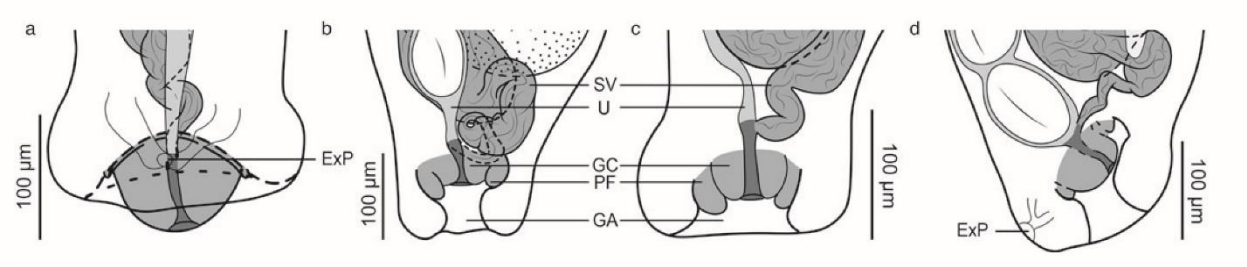


Figure 9. Posterior end of opisthosoma of *Crassiphiala bulboglossa* with vitellarium omitted. (a) hologenophore 1, ventral view, genital cone everted; (b) hologenophore 2, ventral view; (c) voucher 1, lateral view; (d) hologenophore 3, lateral view. Abbreviations: ExP, excretory pore; GA, genital atrium; GC, genital cone; PF, prepucial fold; SV, seminal vesicle; U, uterus.

4.4 Discussion

Our phylogenetic analyses (Fig. 7) demonstrated that members of *Subuvulifer*, *Pseudocrassiphiala*, *Crassiphiala*, *Posthodiplostomoides* and *Uvulifer* form a strongly supported monophyletic group. Although the interrelationships among genera have not been resolved, all genus-level clades were strongly supported and revealed the presence of at least two unknown species-level lineages of *Crassiphiala* and one additional species of *Pseudocrassiphiala*. Unfortunately, specimens representing these three additional species were either adults in poor condition, or metacercariae and thus not suitable for descriptions.

Van Haitsma (1925) erected *Crassiphiala* for diplostomids collected from the intestine of *M. alcyon* in Michigan, USA. Until recently, the genus was viewed as monotypic and limited in its distribution to the Nearctic (Preble & Harwood, 1944; Dubois & Rausch, 1948 Hoffman, 1956; Dubois, 1968; Boyd & Fry, 1971; Scott, 1984; Niewiadomska, 2002; Muzzall *et al.*, 2011), except for a single report by Dubois (1970) who identified *C. bulboglossa* among specimens from an unknown species of kingfisher in Brazil collected by A. Lutz. Based on DNA sequences, Achatz *et al.* (2019b) demonstrated the presence of at least five species-level lineages of *Crassiphiala* throughout the New World (3 in the North America and two in the South America). The present data reveals the presence of at least two additional closely related species-level lineages in the New World. We have provided morphological descriptions for four of these seven species-level lineages, which include representatives of a new genus (*Pseudocrassiphiala* n. gen.) as well as *Crassiphiala* (tables 5, 6). It is worth noting that Crassiphialinae gen. sp. collected from *Biomphalaria straminea* (Dunker) in Belo Horizonte, State of Minais Gerais, Brazil by López-Hernández *et al.* (2019), is potentially conspecific with *C. wecksteini* n. sp. based on the low level of genetic divergence. The two forms differ by only 2.3–3.4% divergence

in partial sequences of *cox*1 (table 7). However, previous studies (Achatz *et al.*, 2021b and references therein) have demonstrated that interspecific variation between diplostomid species may be as low as 3.4% in this fragment of *cox*1. Intraspecific variability of *cox*1 sequences of *Crassiphiala* spp. in our study showed only minimal variation (0.5% in *C. jeffreybelli*, up 0.3% in *C. bulboglossa*, and up to 1% in *C. wecksteini*). The two *cox*1 sequences of *P. tulipifera* differed by only 1.3%, despite originating from distant geographic locations in Brazil.

In the original description of *C. bulboglossa*, Van Haitsma (1925) erroneously referred to an expanded portion of the seminal vesicle as an ejaculatory pouch. An ejaculatory pouch in diplostomids is a muscular/glandular structure that surrounds at least part of the ejaculatory duct (Achatz *et al.*, 2022b). This structure is absent in *Crassiphiala* spp., but present in members of other genera, including *Uvulifer* (Niewiadomska, 2002; Achatz *et al.*, 2019a, 2022b).

Vidyarthi (1938) described *C. ceryliformis* based on specimens collected from the intestine of the pied kingfisher *Ceryle rudis* (Linnaeus) in India. *Crassiphiala ceryliformis* was described as lacking a ventral sucker, but having an ejaculatory pouch (Vidyarthi, 1938). Bhalerao (1942) transferred this species into *Uvulifer* based on the smaller holdfast organ compared to *C. bulboglossa* and stated that the relative holdfast organ size held more taxonomic importance than the presence/absence of the ventral sucker. It is clear that *Uvulifer ceryliformis* (Vidyarthi, 1938) is more morphologically similar to *C. jeffreybelli* n. sp. than to any member of *Uvulifer*. Based on the illustration by Vidyarthi, (1938), the ejaculatory pouch of *U. ceryliformis* is likely a dilated ejaculatory duct, similar to the condition in *C. jeffreybelli* n. sp. Both *U. ceryliformis* and *C. jeffreybelli* n. sp. lack a ventral sucker and have a holdfast organ that does not occupy much of the prosoma width. Based on morphological comparisons, we return *U. ceryliformis* to *Crassiphiala* as *Crassiphiala ceryliformis* (Vidyarthi, 1938).

Table 7. Pairwise comparisons of partial *cox*1 mtDNA gene sequences among *Crassiphiala* spp. and the new genera. Percentage differences are given above the diagonal, and number of variable nucleotide positions are given below the diagonal. Results based on a 386 bp long alignment.

		1. OP688077	**2.** MN193952	**3.** OP688083	**4.** OP688084	**5.** MN193959	**6.** MN193960	**7.** MN179323	**8.** MN193956	**9.** OP688086	**10.** OP688085	**11.** MN193951
1.	*Crassiphiala bulboglossa* OP688077	–	0.3%	11.7%	11.7%	11.9%	12.2%	12.4%	13.7 %	14.2%	14.5%	12.2%
2.	*C. bulboglossa* MN193952*	1	–	11.9%	11.9%	12.2%	12.4%	12.7%	14.0 %	14.5%	14.8%	12.4%
3.	*Crassiphiala wecksteini* n. sp. OP688083	45	46	–	1.0%	0.8%	0.5%	3.4%	13.0 %	10.6%	10.6%	14.0%
4.	*C. wecksteini* n. sp. OP688084	45	46	4	–	0.8%	1.0%	2.3%	14.0 %	10.6%	10.6%	13.7%
5.	*C. wecksteini* n. sp. MN193959‡	46	47	3	3	–	0.8%	3.1%	13.2 %	10.4%	10.4%	14.2%
6.	*C. wecksteini* n. sp. MN193960‡	47	48	2	4	3	–	3.4%	13.5 %	10.6%	10.6%	14.2%
7.	*Crassiphiala* sp. MN179323§	48	49	13	9	12	13	–	15.3 %	10.9%	10.9%	14.8%
8.	*Crassiphiala* sp. lineage 3 MN193956	53	54	50	54	51	52	59	–	17.6%	17.9%	16.6%
9.	*Crassiphiala jeffreybelli* n. sp. OP688086	55	56	41	41	40	41	42	68	–	0.5%	11.4%
10.	*C. jeffreybelli* n. sp. OP688085	56	57	41	41	40	41	42	69	2	–	11.4%
11.	*Crassiphiala* sp. lineage 1 MN193951	47	48	54	53	55	55	57	64	44	44	–

* Previously published as *Crassiphiala* sp. lineage 2 of Achatz et al. (2019b).

‡ Previously published as *Crassiphiala* sp. lineage 5 of Achatz et al. (2019b).

§ Previously published as Crassiphialinae gen. sp. of López-Hernández et al. (2019).

Until 2018, only a single species of *Crassiphiala* and five species of *Uvulifer* were known from kingfishers in the New World. The present study and recent publications (López-Jiménez *et al.*, 2018; Achatz *et al.*, 2019a,b) have revealed four additional species/species-level lineages of *Crassiphiala* and seven additional species/species-level lineages of *Uvulifer* in the New World. The diversity of these diplostomids from kingfishers in the New World is further expanded by the members of *Pseudocrassiphiala* n. gen. (2 species/species-level lineages), *Sphincterodiplostomum* Dubois, 1936 (1 species) and *Posthodiplostomum* Dubois, 1936 (1 species) (Achatz *et al.*, 2021a,b). Based on the current knowledge, it is certain that at least four species of *Uvulifer* as well as additional *Crassiphiala* (2 species) and *Pseudocrassiphiala* n. gen. (1 species) require description when suitable specimens are available.

We have provided the first DNA sequence data from a member of *Subuvulifer* (*S. glandulaxiculus*) and *U. semicircumcisus. Subuvulifer* is a small genus with only three nominal species: *Subuvulifer halcyonae* (Gogate, 1940), *Subuvulifer sabahensis* (Fischthal et Kuntz, 1973) and *S. glandulaxiculus*.

CHAPTER 5:

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

5.1 Introduction

The Diplostomidae Poirier, 1886 is a large, globally distributed family of digeneans, parasitizing the intestines of their tetrapod definitive hosts (Niewiadomska, 2002; Heneberg et al., 2020). The type-genus *Diplostomum* von Nordmann, 1932 (subfamily Diplostominae Poirier, 1886) is highly speciose and globally distributed (Shigin, 1986, 1993; Galazzo et al., 2002; Niewiadomska, 2010; Georgieva et al., 2013; Locke et al., 2015; 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; Locke et al., 2010a, b, 2015; Niewiadomska, 2010; Georgieva et al., 2013; Pérez-del-Olmo et al., 2014; Kudlai et al., 2017; Hoogendoorn et al., 2020; Vivas Muñoz et al., 2021).

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 of *Diplostomum*. 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; García-Varela et al., 2016; Blasco-Costa and Locke, 2017; Achatz et al., 2019b-d, 2020, 2021; Sereno-Uribe et al., 2019a, b; Heneberg et al., 2020; Hoogendoorn et al., 2020; Tkach et al., 2020). Members of these three genera utilize a variety of fishes as second intermediate hosts and typically parasitize fish-eating birds as adults (e.g., Gibson, 1996; Niewiadomska, 2002; Locke et al., 2010a, b, 2015; Georgieva et al., 2013; Rosser et al., 2016a, b). 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 the type-species *Paralaria clathrata* (Diesing, 1850) and *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 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).

Well over 1,000 *cox*1 sequences of *Diplostomum, Tylodelphys* and *Austrodiplostomum* are currently available in GenBank, whereas no DNA sequence data are published for *Paralaria* or *Dolichorchis*. Despite the recent surge of 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.

5.2 Materials and methods

Morphological study

Adult diplostomids were obtained from the intestines of a variety of avian and mammal hosts and larval diplostomids were collected from a variety of snail and fish species in Europe as well as North and South America (Table 9). Digeneans were removed from host, heat-killed, fixed and stained according to our general methods. Staining and light microscopy also followed our general methods. Live digeneans were briefly rinsed in saline, heat-killed with hot water and fixed in 70% 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 (Table 9).

Molecular study

Genomic DNA was extracted and amplified using our general methods. A fragment of 28S rRNA gene was amplified by PCR using the forward primer digL2 and reverse primer 1500R (Tkach et al., 2003). Fragments of *cox*1 were amplified by PCR using the forward primers Plat-diploCOX1F, Cox1_Schist_5', Dipl_Cox_5' and BS_CO1_INT_F with reverse primers Plat-diploCOX1R, acox650R, JB5, Dipl650R, Dipl_Cox_3'and BS_CO1_INT_R (Lockyer et al., 2003; Derycke et al., 2005; Moszczynska et al., 2009; Kudlai et al., 2015; Achatz et al., 2019a, 2022c).

PCR primers were used for sequencing along with forward primers DPL600F and DPL250F and reverse primers DPL700R, DPL350R and DPL1450R. Sequecing, alignment and BI followed our general methods.

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) along with previously published sequences of *Diplostomum* (*n* = 3), *Tylodelphys* (*n* = 1) and *Austrodiplostomum* (*n* = 1) along 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 recognized, 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), along 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), along 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.

To accurately and consistently identify which species-level lineage is referred to throughout the chapter, 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).

5.3 Results

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; Hernández-Mena et al., 2017; Locke et al., 2018; Achatz et al. 2019b–d, 2020, 2021; Queiroz et al., 2020; Tkach et al., 2020), our broader 28S phylogeny (Fig. 10) demonstrated the non-monophyletic nature of the Diplostomidae and Strigeidae.

Diplostomum, *Paralaria*, *Tylodelphys* and *Austrodiplostomum* formed a weakly supported clade. However, the internal topology within this clade was well-resolved. *Diplostomum* + *Paralaria* formed a 100% supported clade; likewise, *Tylodelphys* +

Austrodiplostomum also formed a 100% supported clade. Both of these 100% supported clades had well-supported internal topologies. *Tylodelphys*, as currently recognized, 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. The unidentified diplostomid lineage (Diplostomidae gen. sp. VVT1) formed a separate branch as a part of the extensive basal polytomy of the Diplostomoidea (Fig. 10).

Upon trimming to the length of the shortest sequence, the second 28S alignment limited to *Diplostomum* and *Paralaria* as currently recognized, was 1,106 bp long. The internal topology within this tree was overall moderately resolved (Fig. 11). Similar to the broader 28S phylogeny (Fig. 10), *Diplostomum* sp. VVT5 (= *D. ardeae sensu* Locke et al. (2015); see 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. 11); admittedly, this relationship was not well supported. A number of internal topologies were much better resolved. The two *Diplostomum* spp. (sp. A & 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 4.3 below]; and (iii) a weakly supported large clade consisting of two sub-clades (Fig. 11). The first of these sub-clades included a 100% supported cluster of [*Diplostomum* sp. VVT1 + *Diplostomum scudderi* Olivier, 1941 (94% supported) and

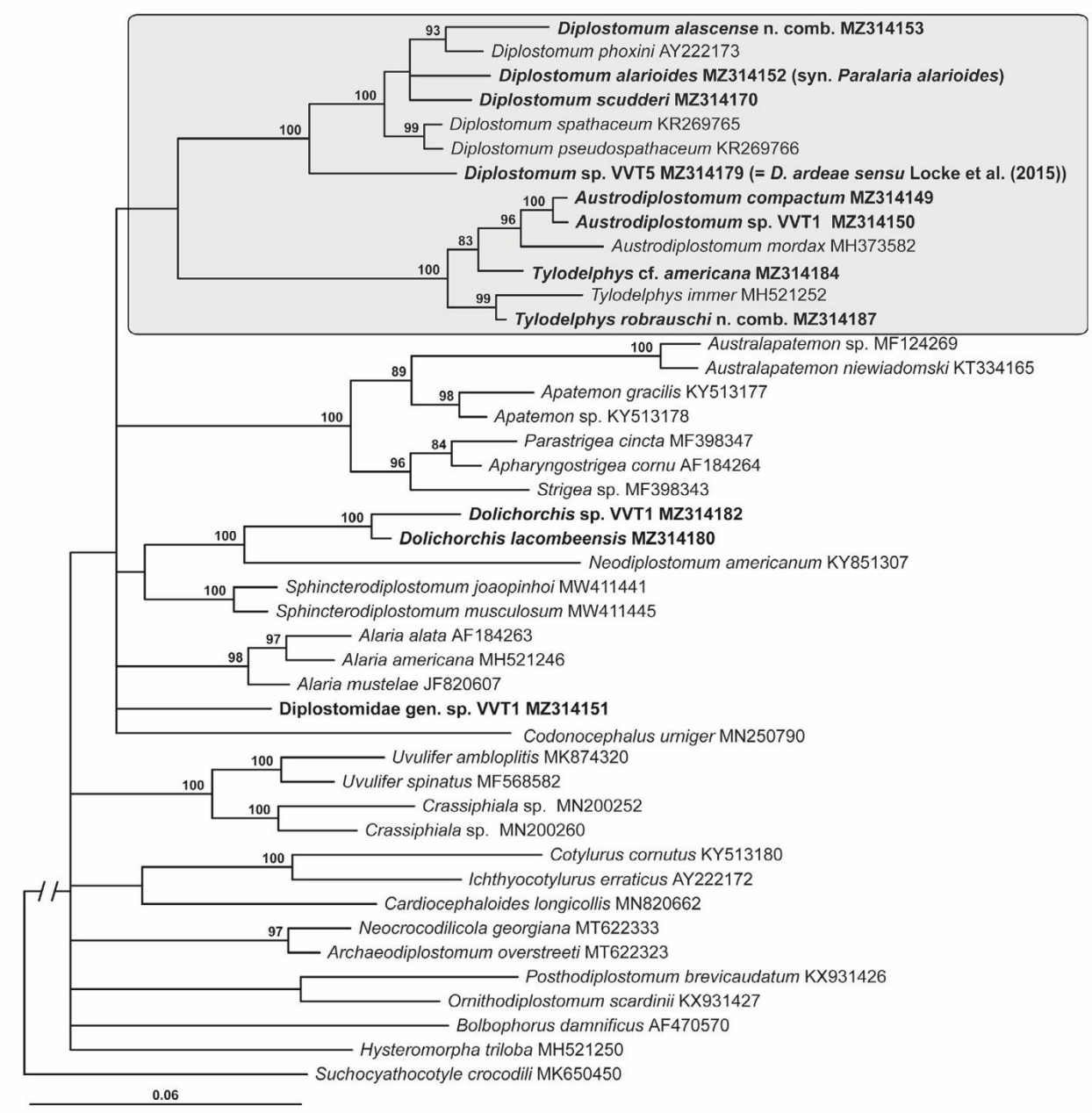


Figure 10. Phylogenetic interrelationships among 43 diplostomoidean taxa including 13 members of *Diplostomum*, *Tylodelphys* and *Austrodiplostomum* (including a former *Paralaria* sp.), two species-level lineages of *Dolichorchis* and an unknown diplostomid based on Bayesian inference analysis of partial 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.

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 characterized 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. 11).

The third 28S alignment was 1,106 bp long and limited to members of *Tylodelphys* and *Austrodiplostomum* taxa, as currently recognized. The phylogenetic tree resulting from the analysis of this alignment contained two clusters (Fig. 12). *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. + an 98% supported clade of [*Tylodelphys* sp. VVT1 + an 98% supported clade of (*Tylodelphys immer* Dubois, 1961 + *Tylodelphys aztecae* García-Varela, Sereno-Uribe, Pinacho-Pinacho, Hernández-Cruz & Pérez-Ponce de León, 2015)]. 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

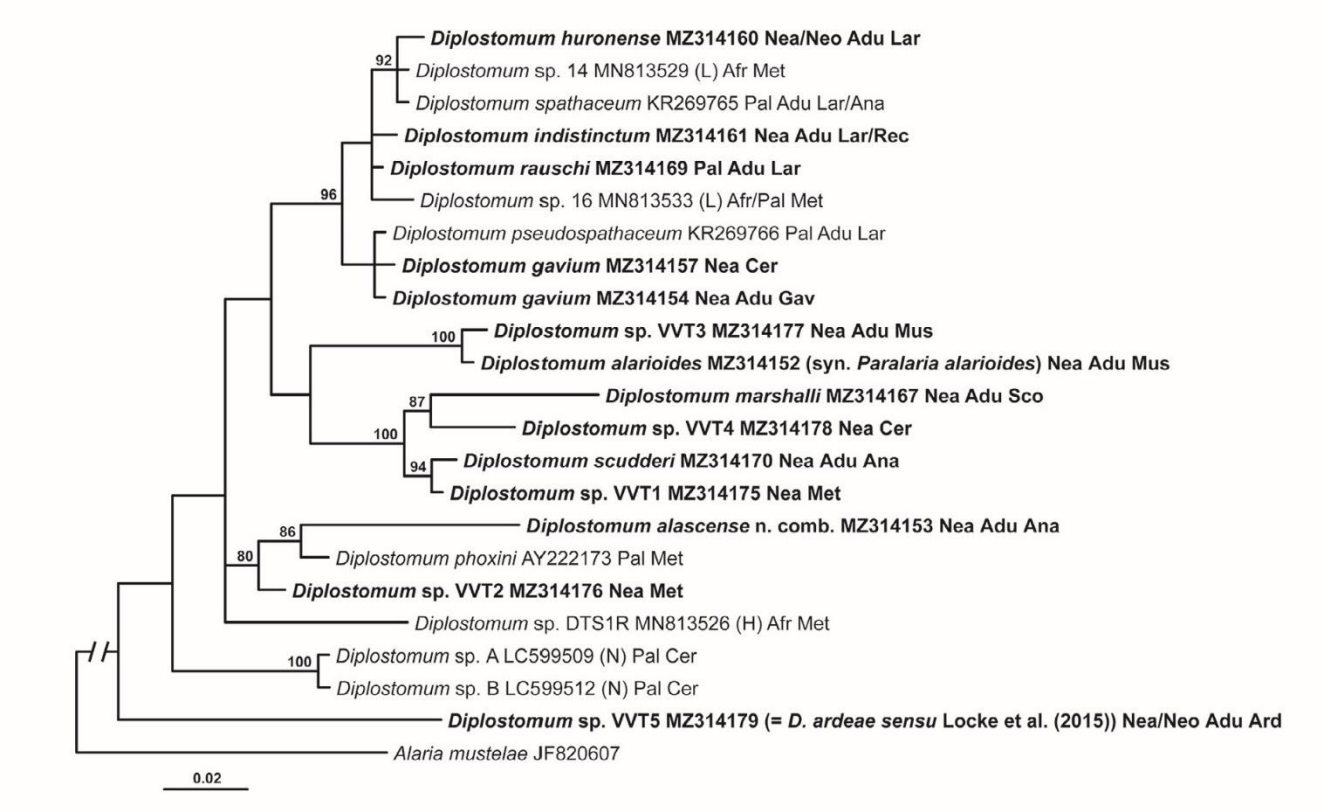


Figure 11. Phylogenetic interrelationships among 21 taxa of *Diplostomum* (including a former *Paralaria* sp.) based on Bayesian inference analysis of partial 28S rRNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale-bar indicates the number of substitutions per site. GenBank accession numbers are provided after the names of species. Reference to origin of species numbering/naming systems is provided in parentheses after GenBank accession numbers followed by biogeographical realm where specimens were collected, life stage of isolate and family of definitive host (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, b; 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.

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. 12).

The *cox*1 alignments were 362 bp long. We provide the phylogenetic tree based on *cox*1 data from *Diplostomum* spp. (Fig. 14) at the end of this chapter in reduced size 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. 13; Fig 14).

The majority of *Diplostomum* spp. formed a 100% supported polytomous cluster with multiple well-supported internal clades, some of them well-resolved (Fig. 14; 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) (Fig. 14). The second major sub-clade also included several species-level lineages and only a single named species, *D. alascense* n. comb.

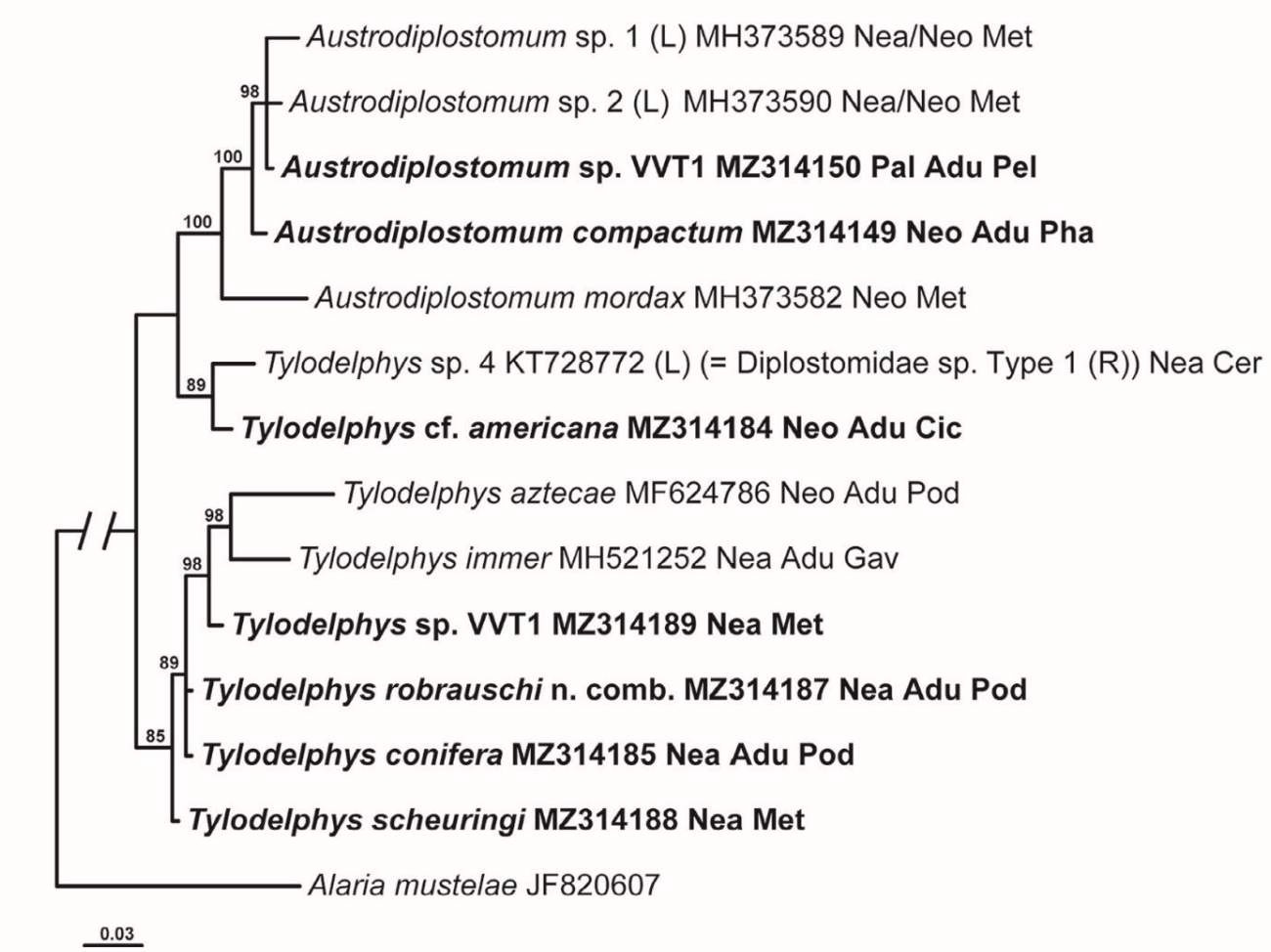


Figure 12. Phylogenetic interrelationships among 13 taxa of *Tylodelphys* and *Austrodiplostomum* spp. based on Bayesian inference analysis of partial 28S rRNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale-bar indicates the number of substitutions per site. GenBank accession numbers are provided after the names of species. Reference to origin of species numbering/naming systems is provided in parentheses after GenBank accession numbers followed by biogeographical realm where specimens were collected, life stage of isolate and family of definitive host (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, b; 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 family of definitive host: Cic, Ciconiidae; Gav, Gaviidae; Pel, Pelecanidae; Pha, Phalacrocoracidae; Pod, Podicipedidae.

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.

The strongly supported 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 (Fig. 14) 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 (Fig. 14). 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).

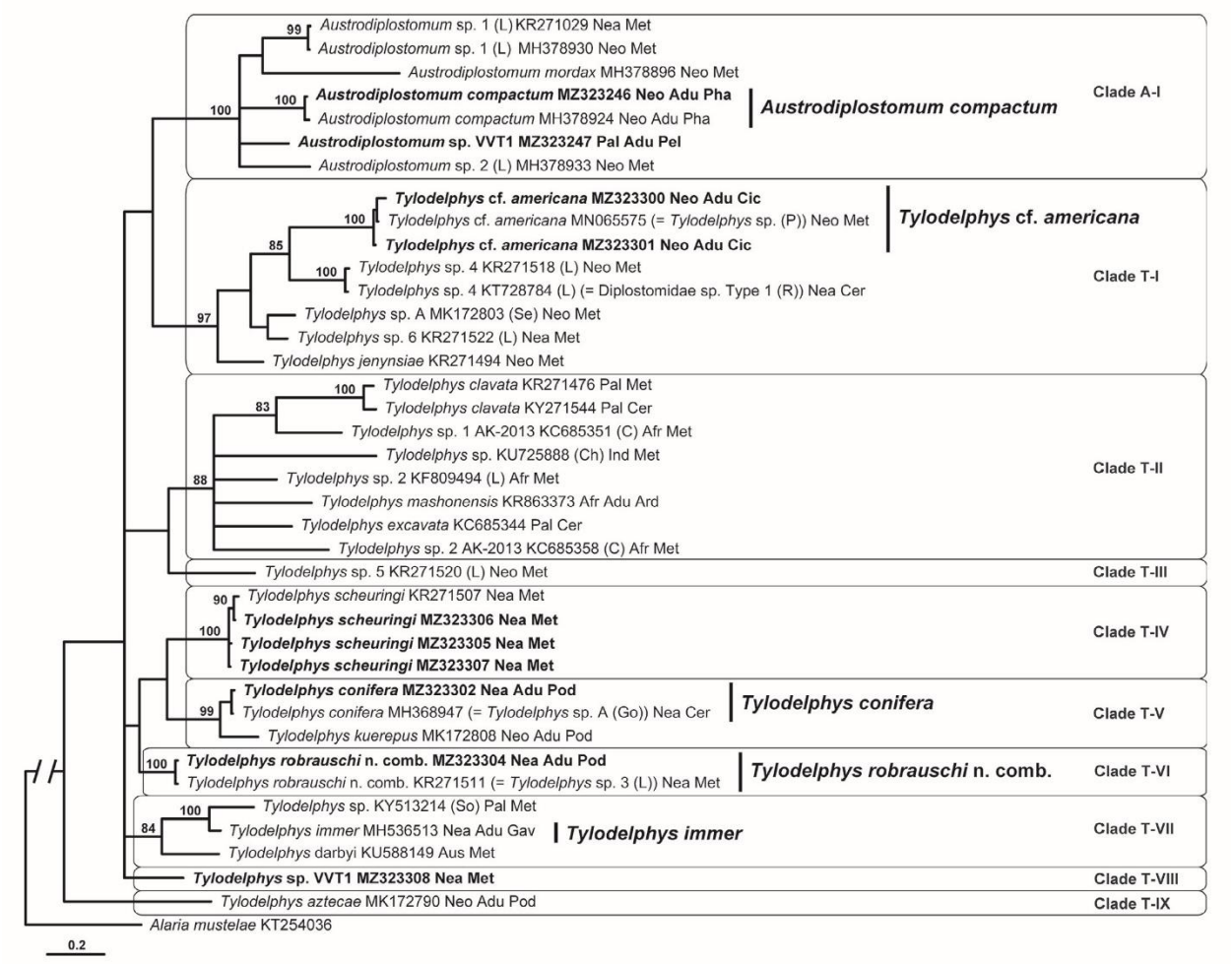


Figure 13. Phylogenetic interrelationships among 27 taxa of *Tylodelphys* and *Austrodiplostomum* spp. based on Bayesian inference analysis of partial *cox*1 mtDNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale-bar indicates the number of substitutions per site. GenBank accession numbers are provided after the names of species. Reference to origin of species numbering/naming systems is provided in parentheses after GenBank accession numbers followed by biogeographical realm where specimens were collected, life stage of isolate and family of definitive host (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); L, Locke et al. (2010a, b; 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 stage: Adu, adult; Cer, cercaria; Met, metacercaria. Abbreviations for family of definitive host: Ard, Ardeidae; Cic, Ciconiidae; Gav, Gaviidae; Pel, Pelecanidae; Pha, Phalacrocoracidae; Pod, Podicipedidae.

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. 13) consisted of a polytomy with nine well-supported clades (clades A-I, T-I–T-VIII) and a sister clade (T-IX) which only contained *T. aztecae*. We only discuss the six clades containing new 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 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. 13). *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 isolates of *T. scheuringi*, while clade T-V contained *Tylodelphys kuerepus* Sereno-Uribe, Andrade-Gómez, Ponce de León & García-Varela, 2019 + [*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.

5.4 Discussion

Generation of new molecular data

Although members of *Diplostomum* are widely spread 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.

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. 10, 11, 14). 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 glands; 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 and Rubio, 2009; Rheingantz et al., 2014; Bouley et al., 2015). We hypothesize 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 (Fig. 14;). *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).

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 figure 2). The differences include the opisthosoma:prosoma length ratio which is 0.65 in our specimens and 0.47–0.51 in *D. ardeae*, along 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 figure 2). *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 and Hundley, 1957 and Supplementary fig. 2) 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. 10, 11; Fig. 14), 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 LaRue (1927) of specimens from the European herring gull *Larus argentatus* Pontoppidan collected at Lake Huron (Supplementary Table S2; Supplementary figure 2). 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 S2; Supplementary figure 2).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, b) 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 S2). 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 figure 2). 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 S2; Supplementary figure 2). 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 S2). 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 9). 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 (Fig. 14). *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 9) as well as the *Diplostomum* sp. VVT2 belong to *D. baeri*. We hypothesize 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 8). 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

Table 8. 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.**	**Accession numbers**	
				28S	***cox1***
A. 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
D. alarioides[a]	*Lontra canadensis*	U.S.A. (Mississippi)	HWML-216521	MZ314152	MZ323249
D. alascense n. comb.	*Mergus merganser*	U.S.A. (Minnesota)	HWML-216522	MZ314153	MZ323250
D. 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

D. 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
D. 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
D. marshalli	*Tringa melanoleuca*	U.S.A. (North Dakota)	HWML-216526	MZ314167	MZ323268
D. pseudospathaceum	*Chroicocephalus genei*	Ukraine (Kherson oblast)	HWML-216527	MZ314168	MZ323269
D. rauschi	*Chroicocephalus genei*	Ukraine (Kherson oblast)	–	MZ314169	MZ323270
D. rauschi	*Hydroprogne caspia*	Ukraine (Kherson oblast)	HWML-216521–HWML-216521	–	MZ323271, MZ323272
D. scudderi	*Lophodytes cucullatus*	U.S.A. (North Dakota)	HWML-216530	MZ314170	MZ323273
D. 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	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
Do. 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
T. cf. *americana*	*Jabiru mycteria*	Brazil (Pantanal)	HWML-216538	MZ314183, MZ314184	MZ323300, MZ323301
T. conifera	*Podiceps grisegena*	U.S.A. (Minnesota)	HWML-216539	MZ314185	MZ323302
T. immer	*Gavia immer*	U.S.A. (North Dakota)	HWML-216540	MZ314186	MZ323303
T. robrauschi n. comb.	*Podilymbus podiceps*	U.S.A. (Minnesota)	HWML-216541	MZ314187	MZ323304
T. 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

[a] Formerly included in *Paralaria*.
Genus abbreviations: *A*, *Austrodiplostomum*; *D*, *Diplostomum*; *Do*, *Dolichorchis*; *T*, *Tylodelphys*
Museum abbreviations: HWML, Harold W. Manter Laboratory; UWSP – PARA, University of Wisconsin - Stevens Point Parasitology Collection

anterior extent of vitellarium (vitellarium extending to about the level of the ventral sucker in *D. m. alascense* vs. 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 S2). 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. S2). 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 9).

Non-monophyly of *Tylodelphys*

Our phylogenetic analyses positioned members of *Austrodiplostomum* nested within *Tylodelphys* (Figs. 10, 12, 13), which indicates 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 characterized by a heavily reduced ventral sucker or no ventral sucker at all, and the lack of 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, b). It is worth

Table 9. *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
D. alarioides[a]	–	Present study
D. alascense	*Diplostomum* sp. 2	Moszczynska et al. (2009)
D. gavium	*Diplostomum* sp. 3	Moszczynska et al. (2009)
	D. baeri	Ubels et al. (2018)
D. marshali	*Diplostomum* sp. A	Gordy and Hanington (2019)
D. huronense	*Diplostomum* sp. 1	Moszczynska et al. (2009)
D. indistinctum	*Diplostomum* sp. 4	Moszczynska et al. (2009)
	D. baeri	Ubels et al. (2018)
D. pseudospathaceum	*D. pseudospathaceum*	Behrmann-Godel (2013); Georgieva et al. (2013)
D. rauschi	*Diplostomum* sp. Lineage 2	Blasco-Costa et al. (2014)
D. scudderi	*Diplostomum* sp. 13	Locke et al. (2015)
	Diplostomum sp. C	Gordy and Hanington (2019)
D. spathaceum	*D. spathaceum*	Georgieva et al. (2013)
	D. spathaceum LIN1	Blasco-Costa et al. (2014)
	D. 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	*D. ardeae sensu* Locke et al. (2015)	Locke et al. (2015)

[a] Formerly included in *Paralaria*.

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. 12) separated *Tylodelphys* spp. into two distinct clades. The first clade (85% support) included the 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 characterized 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. 13), appear to belong to a separate, genus-level lineage. However, as mentioned above, currently available data are insufficient for 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 lacks 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* turn out 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)).

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 S3). 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 (Supplemental Table S3), 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 summarize, 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 10).

Remarks on *Dolichorchis* and the Diplostominae

As previously demonstrated by other authors (e.g., Blasco-Costa and Locke, 2017; Locke et al., 2018; Achatz et al., 2021), the Diplostominae was non-monophyletic in our broader analysis of 28S (Fig. 10). Despite the general morphological similarity of *Tylodelphys* and

Dolichorchis, these two genera were not positioned together in the phylogeny (Fig. 10). It should be noted that the two genera differ in the structure of the anterior testis (asymmetrical in *Dolichorchis* spp. vs. symmetrical in *Tylodelphys* spp.) and often in the distinction between prosoma and opisthosoma (body distinctly bipartite in *Dolichorchis* spp. vs. body typically indistinctly bipartite in *Tylodelphys* spp.).

Table 10. *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**
A. compactum	*A. compactum*	Sereno-Uribe et al. (2019b)
	A. ostrowskiae	O'Hear et al. (2014)
	Austrodiplostomum sp.	Farias et al. (unpublished)
Austrodiplostomum sp. VVT1	–	Present study
T. cf. *americana*	*Tylodelphys* sp.	Pelegrini et al. (2019)
T. conifera	*Tylodelphys* sp. A	Gordy and Hanington (2016)
T. immer	*T. immer*	Locke et al. (2018)
T. robrauschi n. comb.	*Tylodelphys* sp. 3	Locke et al. (2015)
T. scheuringi	*T. scheuringi*	Moszczynska et al. (2009)
Tylodelphys sp. VVT1	–	Present study

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 *cox*1. *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).

Host associations

Our phylogenetic analyses (Figs. 10, 11; Fig. 14) provided evidence of multiple host-switching events among *Diplostomum* species. The majority of adult *Diplostomum* isolates included in our analyses were collected from the Laridae Rafinesque (gulls). However, our analysis also included *Diplostomum* species 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. 10, 11). 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.

The *Diplostomum* spp. from otters and mergansers formed three of the branches within the *Diplostomum* clade, representing separate secondary host-switching events (Fig. 11). 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 of (Fig. 14) 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. 11), 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. 11) 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. 12). Members of the genus included in our analyses were collected from the Podicipedidae Bonaparte (grebes), Gaviidae Coues (loons), Ciconiidae Gray (storks),

Pelecanidae Rafinesque (pelicans) and Phalacrocoracidae Reichenbach (cormorants). In the analysis of 28S (Fig. 12), 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. 12, 13) suggesting a transition from cormorants to pelicans.

Biogeography

Previous studies (e.g., Locke et al., 2015, 2020; Hoogendoorn et al., 2020) have demonstrated that some *Diplostomum* species 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* species 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 further south than Puerto Rico. This provides 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. 10, 11; Fig. 14) along with *D. lunaschiae* in the *cox*1 provide some support for this hypothesis (Figs. 10, 11). This is further supported by the presence of three other clades of *Diplostomum* spp. from the Nearctic in the broader clade of *Diplostomum* (Fig. 11). Most *Diplostomum* spp. from the Palaearctic formed a single, strongly supported clade in our analysis of 28S (Fig. 11). 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 (Fig. 14).

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. 12). 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. 13).

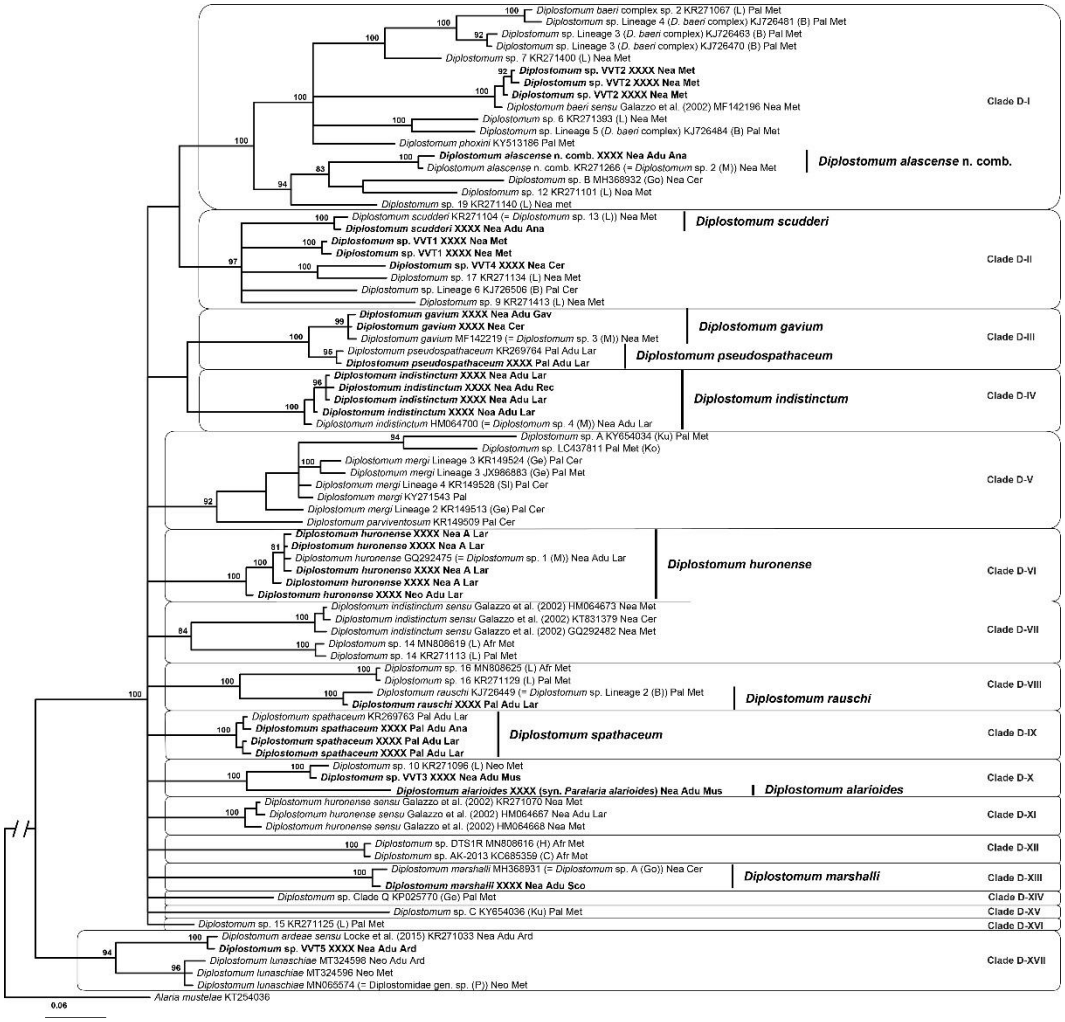


Figure 14. Phylogenetic interrelationships among 80 sequences from members of *Diplostomum* (including a former *Paralaria* sp.) based on Bayesian inference analysis of partial *cox*1 mtDNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale-bar indicates the number of substitutions per site. Reference to origin of species numbering/naming systems are provided in parentheses after GenBank accession numbers followed by biogeographical realms where specimens were collected, life stage of isolate and family of definitive host (for adult isolates and larvae molecularly matched to adult forms). Black bars are positioned besides taxa for which we have provided nominal species identifications based on adult morphology. The previously accepted/published names are provided in parentheses after GenBank accession numbers. Abbreviations for references to the original designations of species-level lineages: B, Blasco-Costa et al. (2014); C, Chibwana et al. (2013); 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); P, Pelegrini et al. (2019); Sl, Selbach et al. (2015). Abbreviations for biogeographical realms: Afr, Afrotropical realm; ANea, 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.

CHAPTER 6:

Molecular phylogeny supports invalidation of *Didelphodiplostomum* and *Pharyngostomoides* (Digenea: Diplostomidae) and reveals a *Tylodelphys* from mammals.

6.1 Introduction

The Diplostomidae Poirier, 1886 is a cosmopolitan family of diplostomoidean digeneans known to parasitize the intestines of a wide diversity of tetrapod definitive hosts. At present, members of 13 genera are known to utilize mammalian definitive hosts (Niewiadomska, 2002; Uhrig *et al*., 2015; Achatz *et al*., 2022c); however, DNA sequence data are only available for adult specimens of two of these genera collected from mammals, *Alaria* Schrank, 1788 and *Diplostomum* von Nordmann, 1832. Members of *Alaria* are well-known, broadly distributed parasites of mammals, while *Diplostomum* spp. are almost exclusively parasitic in avian definitive hosts (e.g., Dubois, 1968; Niewiadomska, 2002; Achatz *et al*., 2022c).

Harkema (1942) erected the genus *Pharyngostomoides* Harkema, 1942 for *Pharyngostomoides procyonis* Harkema, 1942 collected from the common raccoon *Procyon lotor* (Linnaeus) in North Carolina and Texas, U.S.A. Later, Harkema & Miller (1961) established *Parallelorchis* Harkema & Miller, 1961 for their new species *Parallelorchis diglossus* Harkema & Miller, 1961 collected from *Pr. lotor* in Florida, U.S.A. Dubois (1966) synonymized *Parallelorchis* with *Pharyngostomoides*; however, Beckerdite *et al*. (1971) rejected this synonymization. In addition, Beckerdite *et al*. (1971) redescribed *Ph. procyonis* and described *Pharyngostomoides adenocephala* Beckerdite, Miller & Harkema, 1971 collected from *Pr. lotor* in North Carolina. Subsequently, Dubois & Angel (1972) described *Pharyngostomoides dasyuri* Dubois & Angel, 1972 from the eastern quoll *Dasyurus viverrinus* (Shaw) in Tasmania, Australia. The most recent revision of the Diplostomidae by Niewiadomska (2002) maintained the synonymy of *Parallelorchis* with *Pharyngostomoides.*

Didelphodiplostomum Dubois, 1944, another diplostomid genus parasitic in mammals, was erected by Dubois (1944) for the previously described *Proalaria variabile* Chandler, 1932 collected from a Virginia opossum *Didelphis virginiana* (Kerr) in Texas, U.S.A. It is worth noting that Chandler (1932) provided an incorrect suffix for his species; '*delphys'* (uterus) is a Latinized Greek feminine genitive noun and requires a feminine genitive species name. Therefore, we refer to *Didelphodiplostomum variabile* (Chandler, 1932) as *Didelphodiplostomum variabilis* (Chandler, 1932). Later, Dubois (1976) described a second species of the genus, *Didelphodiplostomum nunezae* Dubois, 1976, from a big-eared opossum *Didelphis aurita* Wied-Neuwied (syn. *Didelphis azarae* Temminck) collected in Argentina. No DNA sequence data are currently available for members of *Pharyngostomoides* or *Didelphodiplostomum*.

Herein, we generated 28S rDNA and *cox*1 mtDNA genes for 10 species of *Alaria*, *Pharyngostomoides*, *Didelphodiplostomum* and *Tylodelphys* Diesing, 1850. The 28S sequences were used to determine the phylogenetic position of *Pharyngostomoides* and *Didelphodiplostomum* among other major diplostomoidean lineages. Partial *cox*1 sequences of *Alaria* spp. were used to study the interrelationships among members of the genus.

6.2 Materials & Methods

Several species belonging to *Alaria*, *Pharyngostomoides* and *Didelphodiplostomum* (including type-species of all three genera) were collected from mammalian definitive hosts in North America and Europe; metacercariae of *Tylodelphys excavata* Rudolphi, 1803 were

Table 11. Hosts, geographic origin, GenBank and Harold W. Manter Laboratory (HWML) museum accession numbers of diplostomids collected in our study.

Diplostomid taxa	Host species	Stage	Geographical origin	HWML No.	GenBank accession	
					28S	*cox*1
Alaria alata	*Nyctereutes procyonoides*	A	Ukraine	HWML 216724	OL435536, OL435537	OL439156
Alaria arisaemoides	*Canis latrans*	A	Oregon, U.S.A.	HWML 216725	OL435538	OL439157
Alaria marcianae	*Taxidea taxus*	A	North Dakota, U.S.A.	HWML 216726	OL435539, OL435540	OL439158 – OL439160
A. marcianae	*Procyon lotor*	A	California, U.S.A.	HWML 216727	OL435541	OL439161
Alaria mustelae	*Mustela frenata*	A	North Dakota, U.S.A.	–	OL435542[§]	OL439162
A. mustelae	*Neogale vison*	A	North Dakota, U.S.A.	–	OL435543, OL435544	OL439163 – OL439166
A. mustelae	*Neogale vison*	A	Minnesota, U.S.A.	HWML 216728	–	OL439167
A. mustelae	*Taxidea taxus*	A	North Dakota, U.S.A.	HWML 216729	OL435545	OL439168 – OL439170
A. mustelae	*Thamnophis sirtalis parietalis*	M	Manitoba, Canada	–	–	OL439171
Alaria ovalis comb. nov. *	*Procyon lotor*	A	Mississippi, U.S.A.	HWML 216730	OL435546	OL439172
Alaria procyonis comb. nov. *	*Procyon lotor*	A	Minnesota, U.S.A.	HWML 216731	OL435547	OL439173
Alaria sp. 1	*Thamnophis sirtalis parietalis*	M	Manitoba, Canada	–	OL435548, OL435549	OL439174 , OL439175
Alaria sp. 3	*Puma concolor*	A	Florida, U.S.A.	–	OL435550	OL439176
Tylodelphys excavata	*Pelophylax ridibundus*	Mtc	Ukraine	–	OL435551	OL439177

Tylodelphys variabilis comb. nov. †	*Didelphis virginiana*	A	Arkansas, U.S.A.	HWML 216733	OL435552 ‡, OL435553 ‡	OL439178 , OL439179
T. variabilis comb. nov. †	*Didelphis virginiana*	A	North Carolina, U.S.A.	HWML 216732	OL435554	OL439180

* Previously included in *Pharyngostomoides*.
† Previously included in *Didelphodiplostomum*.
§ The sequence also includes partial 5.8S + ITS2.
‡ The sequence also includes partial 18S + ITS1 + 5.8S + ITS2.
A The sample was an adult
M The sample was a mesocercaria
Mtc The sample was a metacercaria

collected from a frog in Europe (Table 11). Live adult diplostomids were removed, fixed, and stained according to standard methods. Voucher specimens, including hologenophores when possible, are deposited in the collection of the Harold W. Manter Laboratory (HWML), University of Nebraska State Museum, Lincoln, Nebraska, U.S.A.

Genomic DNA was extracted following the protocol described by Tkach & Pawlowski (1999). Fragments of the 28S and *cox*1 genes were amplified using digL2, 1500R, Dipl_Cox_5', Dipl_Cox_3', and Dipl650R (Table 1). Following our standard sequencing and alignment methods, this alignment included newly obtained sequences of *Alaria* (*n* = 6), *Didelphodiplostomum* (*n* = 1), *Pharyngostomoides* (*n* = 2) and *Tylodelphys* (*n* =1) along with previously published sequences of 29 other representatives of the Diplostomidae, 12 representatives of the Strigeidae Railliet, 1919 and two representatives of the Proterodiplostomidae Dubois, 1936 (see Dubois, 1936a). Based on the results of the initial 28S analysis, the interrelationships among *Alaria* and *Pharyngostomoides* spp. were studied using separate alignments of partial 28S and *cox*1 sequences with *Sphincterodiplostomum musculosum* Dubois, 1936 (see Dubois, 1936b) as the outgroup. The 28S alignment limited to only *Alaria* and *Pharyngostomoides* spp. (1,132 bp long; no sites were excluded) included eight newly generated

sequences. The *cox*1 alignment (470 bp long; no sites excluded) included 14 new sequences and 16 previously published sequences. BI analysis was performed following our standard methods.

6.3 Results

Molecular phylogenies

The initial phylogenetic analysis based on 28S demonstrated non-monophyly of the Diplostomidae and Strigeidae (Fig. 15). *Pharyngostomoides* spp. (see discussion below) were positioned within a 91% supported clade of *Alaria* spp. (including the type-species *Alaria alata* (Goeze, 1782)). The 91% supported clade was split into two supported subclades. The first subclade (86% supported) included *A. mustelae* and both former *Pharyngostomoides* spp. (see discussion below). The second subclade (95%) included an 88% supported cluster of *A. alata* + *Alaria* sp. 1 and a 100% supported cluster *Alaria arisaemoides* Augustine & Uribe, 1927 + a clade of [*Alaria marcianae* (La Rue, 1917) + *Alaria* sp. 3 (98% supported)] (Fig. 15). Surprisingly, *Did. variabilis* (= *Tylodelphys variabilis* (Chandler, 1932) comb. nov.; see discussion below) was positioned within a 100% supported cluster of *Tylodelphys* + *Austrodiplostomum* Szidat & Nani, 1951 species (Fig. 15). *Tylodelphys* was non-monophyletic, in part, due to the inclusion of *Austrodiplostomum* spp., as recently demonstrated and discussed by Achatz *et al*. (2022c). *Tylodelphys excavata* was positioned as a sister branch to the larger *Tylodelphys* + *Austrodiplostomum* clade (100% supported). Among the remaining members of the *Tylodelphys* + *Austrodiplostomum* clade, *Did. variabilis* was positioned within an 85% supported clade which contained most other members of *Tylodelphys* (Fig. 15). Considering that details of the interrelationships within *Tylodelphys* + *Austrodiplostomum* clade were recently discussed by Achatz *et al*. (2022c), we do not discuss this clade in detail here. The analysis of

28S limited to Alaria spp. (Fig. 16) had somewhat different topology and lower branch support compared to the initial analysis (Fig. 15). Alaria mustelae was positioned as a sister group to an unsupported clade which consisted of two subclades; the first subclade contained only two former Pharyngostomoides spp. (81% supported). The second subclade (100%) consisted of an 89% supported cluster of A. alata + Alaria sp. 1 and a 100% supported cluster A. arisaemoides + a clade of [A. marcianae + Alaria sp. 3 (99% supported)] (Fig. 16).

The phylogeny of Alaria spp. based on partial *cox*1 sequences had substantially different topology than both 28S phylogenies (Fig. 17). The two former Pharyngostomoides spp. (see discussion below) were positioned in an unsupported clade that was positioned as a sister group to an 81% supported clade containing the remaining members of Alaria. The 81% supported clade consisted of a cluster of A. mustelae isolates (100% supported) and a 100% supported clade which contained two additional subclades. The first subclade (87% supported) contained Alaria sp. 3 + A. marcianae. All isolates of A. marcianae formed a 99% supported clade. The second subclade (92%) included a 98% cluster of [A. alata + Alaria sp. 1] and a 100% supported cluster of A. arisaemoides. Both sequences of Alaria sp. 1 formed a 100% supported clade.

6.4 Discussion

Status of *Pharyngostomoides*

Morphological characteristics of *Pharyngostomoides* spp. in our material conforms to the original descriptions of *Ph. procyonis* and *Ph. ovalis* (Fig. 18E, F). Beckerdite *et al*. (1971) considered *Ph. ovalis* to be a junior synonym of *Ph. procyonis*. In addition, Beckerdite *et al*. (1971) redescribed *Ph. procyonis* and provided an illustration which appears remarkably

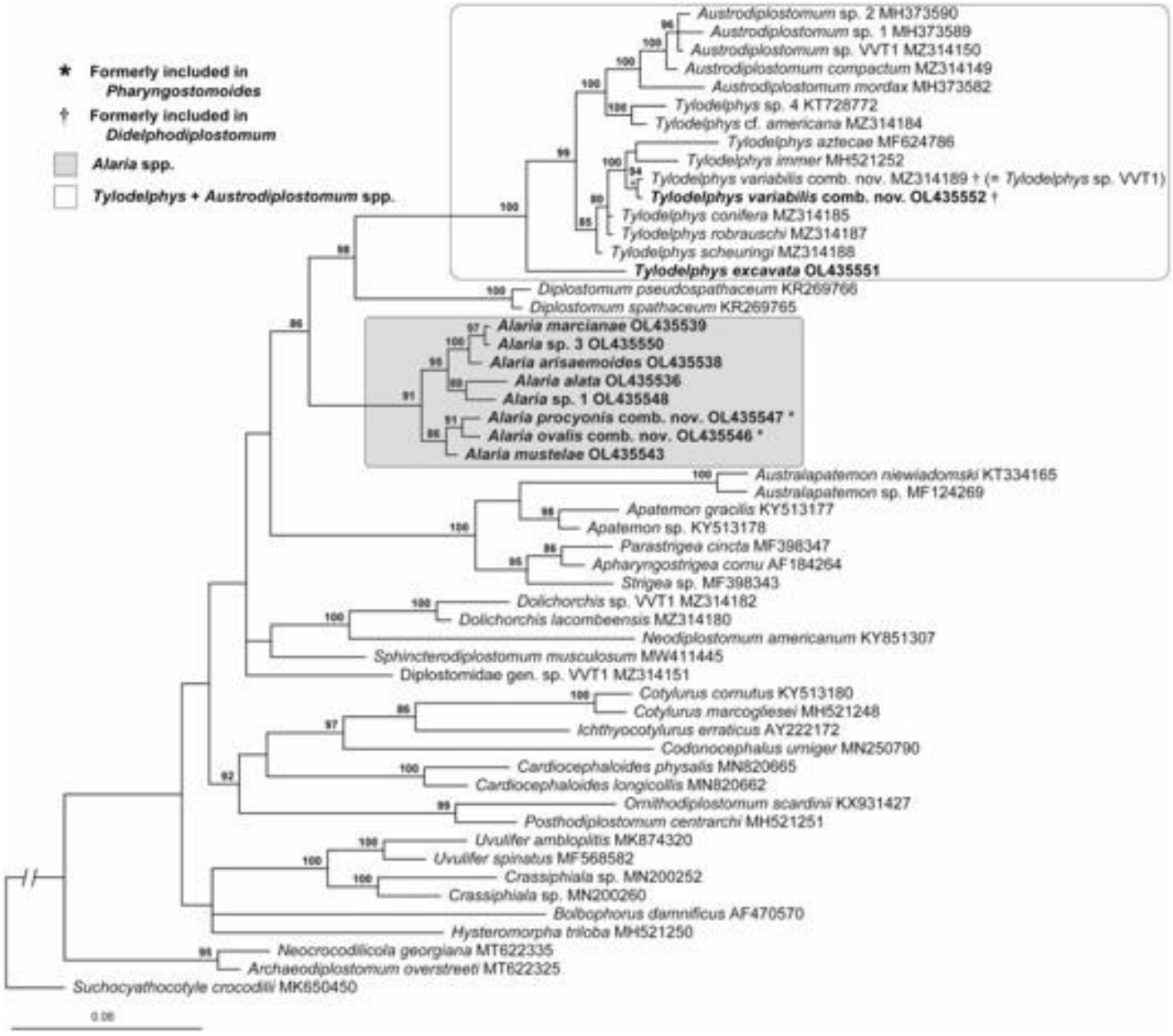


Figure 15. Phylogenetic interrelationships among 54 diplostomoidean taxa based on Bayesian Inference analysis of partial 28S rDNA gene sequences including *Didelphodiplostomum* and *Pharyngostomoides* spp. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale-bar indicates the number of substitutions per site.

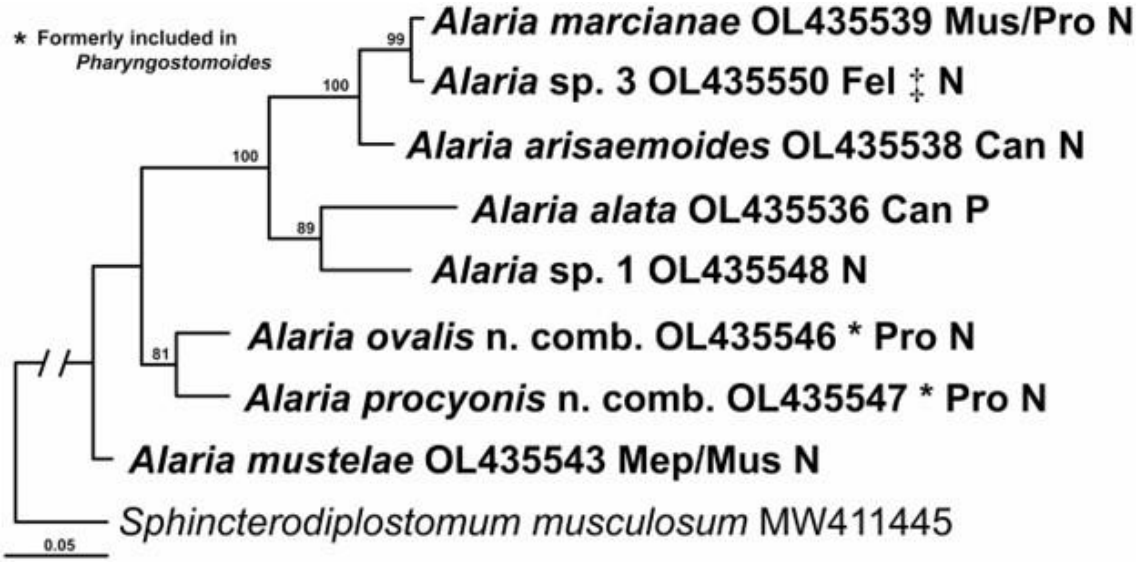


Figure 16. Phylogenetic interrelationships among eight species of *Alaria* (syn. *Pharyngostomoides*) based on Bayesian Inference analysis of partial 28S rDNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale-bar indicates the number of substitutions per site. Biogeographical realm and family of definitive host from which specimens were collected are provided when possible; the information on biogeographical realms and families of definitive hosts is provided only for taxa confirmed with sequence data. Abbreviations of biogeographical realms: N, Nearctic; P, Palearctic. Abbreviations of family of definitive host: Can, Canidae; Fel, Felidae; Mep, Mephitidae; Mus, Mustelidae; Pro, Procyonidae. ‡ All collected specimens are immature.

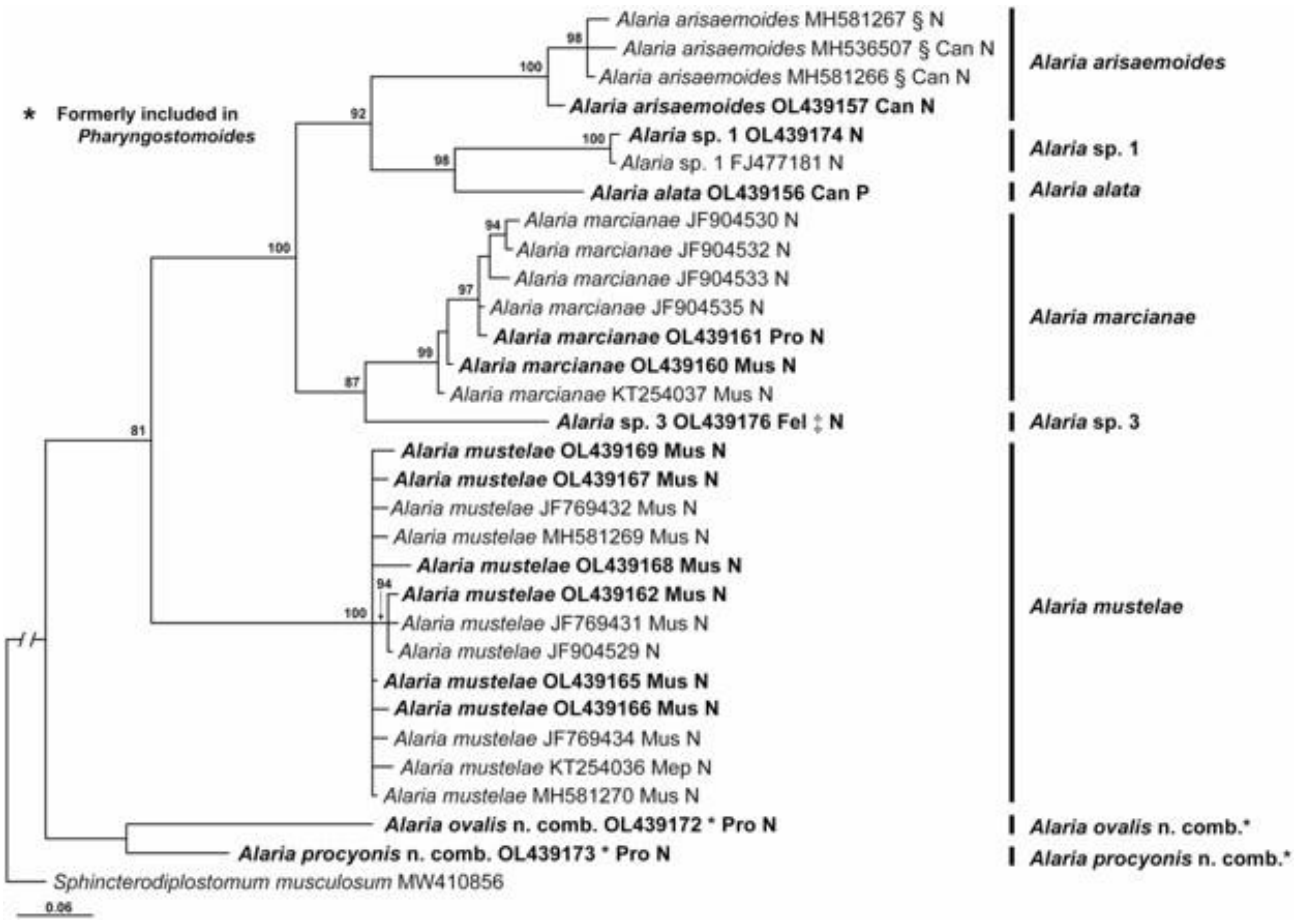


Figure 17. Phylogenetic interrelationships among 31 sequences from members of *Alaria* (syn. *Pharyngostomoides*) based on Bayesian Inference analysis of partial *cox*1 mtDNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale-bar indicates the number of substitutions per site. The information on biogeographical realms and families of definitive hosts is provided only for taxa confirmed with sequence data. Abbreviations of biogeographical realms: N, Nearctic; P, Palearctic. Abbreviations of family of definitive host: Can, Canidae; Fel, Felidae; Mep, Mephitidae; Mus, Mustelidae; Pro, Procyonidae. ‡ All collected specimens are immature. § Previously identified as *A. americana* by Locke *et al*. (2018).

similar to *Ph. ovalis*. Our material of *Ph. procyonis* and *Ph. ovalis* differ by 0.4% and 10% in partial sequences of 28S and *cox*1, respectively (Table 12). The morphology of *Ph. procyonis* and *Ph. ovalis* most obviously differs in general body shape (spatulate in *Ph. procyonis* vs. oval in *Ph. ovalis*), shape of prosoma (anterior end rounded in *Ph. procyonis* vs. anterior end is square-shaped in *Ph. ovalis*), relative sucker sizes (oral sucker similar in size or smaller than ventral sucker in *Ph. procyonis* vs. oral sucker usually larger than ventral sucker in *Ph. ovalis*) and egg size (egg length 82–93 micrometers in *Ph. procyonis* vs. egg length 100–115 micrometers in *Ph. ovalis*). Considering the genetic and morphological differences listed above, we restore *Ph. ovalis.*

Table 12. Pairwise comparisons of 28S sequences among *Alaria* spp. (syn. *Pharyngostomoides*) based on an 1,132 bp long alignment. Percent difference given above diagonal. Number of nucleotide differences provided below diagonal.

		1. OL435539	**2.** OL435550	**3.** OL435538	**4.** OL435536	**5.** OL435548	**6.** OL435546	**7.** OL435547	**8.** OL435543
1.	*Alaria marcianae* OL435539	–	0%	0.3%	1.1%	0.9%	1.3%	1.1%	1.1%
2.	*Alaria* sp. 3 OL435550	0	–	0.3%	1.1%	0.9%	1.3%	1.1%	1.1%
3.	*Alaria arisaemoides* OL435538	3	3	–	1.1%	0.8%	1.2%	1.2%	1.1%
4.	*Alaria alata* OL435536	13	13	12	–	0.8%	1.4%	1.3%	1.3%
5.	*Alaria* sp. 1 OL435548	10	10	9	9	–	1.3%	1.3%	1.2%
6.	*Alaria ovalis* comb. nov. OL435546*	15	15	14	16	15	–	0.4%	0.4%
7.	*Alaria procyonis* comb. nov. OL435547*	13	13	14	15	15	4	–	0.4%
8.	*Alaria mustelae* OL435543	12	12	13	15	14	5	5	–

* Previously included in *Pharyngostomoides*

Pharyngostomoides spp. are readily distinguished from *Alaria* spp. based on the position of the testes (opposite in *Pharyngostomoides* vs. tandem in *Alaria*) (Niewiadomska, 2002; Fig. 18E, F vs. Fig. 18 B–D, G, H). However, our molecular phylogeny based on 28S (Fig. 15) positioned *Ph. procyonis* (type-species) and *Ph. ovalis* among *Alaria* spp., including the type-species *A. alata*. Interestingly, the two *Pharyngostomoides* spp. were positioned in a strongly supported clade with *A. mustelae*, which has typical morphology of *Alaria* spp.; this clade was a sister group to other members of *Alaria*. Based on the phylogenetic position of *Pharyngostomoides* spp. (Fig. 15) and limited morphological differences (i.e., position of testes), we consider *Pharyngostomoides* to be a junior synonym of *Alaria*; therefore, we transfer *Ph. procyonis*, *Ph. ovalis*, *Ph. adenocephala* and *Ph. dasyuri* into *Alaria* as *Alaria procyonis* (Harkema, 1942) comb. nov., *A. ovalis* comb. nov., *Alaria adenocephala* (Beckerdite, Miller & Harkema, 1971) comb. nov. and *Alaria dasyuri* (Dubois & Angel, 1972) comb. nov., respectively. An amended diagnosis of *Alaria* is provided below.

Alaria Schrank, 1788 (after Niewiadomska, 2002, amended)

Diagnosis: Body indistinctly bipartite; prosoma linguiform or spatulate, concave; opisthosoma cylindrical or conical, usually shorter than prosoma. Pseudosuckers present, often forming ear-like projections. Oral and ventral suckers typically small; pharynx small or large. Holdfast organ round to elongate, variable in length; anterior margin reaching pharynx in some species. Ovary oval or reniform, median, pretesticular, at junction of prosoma and opisthosoma. Vitellarium mainly in prosoma, spreading into holdfast organ and extending into opisthosoma in some species. Testes of different size and shape, multi- or bilobed, tandem or opposite; when tandem, anterior asymmetrical, opposite oötype, and, posterior symmetrical, larger. Seminal vesicle with

either ejaculatory pouch or ejaculatory duct with muscular region. Copulatory bursa small or deep. Hermaphroditic duct opening at tip of small genital papilla. Genital pore dorsal, subterminal. In Carnivora Bowdich. Eurasia, North America and South America. Mesocercariae in anurans and branchiobdellid annelids associated with crayfish. Mesocercariae using paratenic hosts in some species. Cercariae with two pairs of pre-acetabular or pre- and postacetabular penetration gland-cells; flame-cell formula 2[(2 + 2 + 2) + (2 + 2 + (2)]) = 24. Metacercariae of 'diplostomulum' type, developing during trans-entero-pulmonary migration in definitive host. Type-species *A. alata* (Goeze, 1782).

Notably, we did not transfer the former member of *Parallelorchis*, *Pa. diglossus*, into *Alaria*. In our opinion, the synonymization of *Parallelorchis* with *Pharyngostomoides* by Dubois (1966) is not supported by morphology. The holdfast organ of the former *Parallelorchis* species is quite different from members of *Alaria* (syn. *Pharyngostomoides*). Harkema & Miller (1961) described the holdfast organ of the former *Parallelorchis* species as a continuation of the ventral surface of the body without a clear constriction point and consists two lateral tongue-like lobes (see description and illustrations provided by Harkema & Miller (1961)). In contrast, the holdfast organ of *Alaria* spp. is very distinct and usually sucker-like as shown in multiple descriptions and seen on some of the photographs on the Fig. 18 (Fig. 18F, G). Based on the difference in holdfast organ structure, we restore the monotypic *Parallelorchis* with its type-species, *Pa. diglossus*. We cannot entirely rule out that the situation might change once molecular data on this interesting taxon becomes available.

Remarks on *Alaria*

The members of *Alaria* in the two phylogenies based on 28S had only slight differences

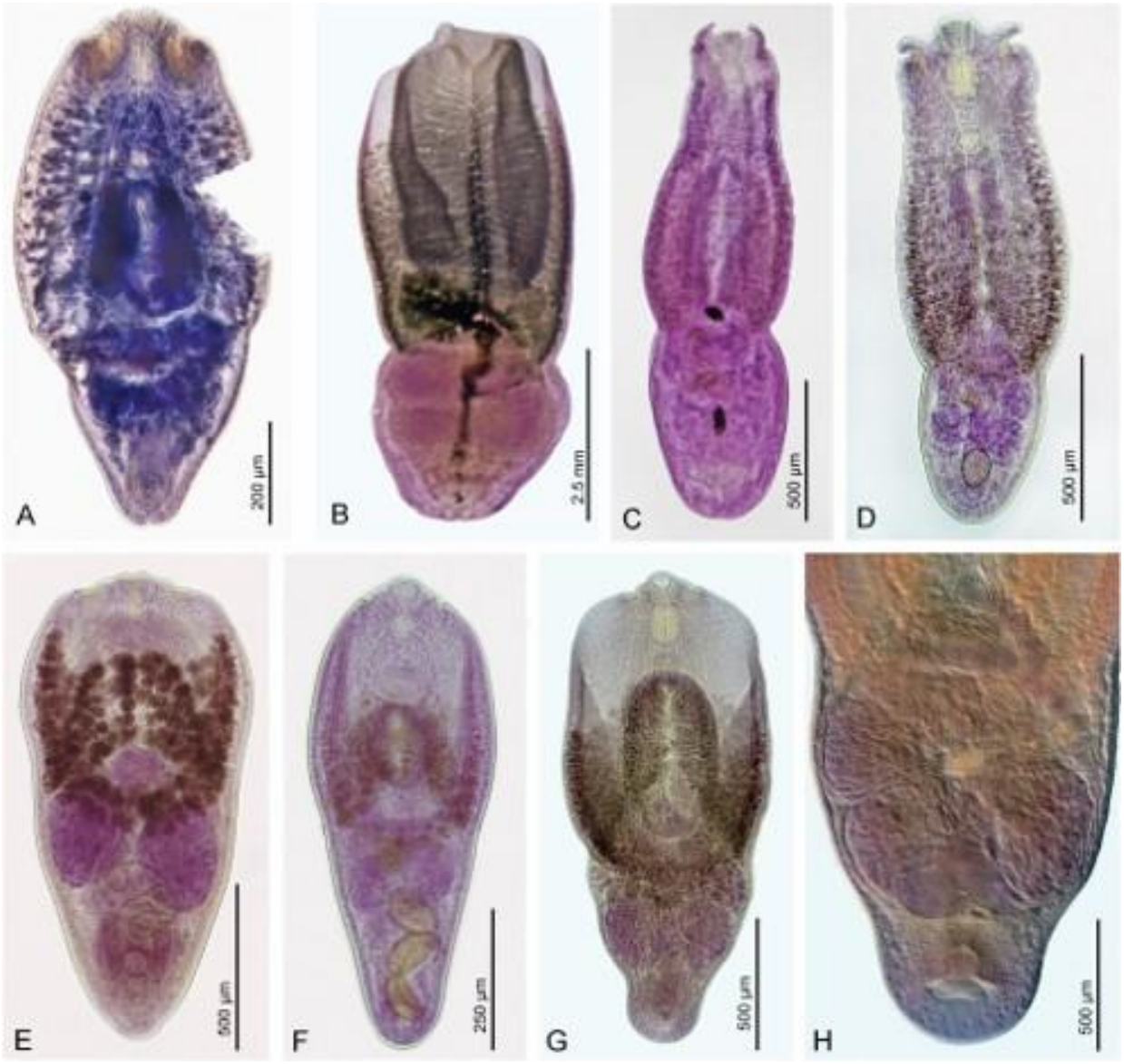


Figure 18. Photographs of (A) *Tylodelphys variabilis* comb. nov. from *Didelphis virginiana*, Arkansas; (B) *Alaria arisaemoides* from *Canis latrans*, Oregon; (C) *Alaria alata* from *Nyctereutes procyonoides*, Ukraine; (D) *Alaria marcianae* from *Taxidea taxus*, North Dakota; (E) *Alaria ovalis* comb. nov., *Procyon lotor*, Mississippi; (F) *Alaria procyonis* comb. nov. from *Procyon lotor*, Minnesota; (G, H) *Alaria mustelae* from *Mephitis mephitis*, North Dakota

in topology (Figs 15, 16). At the same time, the phylogenies of 28S and *cox*1 limited to members of *Alaria* showed more pronounced differences in branch topology (Figs 16, 17). *Alaria mustelae* was positioned as a sister taxon to the other *Alaria* spp. in the second 28S analysis (Fig. 16), while in the *cox*1 phylogeny, *A. ovalis* and *A. procyonis* formed an unsupported clade which was positioned as a sister group to the other members of *Alaria* (Fig. 17). The positions of *A. alata* + *Alaria* sp. 1 and *A. marcianae* + *Alaria* sp. 3 varied between the two analyses as well (Figs 16, 17). Discordance between phylogenies based on ribosomal and mitochondrial data has been well documented among other diplostomoideans (e.g., Brabec et al., 2015; Heneberg et al., 2020; Hoogendoorn et al., 2020; Achatz et al., 2022c*).* Faster mutating genes, such as *cox1*, are more reliable for distinguishing between closely related diplostomoidean species/species-level lineages (Table 12); however, slower mutating genes, such as 28S, remain more suitable for phylogenetic inference at taxonomic levels above genus.

All *Alaria* spp. in the present study, except for *A. alata*, were collected from North America. The nested phylogenetic position of *A. alata* clearly suggests a geographic expansion from the Nearctic into the Palearctic (Figs 15–17).

It is worth noting that our specimens of *A. arisaemoides* (Fig. 18B) conform closely to the original description of the species and subsequent descriptions of the species (e.g., Augustine & Uribe, 1927; Dubois, 1968). However, the *cox*1 sequences of our specimens are only 1.9–2.6% different from material identified as *Alaria americana* Hall & Wigdor, 1918 by Locke *et al*. (2018). The material described by Locke *et al*. (2018) is somewhat different than the original description of *A. americana* described by Hall & Wigdor (1918). For instance, *A. americana* was originally described with vitellarium that does not extend anteriorly beyond the level of the ventral sucker. The vitellarium of *A. americana* from Locke *et al*. (2018) extends anteriorly to

the level of the ventral sucker, similar to the condition in *A. arisaemoides*. In our opinion, the specimens identified as *A. americana* by Locke *et al*. (2018) are likely misidentified specimens of *A. arisaemoides*.

Status of *Didelphodiplostomum*

The analysis of 28S (Fig. 15) positioned *Did. variabilis* within the cluster of *Tylodelphys* and *Austrodiplostomum* species. The morphology of adult *Didelphodiplostomum* and *Tylodelphys* spp. is remarkably similar (Fig. 18A; Dubois, 1968). Furthermore, *Didelphodiplostomum* and *Tylodelphys* have identical flame-cell formulas, 2 [(2 + 2) + (2 + [2])] = 16 (Harris *et al*., 1967; Dubois, 1968, 1970; Niewiadomska, 2002). Dubois (1968) emphasized the remarkable morphological similarity between *Didelphodiplostomum* and *Tylodelphys* species. However, the members of the two genera differ in the shape of anterior testis (asymmetrical in *Didelphodiplostomum* spp. vs. symmetrical in *Tylodelphys* spp.) and the lack of a genital cone in *Didelphodiplostomum* spp. (present in *Tylodelphys* spp., albeit weakly developed in some species).

Our molecular phylogeny (Fig. 15) clearly demonstrates that *Did. variabilis* belongs within one of the two major clades of *Tylodelphys*. Taking into account the results of our phylogenetic analysis (Fig. 15) and rather minor morphological differences between *Didelphodiplostomum* and *Tylodelphys*, we consider *Didelphodiplostomum* to be a junior synonym of *Tylodelphys*. As such, we transfer *Did. variabilis* and *Did. nunezae* into *Tylodelphys* as *T. variabilis* comb. nov. and *Tylodelphys nunezae* (Dubois, 1976) comb. nov., respectively. The partial 28S and *cox*1 sequences of *T. variabilis* and *Tylodelphys* sp. VVT1 of Achatz *et al*. (2022c) are identical. It is clear that the larval specimens of *Tylodelphys* sp. VVT1 from the mole

salamander *Ambystoma talpoideum* Holbrook collected in Mississippi are conspecific with *T. variabilis*. An amended diagnosis of *Tylodelphys* is provided below.

Tylodelphys Diesing, 1850 (after Niewiadomska, 2002, amended)

Diagnosis: Body linguiform, typically indistinctly bipartite; opisthosoma conical or ovoid. Anterior extremity of prosoma not distinctly trilobate; pseudosuckers present. Oral and ventral suckers and pharynx small or large; holdfast organ round or oval, with median slit for opening. Ovary ellipsoid or spherical, submedian, pretesticular, near anterior margin of opisthosoma. Vitellarium in prosoma and opisthosoma, extending anterior to the level of cecal bifurcation in prosoma and posterior to testes in opisthosoma in some species. Testes tandem, typically symmetrical with ventral concavities, forming horseshoe shape; anterior testis symmetrical or asymmetrical. Ejaculatory pouch present or absent. Ejaculatory duct joining uterus forming hermaphroditic duct. Genital cone small or absent, when present, hermaphroditic duct opening terminally. Copulatory bursa with subterminal or (rarely) terminal genital pore. In Accipitridae Vieillot, Ardeidae Leach, Didelphidae Gray and Podicipedidae Bonaparte. Cosmopolitan. Metacercariae of 'diplostomulum' type, in fishes or amphibians. Cercariae with four pre-acetabular penetration gland-cells; flame-cell formula 2[(2 + 2) + (2 + [2])] = 16. Type-species *T. clavata* (von Nordmann, 1832) Diesing, 1850.

Remarks on *Tylodelphys*

Based on our analysis, *Tylodelphys* spp. belong to at least three distinct clades (Fig. 15). Achatz *et al*. (2022c) recently suggested that *Tylodelphys americana* (Dubois, 1936) (see Dubois, 1936b) and *Tylodelphys* sp. 4 may need to be placed within a novel genus. However, the inclusion of the DNA sequence of *T. excavata* in the present analysis has further complicated the

situation. It is possible that *Tylodelphys* as currently recognized may represent a complex of genera and require the establishment of at least two new genera. DNA sequences from adult specimens of *Tylodelphys clavata* (von Nordmann, 1832) are necessary for a conclusive decision regarding the status of *Tylodelphys*.

The majority of *Tylodelphys* spp. and members of the closely related *Austrodiplostomum* and *Diplostomum* are known to primarily parasitize piscivorous birds (Achatz *et al*., 2022c). Achatz *et al.* (2022c) recently revealed the presence of two *Diplostomum* spp. parasitizing North American river otters *Lontra canadensis* (Schreber) in the U.S.A. Based on the results of the present study, *T. variabilis* represents the first species of *Tylodelphys* which secondarily switched from avian to mammalian definitive hosts. Transitions between birds and mammals may happen when hosts occur in the same environments and have overlapping diets; similar to many aquatic birds, otters and raccoons feed on fishes and amphibians.

CHAPTER 7:

Molecular phylogenetic analysis of *Neodiplostomum* and *Fibricola* (Digenea, Diplostomidae) does not support host-based systematics.

7.1 Introduction

Fibricola Dubois, 1932 (Diplostomidae Poirier, 1886: Alariinae Hall et Wigdor, 1918) is a small genus of diplostomid digeneans distributed in North and South America, Africa, Asia and Australia (Barker, 1915; Bisseru, 1957; Seo *et al*., 1964; Kifune and Uyema, 1982; Cribb and Pearson, 1993; Niewiadomska, 2002; Lima *et al*., 2013). Members of *Fibricola* are often reported in ecological and parasite survey studies, most commonly from their frog second intermediate host (e.g., Ulmer, 1970; Premvati and Blair, 1979; Gillilland and Muzzall, 1999;

Goldberg and Bursey, 2001; Goldberg *et al*., 2001; Bolek and Coggins, 2003; Richardson, 2013; Weinstein *et al*., 2019). While most members of *Fibricola* are known to parasitize the intestines of mammals, some *Fibricola* species have also been reported from crocodilians (Bisseru, 1957; Dubois, 1982). Another genus, *Neoparadiplostomum* Bisseru, 1957, was established for two species collected from Nile crocodile *Crocodylus niloticus* Laurenti (Bisseru, 1957). Later, Dubois (1982) synonymized *Neoparadiplostomum* with *Fibricola* and viewed parasitism in crocodilians as accidental infections.

In contrast to *Fibricola* spp., the currently accepted members of the morphologically similar *Neodiplostomum* Railliet, 1919 parasitize intestines of birds with few exceptions. The majority of *Neodiplostomum* spp. known from mammals were collected in the Old World and originally placed into *Fibricola* based on their parasitism in mammals (e.g., *Neodiplostomum seoulensis* (Seo, Rim et Lee, 1964) and *Neodiplostomum minor* (Dubois, 1936)), and later transferred to *Neodiplostomum* (Dubois, 1936; Seo *et al.*, 1964; Cribb and Pearson, 1993; Hong and Shoop, 1994). Notably, *Neodiplostomum vaucheri* Dubois, 1983, described from the frog eating big-eared woolly bat *Chrotopterus auritus* Peters in Peru, was the only member of *Neodiplostomum* from mammals originally assigned into the genus (Dubois, 1983). Noteworthy, *Neodiplostomum seoulensis* has been reported from humans in Korea (Huh *et al.*, 1994). Despite the general trends of parasitism in different groups of definitive hosts (birds vs. mammals), adult *Neodiplostomum* spp. and *Fibricola* spp. are remarkably similar morphologically. In the most recent taxonomic revision of the group, Niewiadomska (2002) admitted that the two genera lack consistent morphological differences that can be used to reliably distinguish one from another. Although Niewiadomska (2002) retained the traditional generic and subfamily status of

Neodiplostomum and *Fibricola*, she emphasized that the resolution of the real relationship between these genera needs to be supported by both morphological and molecular evidence.

At present, DNA sequences are available for six species of *Neodiplostomum* (Woodyard *et al*., 2017; Heneberg *et al*., 2020; Lee *et al*., unpublished), but DNA sequence data from morphologically identified adult *Fibricola* specimens are lacking. Herein, we provide partial sequences of 18S, 28S, and *cox*1 genes for *Fibricola cratera* (Barker et Noll, 1915) and *Fibricola lucidum* (La Rue et Bosma, 1927) from mammals as well as five nominal species of *Neodiplostomum* and an unidentified *Neodiplostomum* species from a bird. We use newly generated and previously available DNA sequences to examine the phylogenetic interrelationships of *Fibricola* and *Neodiplostomum* species and re-evaluate their systematics.

7.2 Materials & Methods

Adult specimens belonging to genera *Fibricola* and *Neodiplostomum* were collected from a variety of mammalian and avian definitive hosts as well as amphibian intermediate hosts in North and South America (Table 13). Specimens were removed, fixed, and stained according to our standard methods. Voucher specimens are deposited in the collection of the Harold W. Manter Laboratory (HWML), University of Nebraska State Museum, Lincoln, NE, U.S.A. and the Museo de Zoología, Pontificia Universidad Católica del Ecuador (QCAZI), Quito, Ecuador. Due to inconsistent reporting of morphological characteristics in descriptions of *Neodiplostomum* spp., we re-measured type and voucher specimens of *Neodiplostomum americanum* Chandler et Rausch, 1947, *Neodiplostomum banghami* Penrod, 1947 and *Neodiplostomum reflexum* Chandler et Rausch, 1947 (syn. *Neodiplostomum delicatum* Chandler et Rausch, 1947) for comparison with specimens collected in the present study. Type and voucher specimens were borrowed for

our study from the Natural History Museum of Geneva and the Smithsonian Institution Museum of Natural History.

For comparative purposes, specimens of following species have been examined from the collection of the Natural History Museum, London (NHM): *Neodiplostomum australiense* (Dubois, 1937) from Australia (co-types, NHM 1950.12.6.18-22), *Neodiplostomum ramachandrani* (Betterton, 1976) from Malaysia (paratypes: NHM 1979.8-3.36, 44-46; NHM 1976.4.21.74; vouchers: NHM 1976.8.4.7-8), *Neodiplostomum spathula* (Creplin, 1829) from Minnesota (vouchers, NHM 1975.1.7.35-42).

Genomic DNA extracted and amplified using our standard methods with primers WormA, WormB, digL2, 1500R, ITSf, 300R, Dipl_Cox_5', and Dipl_Cox_3' (Table 1). For a subset of taxa collected in Mississippi and Arkansas (U.S.A.) the molecular methods described by Woodyard *et al.* (2017) were used. Sequencing was performed following standard methods using PCR primers along with internal primers 18S-8, WB1, DPL600F, DPL700R, d58F (table 1).

Phylogenetic analysis was based on the 18S, 28S and *cox*1 sequence data, in part, to match the data published by Heneberg *et al*. (2020). Interrelationships among members of the genus-level clades of *Fibricola*/*Neodiplostomum* were studied using two *cox*1 datasets based on the presence of two distinct clades of *Neodiplostomum* as seen in the results of our analyses of 18S and 28S as well as the suprageneric analysis of *cox*1 data. Based on the results of the broader phylogenetic analyses (see Results), we opted to not use an outgroup in the phylogenetic analyses of interrelationships within the clade uniting *Fibricola* spp. with the majority of *Neodiplostomum* spp. clade based on *cox*1 data, because of the high level of genetic divergence between members of this clade and other diplostomoidean taxa.

Table 13. Hosts, geographic origin, GenBank and museum accession numbers of *Neodiplostomum* (syn. *Fibricola*) spp. used in this study.

Digenean taxa	Host species	Geographic origin	Museum No.	Accession numbers	
				Ribosomal	*cox*1
Neodiplostomum cf. *cratera* 1 n. comb. *	*Didelphis virginiana*	Arkansas, U.S.A.	HWML-216754	–	OL770020
Neodiplostomum cf. *cratera* 2 n. comb. *	*Didelphis virginiana*	California, U.S.A.	HWML-216765	OL799069, OL799070 (ITS1), OL770124, OL770125 (ITS2),	OL770021 – OL770023
Neodiplostomum cf. *cratera* 2 n. comb. *	*Procyon lotor*	California, U.S.A.	–	OL799097 (28S)	OL770024
Neodiplostomum cf. *cratera* 3 n. comb. *	*Didelphis virginiana*	Mississippi, U.S.A.	HWML-216755	OL799071 (18S–28S)	OL770025
Neodiplostomum cf. *cratera* 3 n. comb. *	*Lithobates pipiens*	North Dakota, USA	–	OL799098 (28S)	OL770026
Neodiplostomum cf. *cratera* 3 n. comb. *	*Procyon lotor*	Minnesota, U.S.A.	HWML-216766	OL799072, OL799073 (ITS2–28S)	OL770027, OL770028
Neodiplostomum cf. *cratera* 3 n. comb. *	*Neogale vison*	Minnesota, U.S.A.	HWML-216767	OL799074 (18S–ITS2)	OL770029
Neodiplostomum cf. *cratera* 3 n. comb. *	*Taxidea taxus*	North Dakota, U.S.A.	–	OL799099 (28S)	OL770030
Neodiplostomum cf. *lucidum* *	*Didelphis virginiana*	Arkansas, U.S.A.	HWML-216752, HWML-216753	OL799075 (18S–28S)	OL770031, OL770032
Neodiplostomum cf. *lucidum* *	*Didelphis virginiana*	Mississippi, U.S.A.	–	–	OL770033 – OL770037
Neodiplostomum cf. *lucidum* *	*Didelphis virginiana*	Nebraska, U.S.A.	HWML-216768	OL799076 (18S–28S)	OL770038, OL764381
Neodiplostomum cf. *lucidum* *	*Didelphis virginiana*	North Carolina, U.S.A.	HWML-216769	OL799100, OL799101 (28S)	OL770039, OL770040
Neodiplostomum cf. *lucidum* *	*Lithobates catesbeianus*	Mississippi, U.S.A.	–	OL799077, OL799078 (ITS1–28S)	OL770041, OL770042
Neodiplostomum cf. *lucidum* *	*Procyon lotor*	California, U.S.A.	HWML-216770	OL799102 (28S)	OL770043
Neodiplostomum microcotyle	*Busarellus nigricollis*	Pantanal, Brazil	HWML-216771	OL799079 (18S–28S)	OL770044
N. microcotyle	*Buteogallus urubitinga*	Pantanal, Brazil	HWML-216772	–	OL770045

Neodiplostomum americanum	*Accipiter cooperii*	North Dakota, U.S.A.	HWML-216773	OL799080 (18S), OL770126 (ITS1–28S)	OL770046
N. americanum	*Bubo virginianus*	Arkansas, U.S.A.	HWML-216774	OL799103 (28S)	OL770047
N. americanum	*Bubo virginianus*	Mississippi, U.S.A.	HWML-216756, HWML-216757, HWML-216760	OL799081–OL799083 (ITS region)	OL770048 – OL770050
N. americanum	*Nerodia fasciata*	Mississippi, U.S.A.	–	OL799084 (ITS1–28S)	OL770051
N. americanum	*Strix varia*	Mississippi, U.S.A.	–	OL799085 (ITS region)	OL770052
N. americanum	*Thalasseus maximus*	Mississippi, U.S.A.	–	OL799086 (ITS1–28S)	OL770053
Neodiplostomum banghami	*Falco columbarius*	North Dakota, U.S.A.	–	OL799087 (18S–28S)	OL770054
N. banghami	*Lithobates sylvatica*	North Dakota, U.S.A.	–	OL799104 (28S)	OL770055
N. banghami	*Thamnophis sirtalis*	North Dakota, U.S.A.	–	OL799105 (28S)	OL770056
Neodiplostomum reflexum	*Bubo virginianus*	North Dakota, U.S.A.	HWML-216775	OL799106 (28S)	OL770057
N. reflexum	*Bubo virginianus*	Mississippi, U.S.A.	HWML-216759	OL799088 (ITS region)	OL770058
N. reflexum	*Buteo jamaicensis*	North Dakota, U.S.A.	–	OL799089 (18S–28S)	OL770059
N. reflexum	*Buteo jamaicensis*	Mississippi, U.S.A.	–	OL799090 (ITS region)	OL770060
N. reflexum	*Strix varia*	Mississippi, U.S.A.	HWML-216758, HWML-216761–216763	OL799091 (18S–28S), OL799092–OL799094 (ITS region)	OL770061 – OL770064
Neodiplostomum vaucheri	*Trachops cirrhosus*	Ecuador	QCAZI 264292	OL799095 (18S–28S), OL799107, OL799108 (28S)	OL770065 – OL770067
Neodiplostomum sp. VVT1	*Bubo virginianus*	North Dakota, U.S.A.	HWML-216776	OL799096 (18S–28S)	OL770068

* – species previously considered to be within *Fibricola.*

The 18S alignment included newly generated sequences of *Fibricola* spp. (*n* = 3) and *Neodiplostomum* spp. (*n* = 5) as well as previously published sequences of *Neodiplostomum* spp. (*n* = 3) and other members of the Diplostomidae (*n* = 21). The 28S alignment included newly generated sequences of *Fibricola* (*n* = 3) and *Neodiplostomum* spp. (*n* = 5) along with a previously published sequence of *N. americanum*. The 28S analysis also included previously published sequences of members of the Diplostomidae (*n* = 17), the Proterodiplostomidae Dubois, 1936 (*n* = 2) and Strigeidae Railliet, 1919 (*n* = 12). The suprageneric *cox*1 alignment included new sequences of *Fibricola* (*n* = 2) and *Neodiplostomum* spp. (*n* = 6) as well as previously published sequences of *Neodiplostomum* spp. (*n* = 7). This alignment also included previously published sequences of other members of the Diplostomidae (*n* = 15). The *cox*1 alignment limited to members of the *Fibricola*/*Neodiplostomum* clade (Clade I) included 21 newly generated sequences. The *cox*1 alignment limited to the second clade of *Neodiplostomum* species (Clade II) included 9 new sequences and 9 previously published sequences.

The general time reversible model with estimates of invariant sites and gamma distributed among-site variation (GTR + I + G) was identified as the best-fitting nucleotide substitution model for the datasets using MEGA7 (Kumar *et al*., 2016). BI analyses were performed using our standard methods.

7.3 Results

Molecular phylogenies

To maintain continuity and consistency in presenting and discussing our results, we are stating herein that we consider *Fibricola* to be a junior synonym of *Neodiplostomum* (see results of the 18S, 28S and suprageneric *cox*1 analyses and discussion below). We refer to *F. cratera*

and *F. lucidum* as *Neodiplostomum cratera* n. comb. (Barker et Noll, 1915) and *Neodiplostomum lucidum* La Rue et Bosma, 1927 throughout the remainder of the text. Justification for the synonymization is provided in the discussion.

The 18S alignment was 1,619 bp long; 22 bases were excluded from the analysis due to ambiguous homology. The phylogenetic tree resulting from the BI analysis of 18S (Fig. 19) demonstrated similar topology to that presented by Heneberg *et al*. (2020). *Neodiplostomum* spp. were positioned in two distinct clades within a larger polytomy of diplostomids. Clade I (100% supported) of *Neodiplostomum* spp. contained *Neodiplostomum* cf. *cratera* 3 (Barker et Noll, 1915) (former type-species of *Fibricola*; see discussion below), *Neodiplostomum* cf. *lucidum* La Rue et Bosma, 1927, *Neodiplostomum spathula* (Creplin, 1829) (former type-species of *Conodiplostomum* Dubois, 1937) + *Neodiplostomum attenuatum* (Linstow, 1906) + *Neodiplostomum microcotyle* Dubois, 1937 + *N. reflexum* + *N. vaucheri* + *Neodiplostomum* sp. VVT1. Clade II (100% supported) of *Neodiplostomum* spp. only contained *N. americanum* + *N. banghami*.

The 28S alignment was 1,135 bp long; three bases were excluded from the analysis due to ambiguous homology. The phylogenetic tree resulting from the BI analysis of 28S demonstrated the non-monophyly of the Diplostomidae and Strigeidae and monophyly of the Proterodiplostomidae (Fig. 20), similar to previous molecular phylogenetic analyses of the Diplostomoidea (e.g., Blasco-Costa and Locke, 2017; Hernández-Mena *et al*., 2017; Achatz *et al*., 2019*b*–*d*, 2020, 2021*a*; Queiroz *et al*., 2020; Tkach *et al*., 2020; Locke *et al*., 2021). All sequences of taxa/lineages representing *Fibricola* formed a 99% supported clade (Clade I) with four *Neodiplostomum* species: *N. microcotyle*, *N reflexum* and *N. vaucheri* as well as unidentified species-level lineage *Neodiplostomum* sp. VVT1. This clade was separated into two strongly

supported sub-clades. The first sub-clade (97%) included sequences of *Fibricola* from mammals + *N. reflexum* from birds. The second sub-clade (96%) contained *Neodiplostomum* sp. VVT1 from great horned owl *Bubo virginianus* (Gmelin) + a weakly supported assemblage of [*N. microcotyle* + *N. vaucheri*]. Clade II of *Neodiplostomum* spp. (100% supported) contained *N. americanum* + *N. banghami* (Fig. 20).

The suprageneric *cox*1 alignment was 285 bp long; the alignment length was limited by the short length of sequences published by Heneberg *et al*. (2020). Similar to the 18S and 28S analyses, *Neodiplostomum* taxa were split among two clades (Fig. 21). Clade I (86% supported) consisted of a large polytomy with poorly resolved internal topology (Fig. 21). The polytomy consisted of *Neodiplostomum spathulaeforme* (Brandes, 1888) (type-species of *Neodiplostomum*) + *Neodiplostomum seoulense* (Seo, Rim et Lee 1964) + a 100% supported clade of [*N. reflexum* + *N.* cf. *cratera* 3 + *N.* cf. *lucidum*] + an 88% supported clade of [*N. vaucheri* + *N. microcotyle* + *Neodiplostomum* sp. VVT1] + an 86% supported clade of [*N. attenuatum* + *N. spathula*] (Fig. 21).

Based on their phylogenetic position in the 18S, 28S and suprageneric *cox*1 analyses, *N. microcotyle, N. reflexum, N. vaucheri* and *Neodiplostomum* sp. VVT1 were included in the focused *cox*1analysis together with former *Fibricola* spp. (Clade I in Figs. 19–21). This alignment was 456 bp long; three bases (1 codon) were excluded from the analysis as an indel. The internal branch topology of the resulting tree (Fig. 22) was somewhat different and better resolved than in the 18S and 28S analyses. *Neodiplostomum microcotyle* + *Neodiplostomum* sp. VVT1 from *B. virginianus* + *N. vaucheri* formed a strongly (100%) supported clade separate from the 100% supported clade of *N. reflexum* + former *Fibricola* lineages.

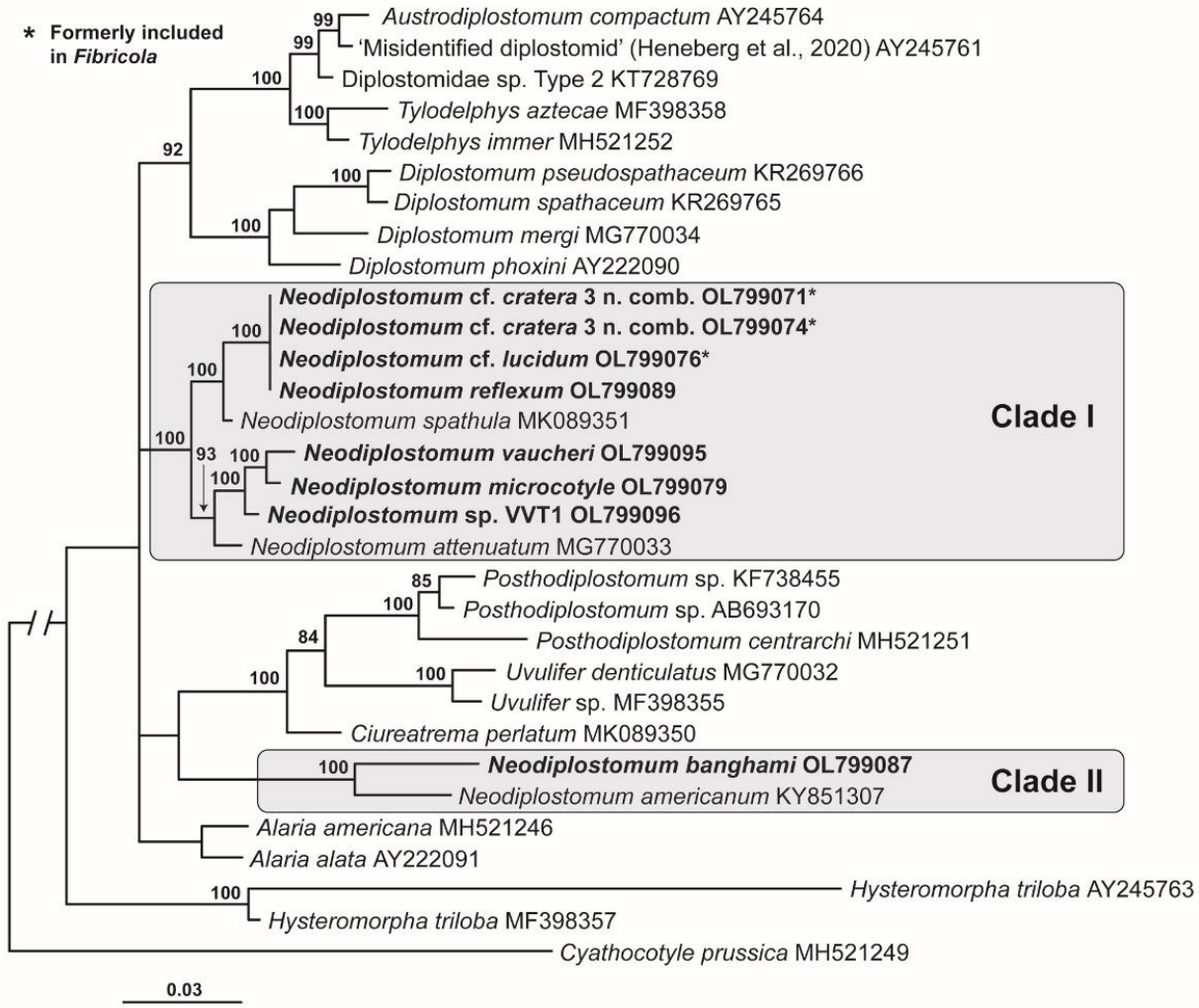


Fig. 19. Phylogenetic interrelationships among the Diplostomidae including *Neodiplostomum* (syn. *Fibricola*) based on Bayesian Inference (BI) analysis of partial 18S rRNA gene sequences. Members of *Neodiplostomum* are indicated by the shaded rectangles. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences are indicated in bold. The scale-bar indicates the number of substitutions per site. GenBank accession numbers are provided after the names of species.

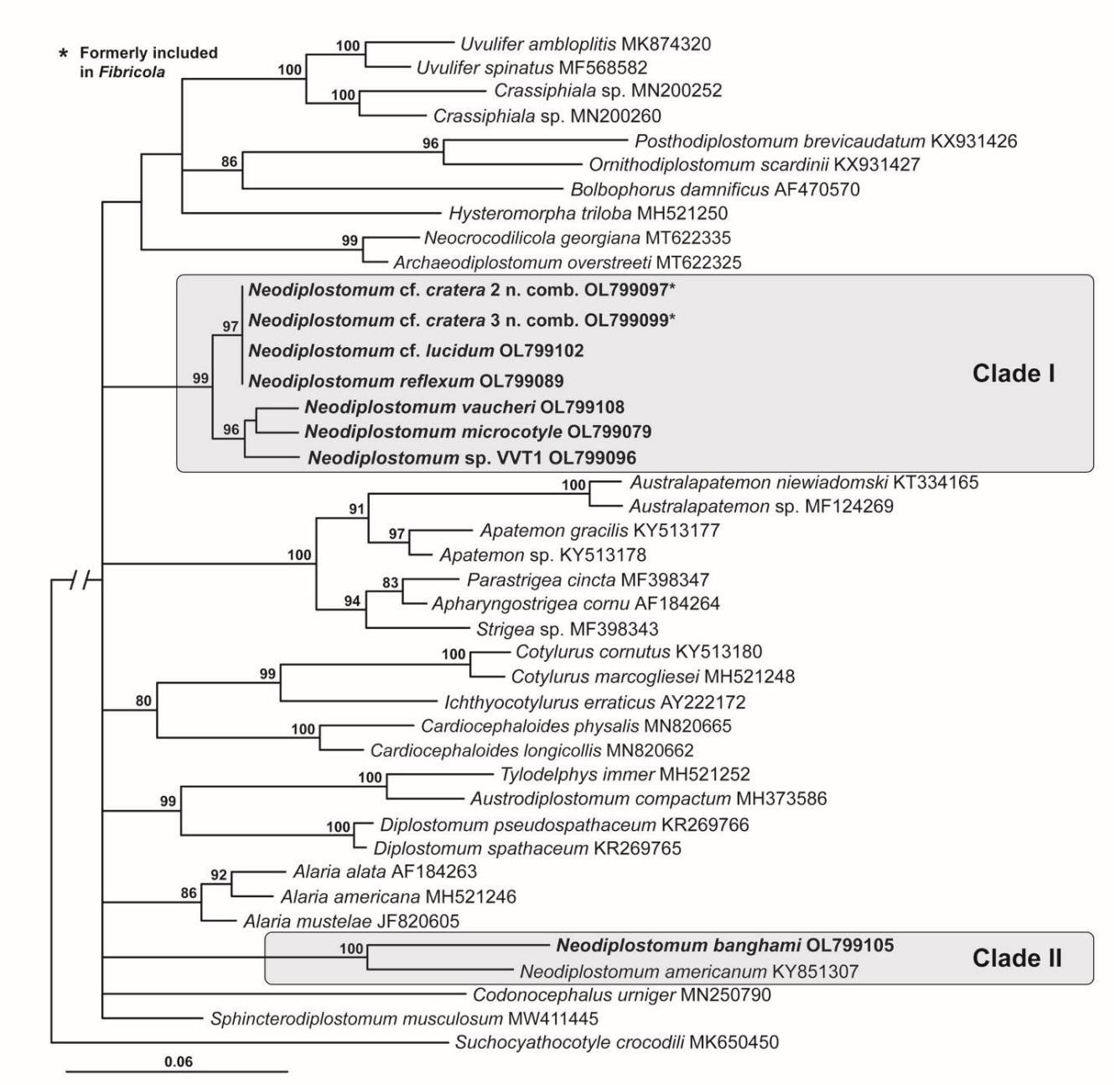


Fig. 20. Phylogenetic interrelationships among diplostomoidean taxa including *Neodiplostomum* (syn. *Fibricola*) based on Bayesian Inference (BI) analysis of partial 28S rRNA gene sequences. Members of *Neodiplostomum* are indicated by the shaded rectangles. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated are indicated in bold. The scale-bar indicates the number of substitutions per site. GenBank accession numbers are provided after the names of species.

Neodiplostomum microcotyle was positioned as a sister group to a weakly supported clade of Neodiplostomum sp. VVT1 + Nvaucheri. All sequences of N. reflexum formed a 100% supported, long-branch clade as a sister clade to a weakly supported clade containing sequences of former Fibricola cf. cratera 3 (Fig. 22). The remaining sequences of former Fibricola formed two clades. One of them was weakly (82%) supported and included N. cf. cratera 1 (formerly F. cf. cratera 1) and specimens that were morphologically identified as N. cf. lucidum (formerly F. cf. lucidum) (Fig. 22). The other was a 100% supported clade of N. cf. cratera 2 (formerly F. cf. lucidum).

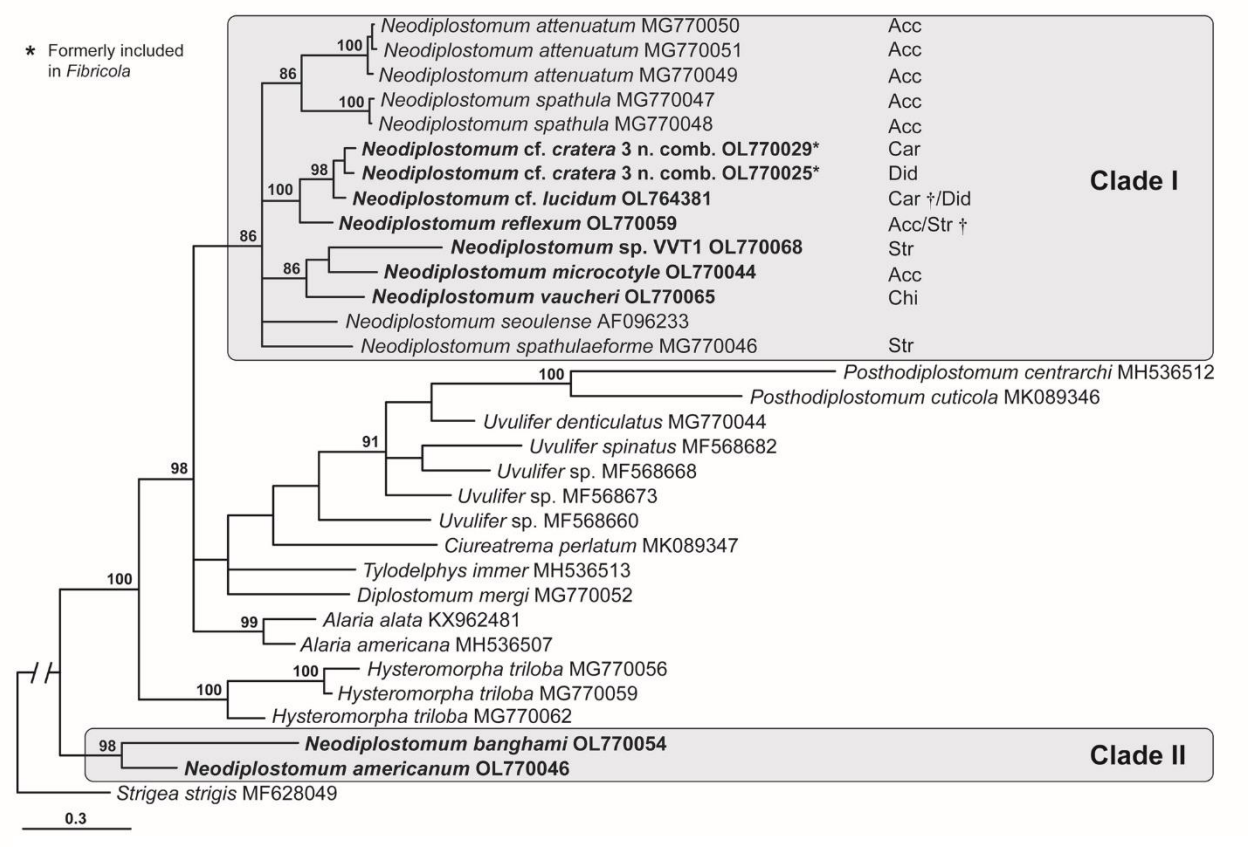


Figure 21. Phylogenetic interrelationships among the Diplostomidae including 16 members of *Neodiplostomum* based on BI analysis of partial *cox*1 sequences. *Neodiplostomum* spp. are indicated by the shaded rectangles. BI posterior probability values lower than 80% not shown. The new sequences are in bold. The scale-bar indicates the number of substitutions per site. GenBank accession numbers are provided after the names of species. The orders of definitive hosts are provided after GenBank accession numbers for *Neodiplostomum* spp. in Clade 1. Abbreviations for orders of definitive host: Acc, Accipitriformes; Car, Carnivora; Chi, Chiroptera; Did, Didelphimorphia; Str, Strigiformes. † – we also sequenced additional conspecific isolates collected from additional orders of definitive hosts.

The *cox*1 alignment of the second *Neodiplostomum* clade (Clade II in Figs 19–21) was 366 bp long. Sequences of *N. americanum* and *N. banghami* formed separate 100% supported clades (Fig. 23).

Genetic variation

Taxa included in Clade I demonstrated low interspecific divergence in 18S sequences (0–1.1%). No differences were detected among 18S sequences of *N.* cf. *cratera* 3 (multiple sequences), *N.* cf. *lucidum* and *N. reflexum*; *Neodiplostomum* cf. *cratera* 3 vs. *N. vaucheri*, *N.* cf. *lucidum* vs. *N. vaucheri* and *N. reflexum* vs. *N. vaucheri* had the greatest level of interspecific divergence in 18S among *Neodiplostomum* spp. in Clade I. *Neodiplostomum americanum* and *N. banghami*, members of the Clade II, differed by 1.6% between their sequences of 18S. No intraspecific

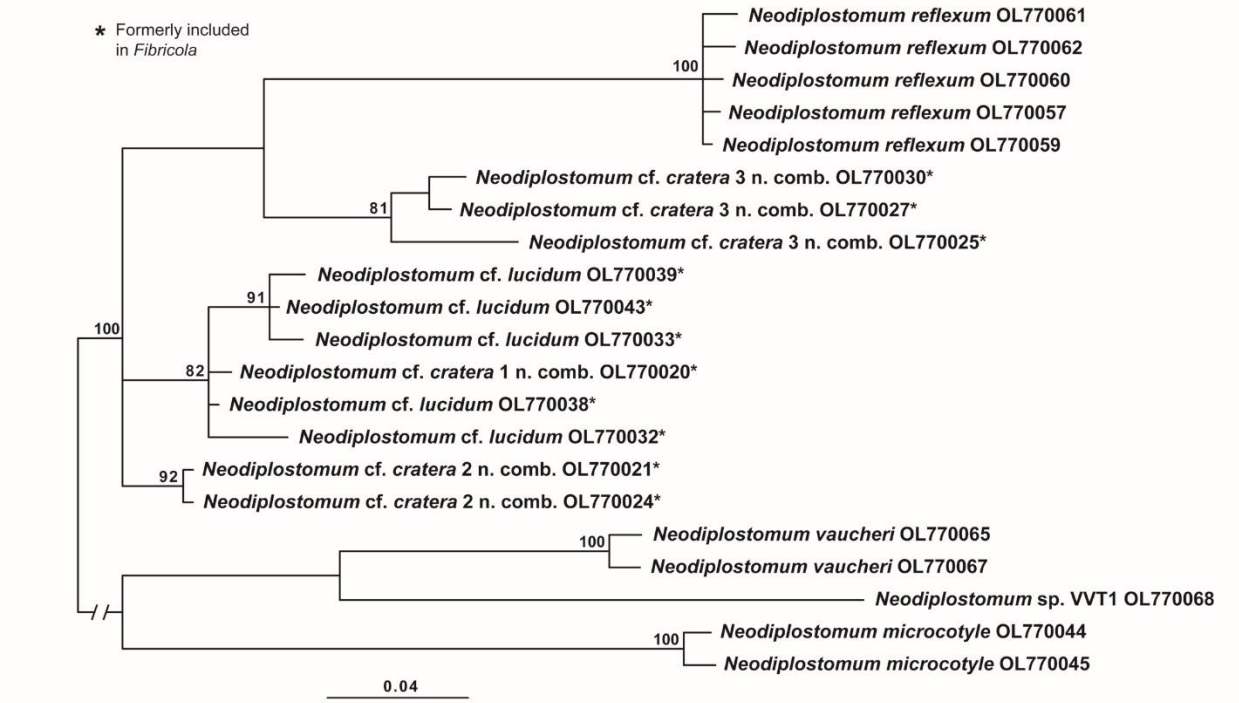


Figure 22. Phylogenetic interrelationships among 20 members of *Neodiplostomum* Clade 1 based on Bayesian Inference (BI) analysis of partial *cox*1 mtDNA gene sequences. Members of the 2 strongly-supported clades of former *Fibricola*. are indicated by the shaded rectangles. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated are indicated in bold. The scale-bar indicates the number of substitutions per site. GenBank accession numbers are provided after the names of species.

Table 14. Pairwise comparisons of partial sequences of the 18S rDNA among *Neodiplostomum* (syn. *Fibricola*) species included in this study based on a 1,602 bp long alignment. Percentage differences are given above diagonal and the number of variable nucleotide positions is given below the diagonal. Taxa previously included in *Fibricola* are denoted by the *.

	1. OL799074	**2.** OL799071	**3.** OL799076	**4.** OL799089	**5.** MK089351	**6.** MG770033	**7.** OL799096	**8.** OL799079	**9.** OL799095	**10.** KY851307	**11.** OL799087
1. *Neodiplostomum* cf. *cratera* 3 n. comb. OL799074*	–	0%	0%	0%	0.4%	0.8%	0.9%	1%	1.1%	2.9%	3.2%
2. *Neodiplostomum* cf. *cratera* 3 n. comb. OL799071*	0	–	0%	0%	0.4%	0.8%	0.9%	1%	1.1%	2.9%	3.2%
3. *Neodiplostomum* cf. *lucidum* OL799076*	0	0	–	0%	0.4%	0.8%	0.9%	1%	1.1%	2.9%	3.2%
4. *Neodiplostomum reflexum* OL799089	0	0	0	–	0.4%	0.8%	0.9%	1%	1.1%	2.9%	3.2%
5. *Neodiplostomum spathula* MK089351	6	6	6	6	–	0.6%	0.6%	0.7%	0.9%	2.6%	2.9%
6. *Neodiplostomum attenuatum* MG770033	13	13	13	13	9	–	0.4%	0.6%	0.7%	2.7%	2.9%
7. *Neodiplostomum* sp. VVT1 OL799096	14	14	14	14	10	7	–	0.2%	0.4%	2.8%	3%
8. *Neodiplostomum microcotyle* OL799079	16	16	16	16	12	9	4	–	0.2%	2.7%	3%
9. *Neodiplostomum vaucheri* OL799095	18	18	18	18	14	11	6	4	–	2.9%	3.1%
10. *Neodiplostomum americanum* KY851307	46	46	46	46	42	43	45	44	46	–	1.6%
11. *Neodiplostomum banghami* OL799087	52	52	52	52	47	46	48	48	49	26	–

variation was detected among 18S sequences of *N.* cf. *cratera* 3 n. comb., *N. reflexum* and *N. americanum*. Complete pairwise comparisons of 18S sequences are provided in Table 14.

The interspecific divergence in 28S sequences of *Neodiplostomum* spp. (Clade I) was similar to differences among 18S sequences (0–1.2%). No differences were detected among 28S sequences of *N.* cf. *cratera* 2 and 3 n. comb., *N.* cf. *lucidum* and *N. reflexum*. The unidentified *Neodiplostomum* sp. VVT1 from *B. virginianus* vs. *N.* cf. *cratera* 2 and 3 n. comb., *Neodiplostomum* sp. VVT1 vs. *N.* cf. *lucidum* and *Neodiplostomum* sp. VVT1 vs. *N. reflexum*

Table 15. Pairwise comparisons of partial sequences of the 28S rDNA among *Neodiplostomum* (syn. *Fibricola*) species included in this study based on a 1,176 bp long alignment. Percentage differences are given above diagonal and the number of variable nucleotide positions is given below the diagonal. Taxa previously included in *Fibricola* are denoted by the *.

	1. OL799097	**2.** OL799071	**3.** OL799102	**4.** OL799089	**5.** OL799079	**6.** OL799108	**7.** OL799096	**8.** KY851307	**9.** OL799105
1. *Neodiplostomum* cf. *cratera* 2 n. comb. OL799097*	–	0%	0%	0%	1.1%	1.1%	1.2%	5%	5.8%
2. *Neodiplostomum* cf. *cratera* 3 n. comb. OL799071*	0	–	0%	0%	1.1%	1.1%	1.2%	5%	5.8%
3. *Neodiplostomum* cf. *lucidum* OL799102*	0	0	–	0%	1.1%	1.1%	1.2%	5%	5.8%
4. *Neodiplostomum reflexum* OL799089	0	0	0	–	1.1%	1.1%	1.2%	5%	5.8%
5. *Neodiplostomum microcotyle* OL799079	13	13	13	13	–	0.9%	1%	5.8%	6.2%
6. *Neodiplostomum vaucheri* OL799108	13	13	13	13	10	–	1.1%	5.4%	6.2%
7. *Neodiplostomum* sp. VVT1 OL799096	14	14	14	14	12	13	–	5.5%	5.6%
8. *Neodiplostomum americanum* KY851307	59	59	59	59	68	64	65	–	3.7%
9. *Neodiplostomum banghami* OL799105	68	68	68	68	73	73	66	43	–

had the greatest level of interspecific divergence in 28S (1.2%) among *Neodiplostomum* spp. in Clade I. In contrast, the interspecific variability among members of *Neodiplostomum* spp. in the Clade II was overall greater; *Neodiplostomum americanum* and *N. banghami* were 3.7% divergent in the sequenced 28S fragment. Notably, no intraspecific variation in sequences of 28S was detected in any of the *Neodiplostomum* taxa with multiple isolates included in the analysis. Complete pairwise comparisons of 28S sequences are provided in Table 15.

Interspecific differences of *cox*1 sequences among *Neodiplostomum* spp. of Clade I, excluding the *N.* cf. *cratera*/*N.* cf. *lucidum* cluster, ranged between 8.6–13.4%. *Neodiplostomum*

vaucheri vs. *Neodiplostomum* sp. VVT1 showed the lowest divergence (8.6–8.8%), whereas *N. reflexum* and *N. microcotyle* had the greatest divergence (12.7–13.4%). The *N.* cf. *cratera*/*N.* cf. *lucidum* cluster demonstrated up to 6.2% divergence among its members. The *cox*1 sequences of *N. americanum* and *N. banghami* (Clade II) differed by 12.3–14.2%. With the exception of *N.* cf. *cratera* lineages, all *Neodiplostomum* spp. in Clade I and II with more than a single sequence available showed no more than 1.6% intraspecific variation in *cox*1 sequences.

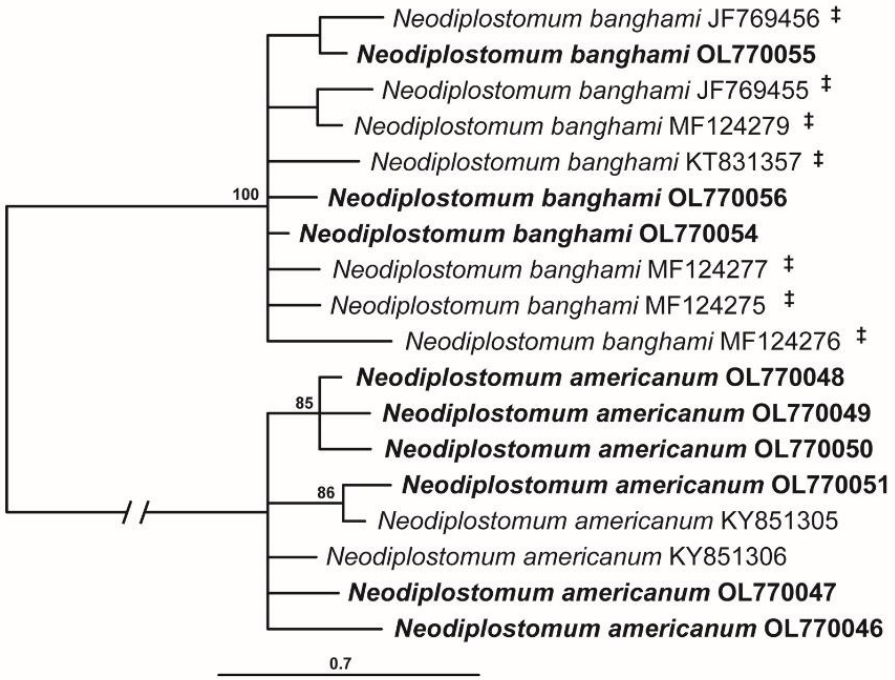


Figure 23. Phylogenetic interrelationships among the 2 species of *Neodiplostomum* Clade 2 based on Bayesian Inference (BI) analysis of partial *cox*1 mtDNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated are indicated in bold. The scale-bar indicates the number of substitutions per site. GenBank accession numbers are provided after the names of species. ‡ – isolates previously identified as *N. americanum* in GenBank

7.4 Discussion

The systematic histories of *Fibricola* and *Neodiplostomum* are complex. Dubois (1932) originally established genus *Fibricola* for *F. cratera* described from muskrat by Barker (1915).

Subsequently, Dubois (1937) added *Fibricola minor* Dubois, 1936 to the genus and noted an error in the topography of the reproductive system organs in the original description of *F. cratera*. Dubois (1937) used parasitism in mammals along with confinement of the vitellarium to the prosoma as the justification for separation between *Fibricola* and *Neodiplostomum* which typically parasitizes birds and has vitellarium in both parts of the body. However, Dubois (1938) noted the vitellarium of *F. cratera* may extend into the opisthosoma to the level of the anterior testis. Miller (1940) later described a second North American species of the genus, *Fibricola laruei* Miller, 1940, from raccoon *Procyon lotor* (Linnaeus) collected in Quebec.

Fibricola texensis Chandler, 1942, was described based on specimens collected from *P. lotor* in Texas. The original description of the species reported its vitellarium extending to variable levels in the opisthosoma (Chandler, 1942). Additionally, Chandler (1942) noted that the vitellarium of *F. laruei* also extended into the opisthosoma, but only to the level of the vitelline reservoir situated between the testes. Zerecero (1943) subsequently described the fourth species of *Fibricola* from North America, *Fibricola caballeroi* Zerecero, 1943, collected from the brown, or Norway, rat *Rattus norvegicus* (Berkenhout) in Mexico.

Dubois (1944) erected *Theriodiplostomum* Dubois, 1944 for *F. texensis* and *N. lucidum* from Virginia opossum collected in Texas, based on vitellarium distributed in both the prosoma and opisthosoma and parasitism in mammals. *Theriodiplostomum* spp. were considered morphologically intermediate forms between *Fibricola* and *Neodiplostomum* (Dubois, 1944).

Chandler and Rausch (1946) described a fifth member of *Fibricola* in North America, *Fibricola nana* Chandler et Rausch, 1946, from American red squirrel *Tamiasciurus hudsonicus* (Erxleben) (syn. *Sciurus hudsonicus*) in Michigan. Importantly, Chandler and Rausch (1946) deemed the use of the distribution of vitellarium and parasitism in either mammals or birds not

tenable for differentiation among genera and rejected *Theriodiplostomum*. Read (1948) agreed with this decision and considered *F. nana* and *F. laruei* synonyms of *F. cratera*. Read (1948) proposed the tendency for greater concentration of vitelline follicles in the prosoma in members of *Fibricola* species as the main distinguishing character from *Neodiplostomum* spp.

Dubois and Rausch (1950) transferred the former *Theriodiplostomum lucidum* (La Rue et Bosma, 1927) to *Fibricola*. In contrast to the previous authors, Pearson (1959) viewed *Fibricola* as a subgenus of *Neodiplostomum*. Odening (1965) maintained *Fibricola* as a subgenus of *Neodiplostomum* based on similarities of larval morphology (i.e., the identical flame cell formula, $2[(1 + 1 + 1) + (1 + 1 + [1])] = 12$).

Several *Fibricola* spp. were previously described from mammalian hosts outside of North America and later transferred to *Neodiplostomum*. For example, *N. seoulensis*, described from *R. norvegicus* collected in Korea, was originally included in *Fibricola* based, in part, on parasitism in mammals. Noteworthy, this species has been reported from humans in Korea (Huh *et al.*, 1994). Hong and Shoop (1994) transferred this species into *Neodiplostomum* based on morphology of adults and metacercariae. Likewise, Cribb and Pearson (1993) transferred three *Fibricola* spp. from Australian mammals into *Neodiplostomum* based on adult morphology.

Despite similarities in larval and adult morphology, Dubois (1970) rejected placement of *Fibricola* as a subgenus of *Neodiplostomum*. In spite of his own statement, Dubois (1983) placed *N. vaucheri* collected from a chiropteran host into *Neodiplostomum*.

While specificity to either mammalian or avian hosts has often been used for differentiation of *Fibricola* and *Neodiplostomum* species, some studies (e.g., Ulmer, 1955; Seo, 1989) demonstrated that *Fibricola* spp. can develop in avian hosts. Nevertheless, the most recent revision of the Diplostomoidea by Niewiadomska (2002) maintained *Fibricola* and

Neodiplostomum as separate genera belonging to different subfamilies (the Alariinae and the Diplostominae Poirier, 1886, correspondingly) based on parasitism in either mammals or birds.

Heneberg *et al*. (2020) demonstrated the non-monophyly of *Neodiplostomum* and proposed *Conodiplostomum* to be a junior synonym of *Neodiplostomum* based on molecular phylogenies. Unfortunately, this solution did not remove the problem of the non-monophyly of *Neodiplostomum*. Members of *Neodiplostomum* consistently formed two distinct clades in our analyses (Figs. 19–21). At present, 18S and 28S sequences of *N. spathulaeforme* (type-species) are not available. The suprageneric analysis of shorter fragment of *cox*1 (Fig. 21) revealed a fairly well supported clade of *Neodiplostomum* (including *N. spathulaeforme*) + former *Fibricola* + the former type-species of *Conodiplostomum* (*N. spathula*). At the same time, the second well-supported clade of *Neodiplostomum* was positioned separately within this phylogeny (Fig. 21) and only contained *N. americanum* + *N. banghami*. Similar patterns related to the constituents of the two *Neodiplostomum* clades (e.g., the position of *Fibricola* within the Clade I) were strongly supported in 18S and 28S analyses (Figs. 19, 20). The position of the type-species of *Neodiplostomum* (*N. spathulaeforme*) in the suprageneric analysis of *cox*1 (Fig. 21) clearly indicates that taxa within Clade I should be considered true *Neodiplostomum*.

Based on our examination of adult morphology (e.g., variable distribution of vitellarium in the prosoma and opisthosoma among and within *Fibricola* species) and previous studies of larval morphology (e. g., Odening, 1965), no morphological characters reliably support the status of *Fibricola* as an independent genus. *Neodiplostomum reflexum* from avian hosts and *F. cratera* lineages from mammals lack any differences among sequences of 18S and 28S, which demonstrates the taxa to be congeneric. Molecular data demonstrate the lack of specificity to mammalian or avian definitive hosts within the *Neodiplostomum* + *Fibricola* clade. Therefore,

we consider *Fibricola* to be a junior synonym of *Neodiplostomum* and transfer the constituent species of *Fibricola* into *Neodiplostomum*. *Fibricola cratera* and *F. caballeroi* are being transferred into *Neodiplostomum* as *N. cratera* n. comb. and *Neodiplostomum caballeroi* Zerecero, 1943, respectively. Noteworthy, *F. lucidum* was originally described as *N. lucidum*; thus, this species is returned to its original genus. Below, we provide an amended diagnosis of *Neodiplostomum* based on the diagnosis by Niewiadomska (2002). Due to the lack of distinct morphological features differentiating *Neodiplostomum* spp. Clade II from true *Neodiplostomum* (Clade I) we temporarily retain the species from the Clade II within *Neodiplostomum*. We anticipate that future detailed studies of their morphology and/or life cycles will provide differentiating characters and may allow placement of the Clade II members into a currently undescribed genus.

Neodiplostomum Railliet, 1919 (After Niewiadomska, 2002 with changes)

Diagnosis: Body distinctly bipartite; prosoma spatulate or oval; opisthosoma cylindrical or oval. Pseudosuckers absent. Oral and ventral suckers and pharynx present. Holdfast organ round or oval, with median slit. Testes of similar size, tandem; anterior in general asymmetrical; posterior symmetrical, often bilobed. Ovary reniform or ellipsoidal, pretesticular, median or submedian, situated close to borderline between prosoma and opisthosoma, rarely near middle of opisthosoma. Vitellarium may extend almost to intestinal bifurcation. Copulatory bursa small or large; genital cone absent; hermaphroditic duct opens directly into bursa. In avian and mammalian definitive hosts. Cosmopolitan. Metacercariae in amphibians; paratenic hosts reptilians and mammals. Cercariae with two pairs of pre- and paracetabular penetration glands; flame-cell formula 2[(1 + 1 + 1) + (1 + 1 + [1])] = 12. Type-species *N. spathulaeforme* (Brandes, 1888).

After the re-evaluation of the validity of *Fibricola* and its constituents in North America, 11 valid named species of *Neodiplostomum* are currently known from North America: *N. cratera* n. comb., *N. lucidum* and *N. caballeroi* n. comb. from mammals as well as *Neodiplostomum accipitris* Dubois et Rausch, 1948, *N. attenuatum*, *Neodiplostomum centuri* Dubois et Macko, 1972, *Neodiplostomum isomegalocotyle* Dubois et Macko, 1972, *Neodiplostomum pearsoni* Dubois, 1962, *N. reflexum*, *N. americanum*, *N. banghami* from birds (e.g., Dubois, 1968, 1982; Dubois and Macko, 1972; present data). As mentioned above, *N. americanum* and *N. banghami* are kept in *Neodiplostomum* only provisionally due to the lack of suitable differentiating morphological characters. The same may potentially apply to *N. accipitris*, *N. centuri*, *N. isomegalocotyle* and *N. pearsoni* for which sequence data are currently lacking.

Notably, our data revealed the presence of three genetically distinct lineages of digeneans morphologically corresponding to *N. cratera* in North America (Fig. 22). One of these lineages appeared in the clade with specimens morphologically corresponding to *N. lucidum*. Our adult specimens of *N.* cf. *cratera* collected from several mammalian hosts throughout the U.S.A., morphologically conform to the original description of *F. cratera* by Barker (1915) from *O. zibethicus* collected in Nebraska and redescribed by Dubois (1937). Since this situation does not affect the main conclusions from the present phylogenetic study, we cautiously designate these forms as *N.* cf. *cratera* 1–3 and *N.* cf. *lucidum*. While the *cox*1 sequences of *N.* cf. *cratera* 1 (GenBank OL770020) were clearly conspecific to sequences of samples that morphologically correspond to *N.* cf. *lucidum*, the *cox*1 sequences of *N.* cf. *cratera* 1 and 2 differ from *N.* cf. *cratera* 3 by 4.6–6.2% of nucleotide positions. Currently, *N. cratera* and *N. lucidum* are differentiated based on the distribution of vitellarium (primarily in prosoma in *N. cratera* vs.

extending far into opisthosoma in *N. lucidum*) (e.g., Dubois, 1968). However, based on our data, this character cannot be used to distinguish between these species.

Our results clearly demonstrated that sequences of *N. americanum* available in Genbank represent two distinct species (Tables 14, 15; Fig. 21). Our specimens of *N. americanum* are conspecific with specimens previously published by Woodyard *et al*. (2017) based on partial sequences of 28S, the ITS region and *cox*1. Furthermore, our specimens and the material of Woodyard *et al*. (2017) conform to the original morphological description of *N. americanum* by Chandler and Rausch (1947). Our morphological examination of voucher specimens of adult *N. americanum* sequenced by Blasco-Costa and Locke (2017) revealed that the taxon was misidentified. Additionally, *cox*1 sequences of *N. americanum* published by Blasco-Costa and Locke (2017) are clearly conspecific with our sequences of *N. banghami* (Fig. 23).

Our *cox*1 phylogeny (Fig. 21) demonstrated at least two independent host-switching events between avian and mammalian hosts in the evolutionary history of *Neodiplostomum*. The clade of *N. reflexum* + a cluster of [*N.* cf. *lucidum* + *N.* cf. *cratera*] suggests a transition from avian definitive hosts (orders Accipitriformes Vieillot and Strigiformes Wagler) to a diversity of mammalian definitive hosts (orders Carnivora Bowdich and Didelphimorphia Gill). The position of *N. vaucheri* in a clade with *N. microcotyle* and *Neodiplostomum* sp. VVT1 confirmed the initial generic position of this species by Dubois (1983) and revealed a transition to bats (order Chiroptera Blumenbach); additional data are needed to determine the directionality of the secondary host-switching event due to the lack of internal support within this clade. The bat in which *N. vaucheri* was found is known to feed on amphibians. This dietary overlap with more traditional hosts of *Neodiplostomum* spp. (birds of prey, carnivorous mammals) created conditions for host switching. It remains to be seen how DNA sequences from other former

Fibricola species that parasitize mammals as adults (e.g., *N. caballeroi* n. comb.) and species from Southeast Asia and Australia (e.g., *N. australiense*) will impact the current picture of the interrelationships of *Neodiplostomum*.

Similar to other previous molecular phylogenetic studies, in our analyses *Neodiplostomum* did not form a clade with other members of the formerly accepted Diplostominae (Figs. 19–21), (e.g., Achatz *et al*., 2019*b*, 2021*a*, *c*; Queiroz *et al*., 2020). Our results, along with other recent molecular phylogenetic studies (e.g., Blasco-Costa and Locke, 2017; Hernández-Mena *et al*., 2017; Locke *et al*., 2018; Achatz *et al*., 2020, 2021*a–d*, 2022c; Sereno-Uribe *et al*., 2019; Queiroz *et al*., 2020; Locke *et al*., 2021), strongly suggest that the most recently accepted subfamilies of the Diplostomidae cannot be considered valid. Achatz et al. (2021*c*) rejected the subfamily system of the Diplostomidae. The data presented in the current work and Achatz et al. (2022c) further corroborates the decision by Achatz et al. (2021*c*).

CHAPTER 8:

New Diplostomid Genus from Crocodilians A New Genus of Diplostomids (Digenea: Diplostomoidea) From the Nile Crocodile In South Africa With A Key To Diplostomid Genera

8.1 Introduction

Crocodilians are an ancient group of vertebrates known to have a highly distinctive digenean fauna (Brochu, 2003; Oaks, 2011; Tellez, 2013). Members of the superfamily Diplostomoidea Poirier, 1886 are among the most commonly reported and most diverse digeneans in crocodilians. The overwhelming majority of diplostomoideans from crocodilians

belong to the Proterodiplostomidae Dubois, 1936, which are characterized by the presence of a paraprostate organ. A few members of the Diplostomidae Poirier, 1886, Cyathocotylidae Mühling, 1896 and Strigeidae Railliet, 1919 are also known from this ancient group of tetrapods (Tellez, 2013; Tkach et al., 2020; Achatz et al., 2021a).

Bisseru (1957) erected the genus *Neoparadiplostomum* Bisseru, 1957 for *Neoparadiplostomum magnitesticulatum* Bisseru, 1957 and *Neoparadiplostomum kafuensis* Bisseru, 1957, partly due to their parasitism in the Nile crocodile *Crocodylus niloticus* Laurenti from Zimbabwe. He placed the new genus in the Proterodiplostomidae due to the presence of sporadic prostate gland cells which he mistook for a paraprostate. Dubois (1968) considered parasitism of the above two diplostomoideans in crocodiles a result of accidental infections and refuted the presence of the paraprostate; consequently, Dubois (1968) transferred both species to the genus *Neodiplostomum* Railliet, 1919 within the Diplostomidae and considered *Neodiplostomum kafuensis* (Bisseru, 1957) to be a junior synonym of *Neodiplostomum butasturinum* (Tubangui, 1932). Later, Dubois (1981) transferred *Neodiplostomum magnitesticulatum* (Bisseru, 1957) into *Fibricola* Dubois, 1932 as *Fibricola magnitesticulatum* (Bisseru, 1957), and subsequently changed the name to *Fibricola magnitesticulatus* (Bisseru, 1957) (Dubois, 1982). Recently, DNA sequence data of the type species of *Fibricola* and *Neodiplostomum* as well as a review of the morphological data led Achatz et al. (2022d) to consider *Fibricola* as a junior synonym of *Neodiplostomum*. Achatz et al. (2022d) transferred nominal *Fibricola* spp., including *Fibricola magnitesticulatus*, into *Neodiplostomum*. Thus, the only mature diplostomids currently known from crocodilians belong to *Neodiplostomum*.

Herein, we describe a new diplostomid genus and species based on adult specimens from a Nile crocodile and metacercariae from Müller's clawed frog *Xenopus muelleri* in South Africa.

In addition, metacercariae of congeneric species are described from Mozambique tilapia *Oreochromis mossambicus* collected in South Africa. Partial sequences of the nuclear large ribosomal subunit (28S) gene were used to study the phylogenetic position of the new diplostomids within the Diplostomoidea. In view of the significant recent changes in the taxonomy of the Diplostomidae at the genus level, we provide a new key to diplostomid genera.

8.2 Materials and Methods

Adult diplostomids were obtained from the intestine of a Nile crocodile from the Crocodile River, Mpumalanga Province, South Africa (25°27'S, 31°58'E) in 2010 (Table I). Metacercariae were obtained and counted from the body cavity of *O. mossambicus*, collected from Lake Nyamithi of the Phongolo River System, and from the lung of *X. muelleri*, collected from the inflow of Lake Nyamithi in the Ndumo Game Reserve, South Africa. Adult diplostomids were removed, fixed and stained according to our standard methods. Metacercariae were directly fixed in 96% molecular grade ethanol or heat-killed in hot saline and fixed in 80% ethanol. Metacercariae were then stained in Mayer's hematoxylin, dehydrated in ethanol, cleared in methyl salicylate and mounted in Damar gum. Type series and morphological vouchers of adults are deposited in the collection of the H. W. Manter Laboratory (HWML), University of Nebraska, Lincoln, Nebraska; vouchers of metacercariae are deposited in the Helminthological Collection of the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences (IPCAS), České Budějovice, Czech Republic.

Photomicrographs of ethanol-preserved metacercariae used for molecular study were taken with a digital camera attached to a Nikon Eclipse Ni compound microscope (Nikon Instruments, Tokyo, Japan). Genomic DNA of adult specimens was extracted using our standard

methods, while DNA from metacercariae were extracted using a KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa).

Amplification and seqsuencing of adult specimens followed our standard methods. The PCR amplifications of 28S were performed using the forward primer digL2 and reverse primer 1500R (Tkach et al., 2003). The *ITS* regions were amplified using forward primers ITSf and D1 and reverse primers 300R and D2 (Littlewood and Olson, 2001; Galazzo et al., 2002; Snyder and Tkach, 2007). The fragment of the *COI* gene was amplified using forward primer Dice1F and reverse primer Dice14R (Van Steenkiste et al., 2015).

The PCR amplicons of metacercariae were purified and sequenced at Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa. The PCR primers were used for sequencing reactions. In addition, internal forward primers DPL250F, DPL600F and 300F and internal reverse primers DPL350R, DPL700R, DPL1300R, DPL1450R and ECD2 (Table 1) were used for sequencing 28S amplicons (Littlewood et al., 2000; Achatz et al., 2019d, 2022c). The newly generated sequences are deposited in GenBank (Table 16).

The 28S sequences were sequenced, aligned and trimmed according to our standard methods. The alignment of new sequences along with 46 previously published sequences of diplostomoideans was 1,119 bp; 32 nucleotide positions with ambiguous homology were excluded from the analyses. *Suchocyathocotyle crocodili* (Yamaguti, 1954) was used as outgroup in the analysis, based on the tree topology published by Achatz et al. (2019d). This analysis included representatives of all diplostomid and strigeid genera with available 28S sequences that are at least 1,100 bp long, to be compatible with the remainder of the dataset. Only two representatives of the Proterodiplostomidae (*Archaeodiplostomum overstreeti* Tkach, Achatz and Pulis, 2020 and *Neocrocodilicola georgiana* (Byrd and Reiber, 1942)) were included in the

analysis considering that their monophyly has been well-documented (Tkach et al., 2020). BI analysis was performed according to our standard methods.

Table 16. Hosts, GenBank accession numbers and museum accession numbers assigned by the Harold W. Manter Laboratory (HWML) and Helminthological Collection of the Institute of Parasitology of the Czech Academy of Sciences, České Budějovice, Czech Republic (IPCAS) for members of *Neofibricola* n. gen. from South Africa that are described in this study.

***Neofibricola* spp.**	**Life stage**	**Host species**	**Museum No.**	**Accession numbers**		
				28S	***ITS* regions**	***COI***
Neofibricola smiti n. sp.	A	*Crocodylus niloticus*	HWML 216803, 216804	ON482326−ON482328	ON482326	−
Neofibricola smiti n. sp.	M	*Xenopus muelleri*	IPCAS D-855	ON482329	ON482331, ON482332	ON455355−ON455357
Neofibricola sp.	M	*Oreochromis mossambicus*	IPCAS D-856	ON482330	ON482333	ON455358, ON455359

A–The specimen was an adult
M–The specimen was a metacercaria

8.3 Results

Molecular phylogeny

The phylogeny resulting from the analysis of 28S (Fig. 24) demonstrated non-monophyly of the Diplostomidae and Strigeidae. The members of the new genus (*Neofibricola* n. gen.) were positioned in a weakly supported clade with 3 other diplostomid genera. Within this clade, the *Sphincterodiplostomum* spp. were positioned together (99% supported) as a sister group to a 93%

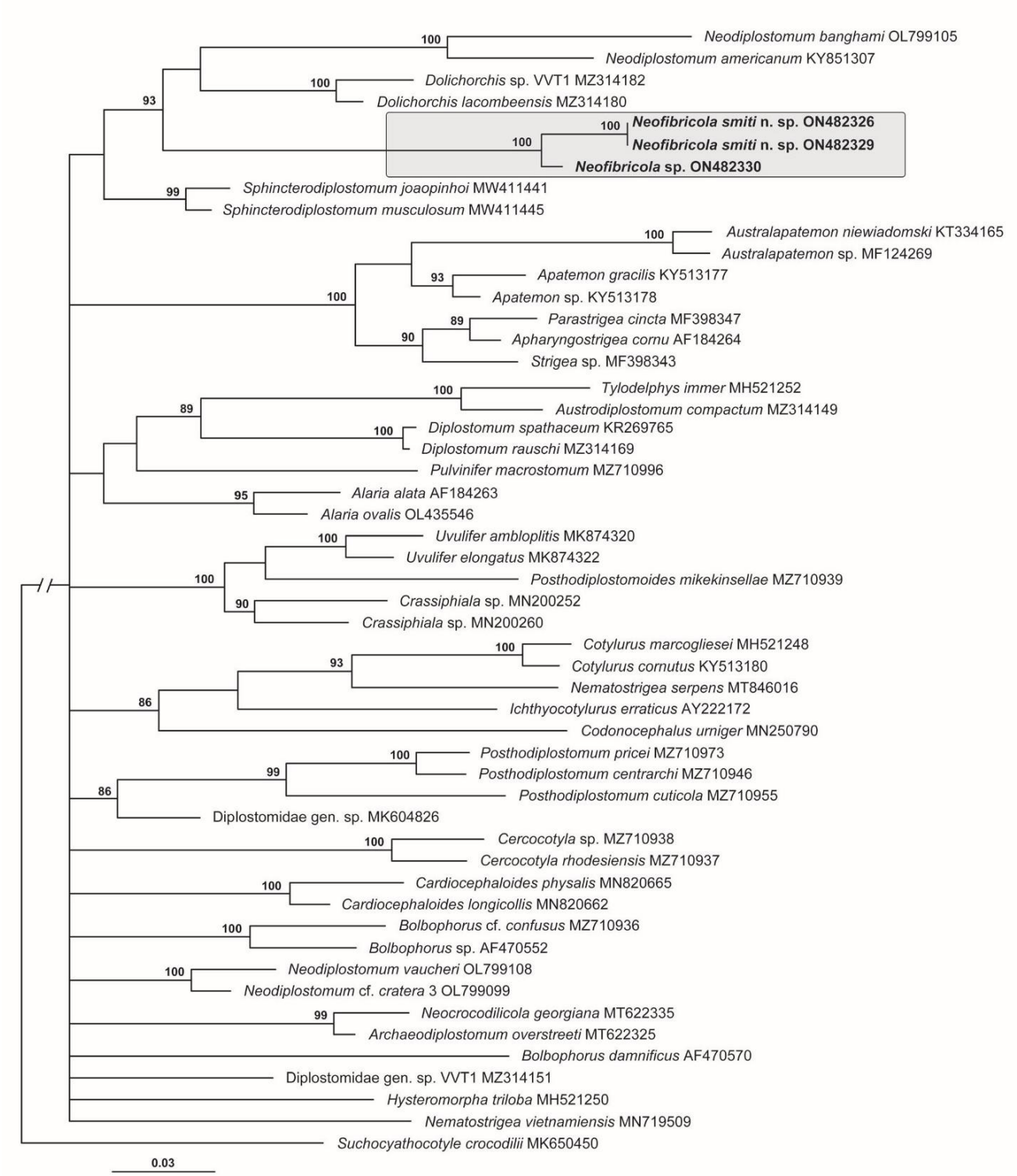


Figure 24. Phylogenetic interrelationships among 51 diplostomoideans based on Bayesian Inference analysis of partial 28S rDNA gene sequences. Bayesian inference posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale-bar indicates the number of substitutions per site. The members of the new genus are within the shaded box. GenBank accession numbers are provided after names of taxa.

supported subclade including the remaining diplostomids. The new species of *Neofibricola* (100% supported clade) were positioned separately from a weakly supported clade of *Neodiplostomum* spp. (100% supported) + *Dolichorchis* spp. (100% supported).

Table 17. Morphometric characters of *Neofibricola* spp. Ranges provided followed by mean in parentheses.

Species	*N. smiti* n. sp.		*Neofibricola* sp.
Host	*Crocodylus niloticus*	*Xenopus muelleri*	*Oreochromis mossambicus*
Life stage	Adult	Metacercaria	Metacercaria
Number of specimens (*n*)	8	6	8
Body length	1,828–2,321 (2,065)	884–1,131 (984)	1,942–2,693 (2,429)
Prosoma length	1,025–1,425 (1,224)	800–1,035 (903)	1,769–2,422 (2,213)
Prosoma width	314–471 (374)	316–361 (335)	338–637 (464)
Opisthosoma length	727–962 (836)	118–147 (139)	288−377 (339)
Opisthosoma width	220–309 (262)	118–122 (120)	166−226 (182)
Prosoma:opisthosoma length ratio	1:1.1–1.6 (1:1.5)	1:5.4–7.6 (1:6.5)	1:4.9–7.9 (1:6.6)
Oral sucker length	40–53 (47)	41–60 (51)	58–72 (66)
Oral sucker width	37–54 (43)	36–49 (46)	39–54 (46)
Ventral sucker length	84–117 (105)	67−74 (70)	130−149 (137)
Ventral sucker width	115–136 (123)	71–103 (90)	117−171 (141)
Oral sucker:ventral sucker width ratio	1:0.3–0.4 (1:0.4)	1:1.4–2.6 (1:2.1)	1:2.8–3.7 (1:3.1)
Holdfast organ length	256–363 (314)	131–175 (153)	338–392 (367)
Holdfast organ width	98–155 (115)	69–93 (84)	130–184 (155)
Pharynx length	35–42 (39)	45–57 (49)	43–48 (46)
Pharynx width	24–32 (28)	34–47 (39)	33–39 (35)
Esophagus length	61–94 (74)	44–70 (63)	137–229 (161)
Anterior testis length	145–196 (173)	18–36 (27)	48–70 (62)
Anterior testis width	173–222 (193)	25–35 (29)	41–65 (53)
Posterior testis length	171–239 (216)	21–32 (27)	52–70 (59)
Posterior testis width	162–237 (191)	30–35 (32)	63–80 (67)
Ovary length	52–72 (65)	14–18 (17)	26–39 (32)
Ovary width	73–111 (85)	16–18 (17)	25–45 (33)
Egg length	96–128 (108)	–	–

Descriptions

Neofibricola n. gen.

Diagnosis

DIPLOSTOMIDAE. Body distinctly bipartite; prosoma flattened, elongate, similar in length to, or longer than, cylindrical opisthosoma. Oral and ventral suckers present. Holdfast organ elliptical, with median slit. Pseudosuckers absent. Pharynx present; ceca reach near posterior margin of posterior testis or somewhat more posteriorly. Testes 2, tandem. Seminal vesicle compact, winding. Ejaculatory pouch present. Ejaculatory duct joins distal part of metraterm to form a short hermaphroditic duct. Hermaphroditic duct opens into muscular genital atrium. Genital atrium with muscular sphincter, with subterminal opening on dorsal side. Ovary pretesticular; oötype intertesticular. Vitellarium mainly distributed in prosoma, some follicles may extend into opisthosoma; vitelline reservoir intertesticular. Excretory pore terminal. In crocodilians. Afrotropics.

Type species: Neofibricola smiti n. sp.

Other species: Neofibricola sp.

Etymology: The name of the new genus refers to the morphological similarity to the former genus *Fibricola*.

Remarks

Neofibricola n. gen. belongs to the Diplostomidae based on the presence of a sucker-like holdfast organ (Figs. 2A, 2D, 2E, 3A, 3G), flattened prosoma and absence of a paraprostate. The new genus can be distinguished from the majority of other diplostomid genera by the presence of an ejaculatory pouch (Figs. 2C&D, 3B&C). This structure, or a similar structure, is also found in some members of *Alaria* Schrank, 1788, *Pseudodiplostomum* Yamaguti, 1934, *Tylodelphys*

Diesing, 1850, *Uvulifer* Yamaguti, 1934 and *Scolopacitrema* Sudarikov and Rykovsky, 1958, however, the muscular nature of the genital atrium and the presence of a genital atrial sphincter (Figs. 2A&B, 3D&E) distinguishes the new genus from the aforementioned 5 diplostomid genera.

Neofibricola smiti n. sp.

Description

Adult (Figs. 2A–C, 3A–E)

Based on 8 adult specimens; measurements of holotype in text; measurements of entire series given in Table 17. Body 1,925 long, consists of distinct prosoma and opisthosoma; prosoma elongate, 1,025 long, widest at level of holdfast organ, 350; opisthosoma elongated, almost cylindrical, 900 × 275. Prosoma:opisthosoma length ratio 1:1.1. Forebody 26% of body length. Oral sucker subterminal, 43 × 41. Pseudosuckers absent. Ventral sucker larger than oral sucker, 100 × 115, located near mid-length of prosoma; oral:ventral sucker width ratio 1:0.4. Holdfast organ posterior to ventral sucker, oval, 256 × 108. Proteolytic gland not well-observed. Prepharynx absent. Pharynx subspherical, 36 × 26. Esophagus 64 long. Cecal bifurcation in anterior-most 15% of prosoma length. Ceca slender, extend to near level of seminal vesicle.

Testes 2, tandem, entire, anterior testis 180 × 222, posterior testis 229 × 220. Seminal vesicle post-testicular, winding. Thick-walled, spherical, muscular, glandular ejaculatory pouch present. Ejaculatory duct joins distal part of metraterm to form short hermaphroditic duct. Hermaphroditic duct opens into muscular genital atrium. Genital atrium with muscular sphincter, with subterminal opening on dorsal side.

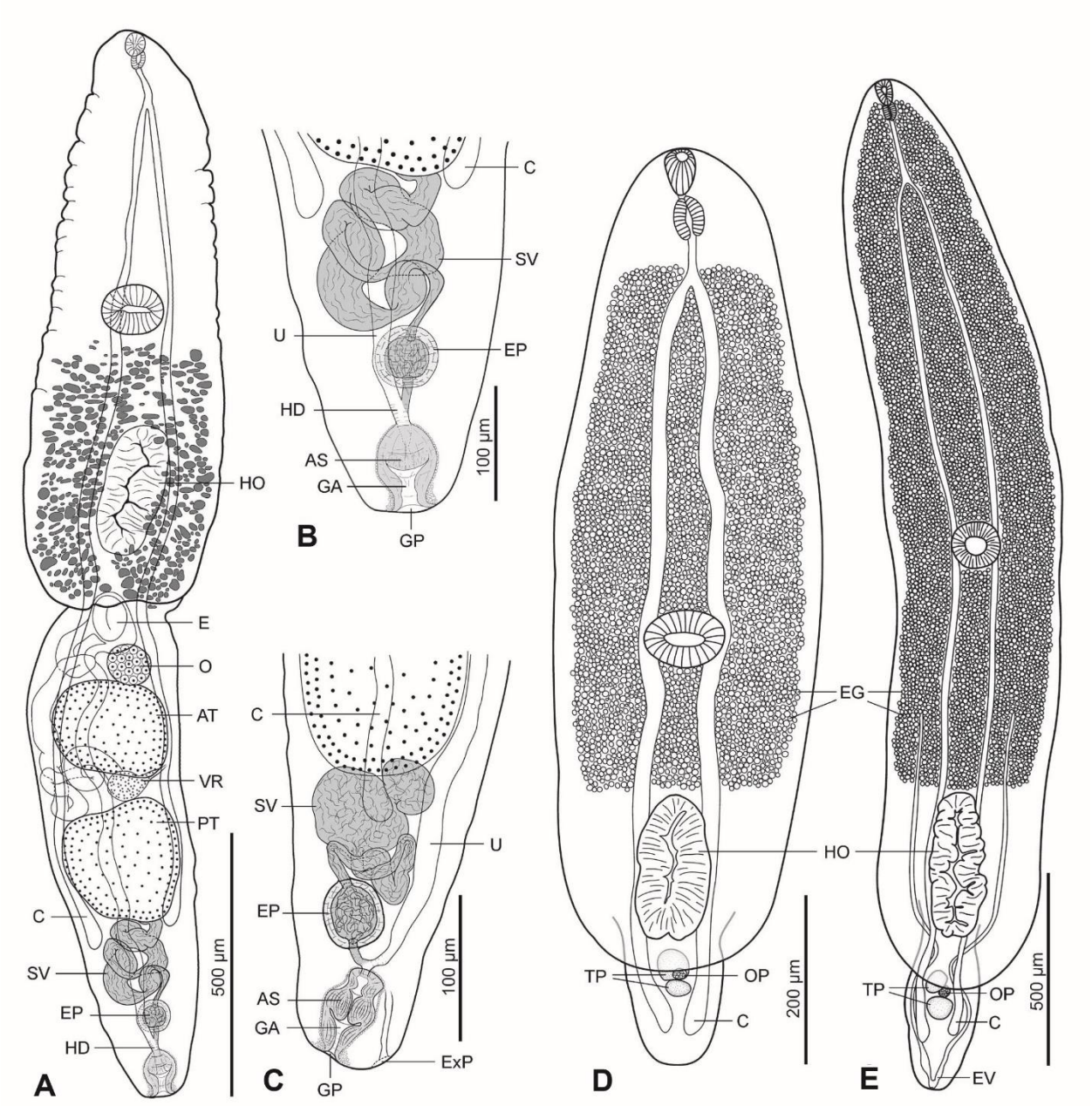
Ovary pretesticular, subspherical, 72 × 81. Oötype, Mehlis' gland and uterine seminal receptacle intertesticular. Vitelline follicles extend anteriorly to near level of ventral sucker and

posteriorly to posterior margin of prosoma or, in some cases, to level of anterior margin of anterior testis. Vitelline reservoir intertesticular. Uterus ventral to gonads, extends anteriorly to near prosoma-opisthosoma junction before turning and extending posteriorly. Uterus contains 1 egg in holotype, 101 × 65, up to 10 in paratypes. Excretory vesicle not well-observed; excretory pore terminal.

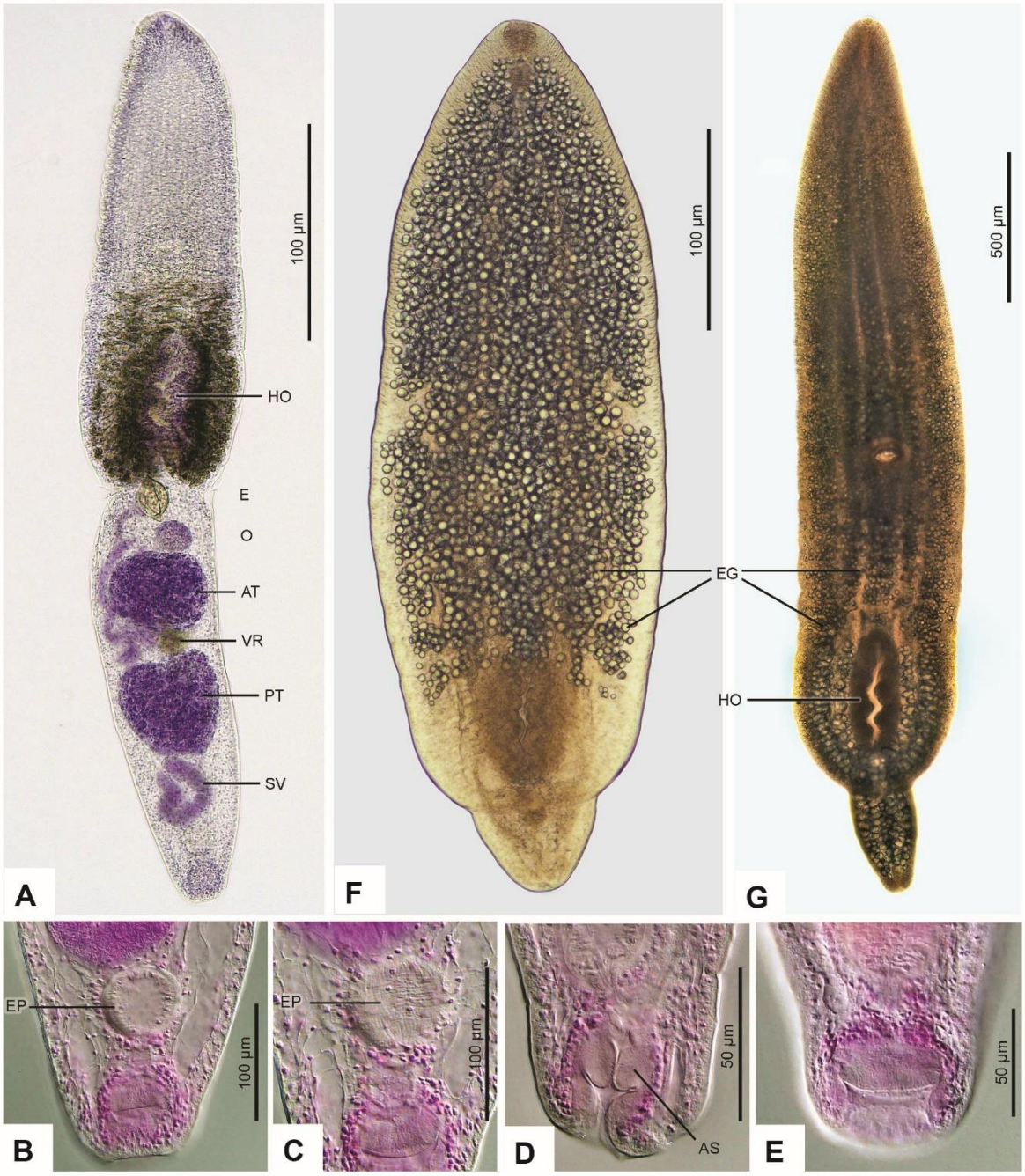
Description

Metacercaria (Figs. 2D, 3F)

Based on 6 excysted metacercariae; range provided, followed by mean in parentheses. Encysted in thin-walled cysts. Body 884−1,131 (984) long, consists of distinct prosoma and opisthosoma. Prosoma elongate-oval, dorsoventrally flattened, 800−1,035 × 316−361 (903 × 335), with maximum width anterior to ventral sucker. Opisthosoma elongate, cylindrical, 118−147 × 118−122 (139 × 120). Prosoma:opisthosoma length ratio 1:5.4−7.6 (1:6.5). Pseudosuckers absent. Oral sucker subterminal, elongate-oval, 41−60 × 36−49 (51 × 46). Ventral sucker transversely oval, 67−74 × 71−103 (70 × 90), larger than oral sucker; oral:ventral sucker width ratio 1:0.4−0.7 (1:0.5). Distance from ventral sucker to anterior end of prosoma, 446−600 (515), and to posterior end of prosoma, 255−383 (317). Holdfast organ large, elongate oval, 131−175 × 69−93 (153 × 84), in posterior part of prosoma. Holdfast organ length equal to 14−20% (17%) of prosoma length. Distance from holdfast organ to ventral sucker 87−184 (132). Prepharynx absent or short, 13 (*n* = 1); pharynx muscular, well developed, elongate-oval, 45−57 × 34−47 (49 × 39), similar in size to oral sucker; pharynx to oral sucker length ratio 1:0.84−1.2 (1:1.1); esophagus short, narrow, 44−70 (63); ceca long, slender, extend to near posterior end of opisthosoma.



Figures 25. *Neofibricola* spp. (**A**) Adult *Neofibricola smiti* n. sp., holotype, ventral view. (**B**) Adult *Neofibricola smiti* n. sp., holotype, ventral view of posterior portion of opisthosoma. (**C**) Adult *Neofibricola smiti* n. sp., paratype, lateral view of posteror portion of opisthosoma. (**D**) Excysted metacercaria of *Neofibricola smiti* n. sp. (**E**) Metacercaria of *Neofibricola* sp. Abbreviations: AS, atrial sphincter; AT, anterior testis; C, ceca; E, egg; EG, excretory granules; EP, ejaculatory pouch; EV, excretory vesicle; ExP, excretory pore; GA, genital atrium; GP, genital pore; HD, hermaphroditic duct; HO, holdfast organ; O, ovary; OP, ovary primordium; PT, posterior testis; SV, seminal vesicle; TP, testes primordium; U, uterus; VR, vitelline



Figures 26 *Neofibricola* spp. (**A**) Holotype of *Neofibricola smiti* n. sp.. (**B**) Holotype of *Neofibricola smiti* n. sp., posterior end showing spherical expansion of the distal part of the seminal vesicle. (**C**) Holotype of *Neofibricola smiti* n. sp., posterior end showing longitudinal and circular musculature of the expansion of the the distal part of the seminal vesicle. (**D**) Paratype of *Neofibricola smiti* n. sp., lateral view of the posterior end showing sphincter in the genital atrium. (**E**) Paratype of *Neofibricola smiti* n. sp., ventral view of the posterior end with the focal plane positioned inside the genital atrium. (**F**) Photohologenophore of *Neofibricola smiti* n. sp. metacercariae, ventral view. (**G**) Photohologenophore of *Neofibricola* sp. metacercariae, ventral view. Abbreviations: AS, atrial sphincter; AT, anterior testis; E, egg; EG, excretory granules; EP, ejaculatory pouch; HO, holdfast organ; O, ovary; PT, posterior testis; SV, seminal vesicle; VR, vitelline reservoir

Genital primordia located in anterior half of opisthosoma. Testes primordia tandem, entire, contiguous, median; anterior testis subspherical, 18−36 × 25−35 (27−29) (*n* = 3), posterior testis transversely oval, 21−32 × 30−35 (27 × 32) (*n* = 3). Ovary primordium subspherical, entire, smaller than testes, 14−18 × 16−18 (17 × 17), between testes or overlaps anterior testis ventrally. Excretory vesicle not observed. Excretory granules medium-sized, numerous, occupy most of prosoma anterior to holdfast organ (Figs. 2D, 3F). Excretory pore terminal.

Taxonomic summary

Type host: Nile crocodile *Crocodylus niloticus* Laurenti (Reptilia: Crocodylidae).

Second intermediate host: Müller's clawed frog *Xenopus muelleri* (Peters) (Amphibia: Pipidae).

Site of infection in definitive host: Small intestine.

Site of infection in second intermediate host: Lungs.

Type locality: Crocodile River, Mpumalanga Province, South Africa (25°27'S, 31°58'E).

Other locality: Inflow of Lake Nyamithi of the Phongolo River System, South Africa (26°53'59"S, 32°15'46"E).

Type specimens deposited: The type series consists of 8 adult specimens deposited in the HWML. Holotype: HWML 216803, labeled ex. *Cr. niloticus*, small intestine, Crocodile River, Mpumalanga Province, South Africa (25°27'S, 31°58'E), 12 July 2010, coll. K. Junker. Paratypes*:* HWML 216804 (lot of 7 slides), labels identical to the holotype.

Vouchers of metacercariae deposited: IPCAS-D-855.

Prevalence in second intermediate host: 46.2% (6 out of 13 *X. muelleri* infected).

Intensity of infection in second intermediate host: 15–36 metacercariae per frog.

Representative DNA sequences: ITS region: ON482326, ON482331, ON482332, 28S: ON482326−ON482329, *COI*: ON455355−ON455357.

Zoobank registration: urn:lsid:zoobank.org:act:64A0A182-3D8F-4EC3-8A54-826729CAAF3F

Etymology: The species epithet is given in honor of Prof. Nico Smit in recognition of his numerous contributions to parasitology and particularly the knowledge of parasites of aquatic animals in South Africa.

Neofibricola sp.

Description

Metacercaria (Figs. 2E, 3G)

Based on 8 metacercariae; range provided, followed by mean in parentheses. Not encysted. Body 1,942−2,693 (2,429) long, consists of distinct prosoma and opisthosoma. Prosoma elongate, narrow, dorsoventrally flattened, 1,769−2,422 × 338−637 (2,213 × 464), with maximum width just anterior to ventral sucker. Opisthosoma elongate, cylindrical, 288−377 × 166−226 (339 × 182). Prosoma:opisthosoma length ratio 1:4.9−7.9 (1:6.6). Pseudosuckers absent. Oral sucker subterminal, elongate-oval, 58−72 × 39−54 (66 × 46). Ventral sucker subspherical or transversely oval, 130−149 × 117−171 (137 × 141), distinctly larger than oral sucker; oral:ventral sucker width ratio 1:0.3−0.4 (1:0.3). Distance from ventral sucker to anterior end of prosoma, 896−1,247 (1,116), and to posterior end of prosoma, 742−1,086 (963). Holdfast organ large, elongate oval, 338−392 × 130−184 (367 × 155), in posterior part of prosoma. Holdfast organ length equal to 15−21% (17%) of prosoma length. Distance from holdfast organ to ventral sucker, 266−577 (468). Prepharynx absent or short, 4−12 (5); pharynx muscular, well developed, elongate-oval, 43−48 × 33−39 (46 × 35); esophagus short, narrow, 137−229 (161); ceca long, slender, extend to near posterior end of opisthosoma.

Genital primordia located in first half of opisthosoma. Testes primordia entire, tandem, separated by primordium of ovary, median; anterior testis elongate-oval, 48−70 × 41−65 (62 × 53), posterior testis transversely oval, 52−70 × 63−80 (59 × 67). Ovary primordium subspherical, entire, smaller than testes, 26−39 × 25−45 (32 × 33), between testes or overlaps anterior testis ventrally. Excretory vesicle V-shaped. Excretory granules medium-sized, numerous, occupy most of prosoma between posterior margin of oral sucker and anterior margin of holdfast organ (Figs. 2E, 3G). Excretory pore terminal.

Taxonomic Summary

Second intermediate host: Mozambique tilapia *Oreochromis mossambicus* (Peters) (Actinopterygii: Cichlidae).

Site of infection in second intermediate host: Body cavity.

Locality: Lake Nyamithi of the Phongolo River System (26°53'35"S, 32°17'35"E).

Specimens deposited: IPCAS-D-856.

Prevalence in second intermediate host: 37.5% (3 out of 8 *O. mossambicus* infected).

Intensity of infection in second intermediate host: 2–51 metacercariae per fish.

Representative DNA sequences: ITS region: ON482333, 28S: ON482330, *COI*: ON455358, ON455359.

Remarks

Adult specimens have only been collected for one of the two members of *Neofibricola* (Figs. 2A, 3A). In our opinion, larval morphology should not be relied upon for species-level differentiations. The sequences of the 2 species differ by 0.9% (12 out of 1,288 nucleotides) in 28S, 4.9% (61 out of 1,240 nucleotides) in the *ITS* region and 14.6−15.4% (72−76 out of 492

nucleotides) in *COI*. Once the adult stage of *Neofibricola* sp. is found, adult morphology will likely provide morphological characters suitable for species differentiation.

8.4 Discussion

Remarks on the Diplostomidae

Since Niewiadomska (2002) provided the previous key to diplostomid genera, the Diplostomidae has undergone substantial systematic changes. Recently, Achatz et al. (2021b) abandoned the use of diplostomid subfamilies, based on strong molecular phylogenetic evidence. As a result of morphological studies and molecular phylogenies, *Ornithodiplostomum* Dubois, 1936 and *Mesoophorodiplostomum* Dubois, 1936 were synonymized with *Posthodiplostomum* Dubois, 1936, *Didelphodiplostomum* Dubois, 1944 was synonymized with *Tylodelphys* Diesing, 1850, and *Pharyngostomoides* Harkema, 1942 was synonymized with *Alaria* Schrank, 1788 (Achatz et al. 2021b; Achatz et al., 2022b). In addition, *Conodiplostomum* Dubois, 1937 and *Fibricola* were synonymized with *Neodiplostomum* (Heneberg et al., 2020; Achatz et al., 2022d). *Parallelorchis* Harkema and Miller, 1961 was restored based on morphological data (Achatz et al., 2022b).

Molecular phylogenetic analyses have demonstrated the non-monophyly of *Bolbophorus* and *Tylodelphys* (e.g., Locke et al., 2015; Achatz et al., 2021b, 2022c), although adult morphology did not provide sufficient evidence to separate these genera. Knowledge of the morphology of other life cycle stages and life history characteristics remains limited for many members of non-monophyletic diplostomid genera.

New key to diplostomid genera

Niewiadomska (2002) provided keys to the 4 subfamilies of the Diplostomidae existing at the time and used definitive host groups (mammals vs. birds) were used as the first

distinguishing characteristic in the keys to subfamilies. Notably, this step separated the previously accepted Alariinae Hall and Wigdor, 1918 parasitic in mammals from the 3 other diplostomid subfamilies parasitic in birds, including the Diplostominae Poirier, 1886. As has been demonstrated previously (Dubois, 1983, Achatz et al., 2022b, 2022c, 2022d), some members of at least 3 genera belonging to the previously accepted Diplostominae (*Diplostomum* von Nordmann, 1832, *Tylodelphys* and *Neodiplostomum*) parasitize both birds and mammals.

The abandonment of diplostomid subfamilies by Achatz et al. (2021b) as well as the recent descriptions of new genera, synonymizations and restorations created several problems for the use of the key to diplostomid genera by Niewiadomska (2002). Therefore, we provide a new key to diplostomid genera based primarily on adult morphology.

Key to the genera of the Diplostomidae Poirier, 1886

1a. Holdfast organ not sucker-like **2**

1b. Holdfast organ sucker-like **5**

2a. Pseudosuckers present. Holdfast organ distinct **3**

2b. Pseudosuckers absent. Holdfast organ indistinct ..... *Codonocephalus* Diesing, 1850

3a. Holdfast organ with 3 massive lobes (2 lateral and 1 posteromedian) ..... *Allodiplostomum* Yamaguti, 1935

3b. Holdfast organ with only 2 lateral projections **4**

4a. Muscular vaginal sphincter present ..... *Pseudoscolopacitrema* Palmieri, Krishnasamy and Sullivan, 1979

4b. Muscular vaginal sphincter absent ..... *Parallelorchis* Harkema and Miller, 1961

5a. Pseudosuckers present **6**

5b. Pseudosuckers absent **26**

6a. Dorsal tubular invagination at level of posterior testis equipped with muscular sphincter present ..... *Sphincterodiplostomum* Dubois, 1936

6b. Dorsal tubular invagination of body wall at level of posterior testis equipped with muscular sphincter absent **7**

7a. Genital cone with semicircular ventral pad projecting from dorsal region of anterior wall of genital atrium. Large unicellular gland-cells with ducts opening on ventral surface around ventral sucker present ..... *Adenodiplostomum* Dubois, 1937

7b. Genital cone without semicircular ventral pad. Large unicellular gland-cells with ducts opening around ventral sucker absent **8**

8a. Genital cone with a preputial or prepuce-like fold **9**

8b. Genital cone without a preputial or prepuce-like fold or genital cone absent **11**

9a. Prosoma elongated, generally flattened, without deep concavity ..... *Posthodiplostomoides* Williams, 1969

9b. Prosoma oval or cochleariform, concave (bowl-like) **10**

10a. Body strongly retroflexed. Prosoma similar in length to opisthosoma ..... *Neoharvardia* Gupta, 1963

10b. Body not strongly retroflexed. Prosoma much shorter than opisthosoma ..... *Subuvulifer* Dubois, 1952 (Syns *Choanochenia* Yang, 1959; *Cotylostoma* Yang, 1965; *Neochoanochenia* Yang, 1965)

11a. Prosoma pouch-shaped. Holdfast organ is located inside pouch formed by prosoma .... *Procyotrema* Harkema and Miller, 1959

11b. Prosoma is not pouch-shaped **12**

12a. Muscular bulb in genital atrium present ..... *Bolbophorus* Dubois, 1935

12b. Muscular bulb in genital atrium absent **13**

13a. Vitellarium only distributed within opisthosoma ..... *Pulvinifer* Yamaguti, 1933 (Syn. *Laterostrigea* Yang, 1962)

13b. Vitellarium in prosoma and opisthosoma or only prosoma **14**

14a. Body strongly retroflexed ..... *Harvardia* Baer, 1932

14b. Body not strongly retroflexed **15**

15a. Ventral sucker present **16**

15b. Ventral sucker absent **24**

16a. Genital atrium with internal muscular sphincter ..... *Cynodiplostomum* Dubois, 1936

16b. Genital atrium without internal muscular sphincter **17**

17a. Vitellarium mainly in prosoma, some follicles may extend into opisthosoma as far as level of ovary **18**

17b. Vitellarium well-distributed in both parts of the body, typically reaching to near posterior end of opisthosoma **19**

18a. Pseudosuckers auricular (testes tandem) or invaginated (testes opposite). Ejaculatory pouch present or absent ..... *Alaria* Schrank, 1788 (Syns *Conchosomum* Railliet, 1896; *Pharyngostomoides* Harkema, 1942)

18b. Pseudosuckers invaginated (testes tandem). Ejaculatory pouch absent ..... *Paralaria* Krause, 1914 (Syn. *Enhydridiplostomum* Dubois, 1944)

19a. Genital cone relatively large, occupies approximately 25% of body length. Anterior testis asymmetrical ..... *Glossodiplostomoides* Bhalerao, 1942 (Syn. *Pseudoglossodiplostomum* Dubois, 1944)

19b. Genital cone smaller or absent. Anterior testis symmetrical or asymmetrical **20**

20a. Ejaculatory pouch present. Anterior testis asymmetrical. Body distinctly bipartite .... *Tylodelphys* Diesing, 1850, part. (Syns *Didelphodiplostomum* Dubois, 1944; *Glossodiplostomum* Dubois, 1932; *Prodiplostomum* Ciurea, 1933)

20b. Ejaculatory pouch absent. Anterior testis symmetrical or asymmetrical. Body distinctly or indistinctly bipartite **21**

21a. Genital cone distinct **22**

21b. Genital cone indistinct **23**

22a. Body distinctly bipartite. Anterior testis asymmetrical ..... *Dolichorchis* Dubois, 1961

22b. Body typically indistinctly bipartite. Anterior testis symmetrical .... *Tylodelphys* Diesing, 1850, part. (Syns *Didelphodiplostomum* Dubois, 1944; *Glossodiplostomum* Dubois, 1932; *Prodiplostomum* Ciurea, 1933)

23a. Body usually distinctly bipartite. Anterior portion of prosoma not trilobate or weakly trilobate ..... *Diplostomum* von Nordmann, 1832 (Syns *Hemistomum* Diesing, 1850; *Proalaria* La Rue, 1926)

23b. Body indistinctly bipartite. Anterior portion of prosoma strongly trilobate .... *Hysteromorpha* Lutz, 1931

24a. Genital atrium bell-shaped ..... *Bursatintinnabulus* Tehrany, Dronen and Wardle, 1999

24b. Genital atrium not bell-shaped **25**

25a. Vitellarium digitiform. Genital atrium sucker-like ..... *Bursacetabulus* Dronen, Tehrany and Wardle, 1999

25b. Vitellarium follicular. Genital atrium not sucker-like ..... *Austrodiplostomum* Szidat and Nani, 1951

26a. Ventral sucker absent **27**

26b. Ventral sucker present **28**

27a. Genital atrium contains large, muscular sucker-like structure. Genital cone absent .... *Cercocotyla* Yamaguti, 1939 (Syn. *Pseudocercocotyla* Yamaguti, 1971)

27b. Genital atrium without muscular sucker-like structure. Genital cone present .... *Crassiphiala* Van Haitsma, 1925

28a. Vitellarium only in opisthosoma **29**

28b. Vitellarium in both prosoma and opisthosoma or primarily in prosoma **30**

29a. Genital cone without preputial fold. Genital atrium width less than one third of opisthosoma width ..... *Pseudodiplostomum* Yamaguti, 1934

29b. Genital cone half-enclosed in preputial fold. Genital atrium width greater than one third of opisthosoma width, often occupies most of opisthosoma width at level of genital atrium .... *Uvulifer* Yamaguti, 1934 (Syn. *Prochoanochenia* Yang, 1965)

30a. Posterior part of opisthosoma consists of ventral and dorsal conical protuberances .... *Podospathalium* Dubois, 1932

30b. Posterior part of opisthosoma not divided into 2 conical protuberances **31**

31a. Genital atrium with muscular, sucker-like structure ..... *Scolopacitrema* Sudarikov and Rykovsky, 1958

31b. Genital atrium without sucker-like structure **32**

32a. Genital cone surrounded by a preputial fold ..... *Posthodiplostomum* Dubois, 1936 (Syns *Choanouvulifer* Lung, 1966; *Mesoophorodiplostomum* Dubois, 1936; *Ornithodiplostomum* Dubois, 1936; *Prolobodiplostomum* Baer, 1959)

32b. Genital cone without a preputial or prepuce-like fold or genital cone absent **33**

33a. Genital cone large, occupies about half of opisthosoma width. Body bottle-shaped .... *Bursotrema* Szidat, 1960

33b. Genital cone smaller or absent. Body not bottle-shaped **34**

34a. Ejaculatory pouch present. Genital atrium with well-developed internal muscular sphincter ..... *Neofibricola* n. gen.

34b. Ejaculatory pouch absent. Genital atrium without internal muscular sphincter **35**

35a. Prosoma cup-shaped. Holdfast organ linguiform ..... *Prudhoella* Beverley-Burton, 1960

35b. Prosoma not cup-shaped. Holdfast organ not linguiform **36**

36a. Testes opposite. Holdfast organ massive, occupying almost entire concavity of prosoma .... *Pharyngostomum* Ciurea, 1922

36b. Testes tandem. Holdfast organ not massive, does not typically occupy most of prosoma **37**

37a. Oral sucker with equatorial muscular ring ..... *Lophosicyadiplostomum* Dubois, 1936

37b. Oral sucker without equatorial muscular ring **38**

38a. Metacercaria of neascus type. Second intermediate hosts are fishes ..... *Ciureatrema* Heneberg, Sitko and Těšínský, 2020

38b. Metacercaria of diplostomulum/neodiplostomulum type. Second intermediate hosts are amphibians ..... *Neodiplostomum* Railliet, 1919

(Syns *Conchogaster* Lutz, 1928; *Conodoiplostomum* Dubois, 1937; *Fibricola* Dubois, 1932; *Neodiplostomoides* Vidyarthi, 1938; *Neoparadiplostomum* Bisseru, 1957; *Theriodiplostomum* Dubois, 1944; *Triplostomum* Lutz, 1928)

CHAPTER 9:

Conclusions

Our molecular and morphological studies of diplostomoideans produced a wealth of novel data which allowed us to make significant contributions into taxonomy, systematics, phylogenetics of diplostomid digeneans, as well as into knowledge of their life cycles, host-parasite associations and historical biogeography. We provided new ribosomal and mitochondrial DNA sequence data for a broad array of diplostomid taxa with a focus on newly collected, high quality samples. Our study, along with other recent research, have demonstrated that considerable number of diplostomoidean taxa are likely still awaiting their discovery. By describing several new species and two new genera we partly covered that gap in our knowledge. Our phylogenetic analyses have demonstrated several host-switching events and allowed to propose possible directions of diplostomiden dispersal throughout their evolutionary history. We have abandoned the use of the subfamily system within the Diplostomoidea and synonomized several taxa based on combined molecular phylogenetic and morphological evidence. We have also proposed a new key to identification of the genera in the large, cosmopolitan family Diplostomidae. Despite the achieved progress, it is evident that additional thorough morphological and molecular studies of a broader diversity of adult and larval diplostomoideans are required to properly re-evaluate the system of this evolutionarily fascinating and practically important group of digeneans.

Conclusion 1

Our study has thus significantly expanded the available sequence data from morphologically identified adult stages of *Diplostomum* and *Tylodelphys*. Our data demonstrated that *Paralaria alarioides* belongs to *Diplostomum*. Importantly, molecular phylogenetic analyses have demonstrated the non-monophyly of *Tylodelphys* and suggested the need to eventually establish a novel genus which contains *T.* cf. *americana*.

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*.

Conclusion 2

The results of our molecular phylogenetic analysis and morphological sudies convincingly demonstrated non-monophyly of two major subfamilies of the Diplostomidae, therefore we proposed abandonment of the subfamilies in the system of the Diplostomidae. We synonymized *Ornithodiplostomum* and *Mesoophorodiplostomum* with *Posthodiplostomum*. Newly generated sequence data significantly enhanced the current picture of the phylogenetic interrelationships within the Diplostomidae and expanded the reference database for future studies.

Conclusion 3

The fauna of diplostomids parasitic in New World kingfishers is likely much richer than currently known. Until 2018, only a single species of *Crassiphiala* and 5 species of *Uvulifer* were known from kingfishers in the New World. The present study and recent publications (López-Jiménez *et al.*, 2018; Achatz *et al.*, 2019a,b) have revealed 4 additional species/species-level lineages of *Crassiphiala* and 7 additional species/species-level lineages of *Uvulifer* in the New World. The diversity of these diplostomids from kingfishers in the New World is further expanded by the members of *Pseudocrassiphiala* n. gen. (2 species/species-level lineages), *Sphincterodiplostomum* Dubois, 1936 (1 species) and *Posthodiplostomum* Dubois, 1936 (1 species) (Achatz *et al.*, 2021a,b).

Conclusion 4

Our results clearly demonstrated that *Pharyngostomoides* and *Didelphodiplostomum* should be considered junior synonyms of *Alaria* and *Tylodelphys*, respectively. Our study has shown that two of the 13 diplostomid genera known to parasitize mammals as adults are not valid. However, we have also revealed one genus of primarily avian parasites (*Tylodelphys*) to include species that parasitize mammals, similar to the situation in *Diplostomum*.

Conclusion 5

Based on our morphological and molecular study we described the new diplostomid genus *Neofibricola* and new species *Neofibricola smiti* parasitizing Nile crocodiles in South Africa. The DNA sequence data strongly suggest the presence of a second member of this new genus represented by metacercariae in our collection. We hypothesize that additional, yet-to-be described diplostomids parasitize crocodilians throughout the world. This assumption is based on

the discovery of at least 3 diplostomids from crocodiles in Africa, and the fact that for millennia crocodilians shared habitats with a variety of piscivorous avian and mammalian hosts of diplostomids. These ecological overlaps may certainly lead to host switching events, especially considering that in tropical and subtropical climates the body temperatures of crocodilians may be maintained close to that of warm-blooded vertebrates.

Future directions

Despite recent advances in obtaining DNA sequence data (particularly of the 28S gene) from a variety of diplostomid taxa, future efforts should be focused on obtaining sequences from 20 nominal genera still lacking sequence data (*Adenodiplostomum, Allodiplostomum, Bursacetabulus, Bursatintinnabulus, Bursotrema, Cynodiplostomum, Glossodiplostomoides, Harvardia, Lophosicyadiplostomum*, *Neoharvardia*, *Paralaria*, *Parallelorchis, Pharyngostomum*, *Podospathalium*, *Procyotrema*, *Prudhoella*, *Pseudodiplostomum, Pseudoscolopacitrema, Scolopacitrema*, *Subuvulifer*) and as many species as possible. The latter is important to resolve the systematic problems concerning the current non-monophyletic genera and better understand the directionality of evolutionary host-switching events in this group of digeneans.

Future studies should focus on obtaining DNA sequence data from well-fixed adult specimens that allow for proper identification and morphological study. 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, b, 2015; Gordy and Hanington, 2019). Furthermore, of particular interest is species identification within the two species complexes: the

D. baeri species complex and the *D. mergi* species complex. Finally, many lineages of *Tylodelphys* with sequence data reported from larval stages, predominantly metacercariae, still await further taxonomic scrutiny.

Broader sampling from insufficiently studied hosts (e.g., crocodilians, kingfishers) and geographical regions (e.g. Afrotropics and Australasia) is critical for the improvement of our understanding of the diversity and evolution of the Diplostomidae.

CHAPTER 10

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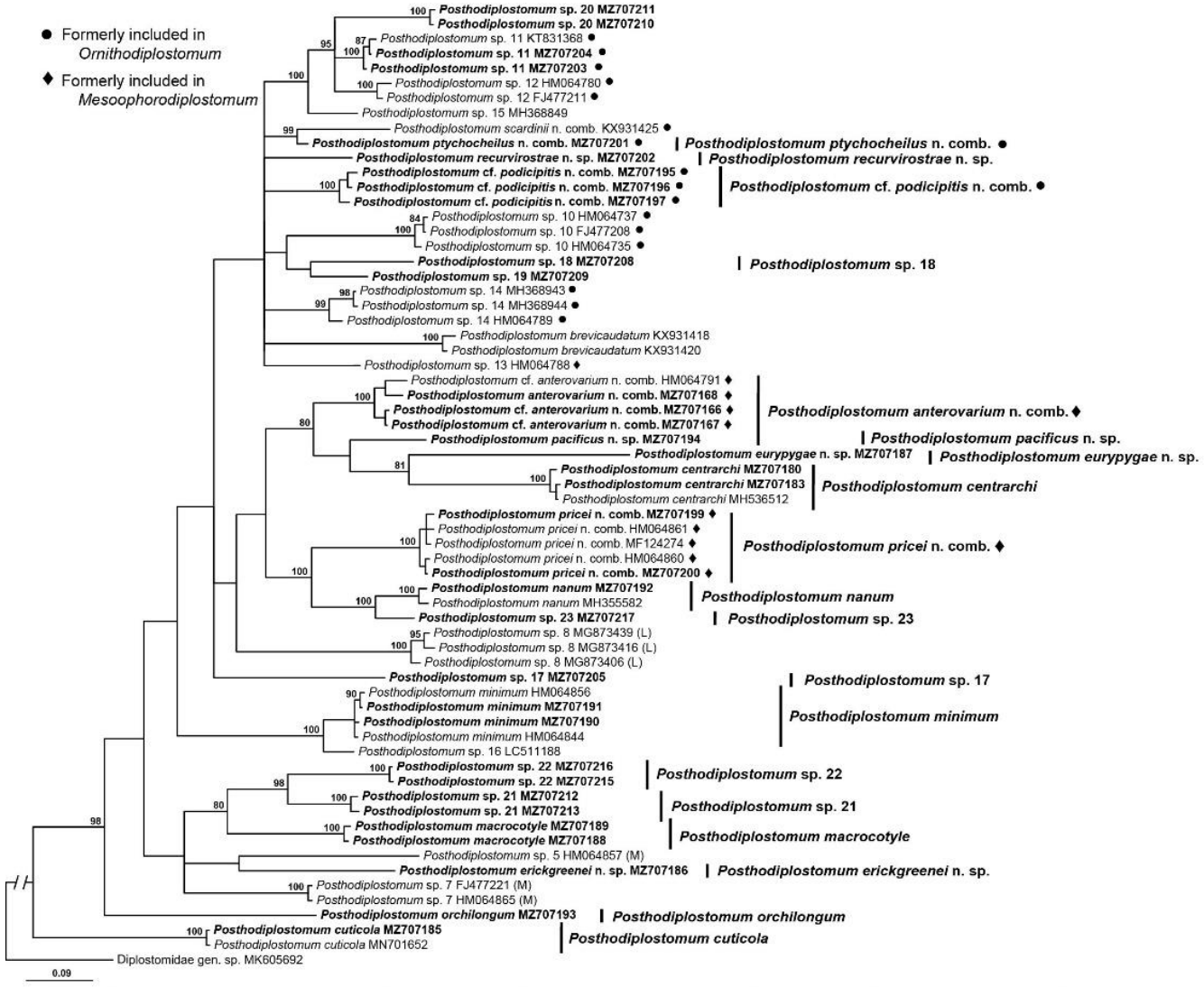
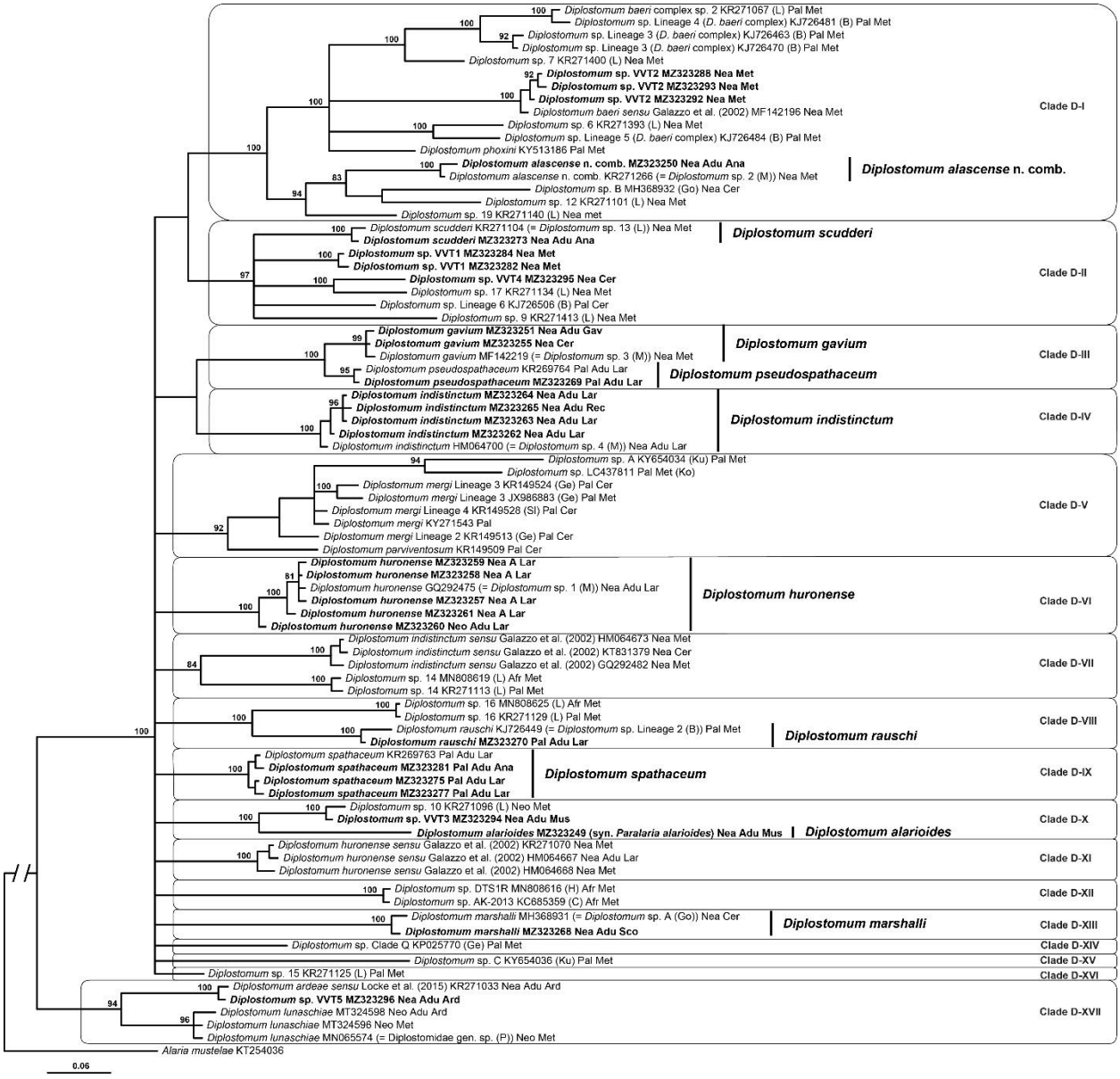


Supplementary Figure S1. Phylogenetic interrelationships among 64 sequences from members of Posthodiplostomum (syns. *Ornithodiplostomum* and *Mesoophorodiplostomum*) based on Bayesian Inference (BI) analysis of partial *cox*1 mtDNA gene sequences. Bayesian inference posterior probability values lower 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. Reference to origin of species numbering/naming systems are provided in parentheses after GenBank accession numbers. Black bars are positioned besides taxa for which we have collected adult specimens. Abbreviations for references to the original designations of species-level lineages:L, Locke et al. (2010);M, Moszczynska et al. (2009).



Supplementary Figure S2. Phylogenetic interrelationships among 80 sequences from members of *Diplostomum* (including a former *Paralaria* sp.) based on Bayesian inference analysis of partial *cox*1 mtDNA gene sequences. Abbreviations for references to the original designations of species-level lineages: B, Blasco-Costa et al. (2014); C, Chibwana et al. (2013); 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); P, Pelegrini et al. (2019); Sl, Selbach et al. (2015). Abbreviations for biogeographical realms: Afr, Afrotropical realm; ANea, 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

Supplementary Table S1. *Posthodiplostomum* 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 *cox1* sequences in GenBank. Species with less than 4.1% divergence in *cox1* sequences were considered conspecific. Host, life cycle stage, biogeographical origin and references to the original designations of species-level lineages are provided. Site of metacercarial infection in second intermediate host is provided in parentheses after host names when possible. * experimental infection

Taxon	Host	Life cycle	Biogeographical origin	Corresponding previously accepted species/species-	Host	Life cycle	Biogeographical origin	Reference
Po. cf. *antrocavarium* n. comb.	*Lepomis cyanellus* (liver)	Metacerca	Nearctic	*Posthodiplostomum* sp. 1	*Amblopites rupestris*	Metacerca	Nearctic	Locke et al. (2010)
	Lepomis gibbosus (liver)	Metacerca	Nearctic		*Lepomis gibbosus*	Metacerca	Nearctic	Moszczynska et al. (2009)
				Posthodiplostomum sp. 2	*Lepomis cyanellus* (liver)	Metacerca	Nearctic	Boone et al. (2018)
					Lepomis gibbosus	Metacerca	Nearctic	Moszczynska et al. (2009)
					Lepomis gibbosus (viscera)	Metacerca	Nearctic	Locke et al. (2010)
					Lepomis humilis (kidney, liver)	Metacerca	Nearctic	Boone et al. (2018)
					Pomoxis nigromaculatus (liver)	Metacerca	Nearctic	Boone et al. (2018)
					Larus delawarensis *	Adult	Nearctic	Locke et al. (2010)
Po. antrocavarium n. comb.	*Pelecanus erythrorhynchos*	Adult	Nearctic	No Match				
Po. centrarchi	*Amblopites rupestris*	Metacerca	Nearctic	*Posthodiplostomum centrarchi*	*Lepomis gibbosus* (abdominal cavity,	Metacerca	Palearctic	Cech et al. (2020)
	Lepomis cyanellus (liver, skin),	Metacerca	Nearctic		*Lepomis microlophus*	Metacerca	Neotropics	Locke et al. (2018)
	Lepomis macrochirus (heart, liver, mesentery, s	Metacerca	Nearctic		*Ardea herodias*	Adult	Nearctic	Locke et al. (2018)
	Anhinga anhinga	Adult	Nearctic		*Gallus gallus domesticus* *	Adult	Palearctic	Cech et al. (2020)
	Ardea alba	Adult	Nearctic	*Posthodiplostomum* sp. 3	*Amblopites rupestris* (viscera)	Metacerca	Nearctic	Locke et al. (2010)
	Ardea herodias	Adult	Nearctic		*Lepomis cyanellus* (head, heart, kidney,	Metacerca	Nearctic	Boone et al. (2018)
	Megaceryle alcyon	Adult	Nearctic		*Lepomis gibbosus*	Metacerca	Nearctic	Moszczynska et al. (2009)
					Lepomis gibbosus (viscera)	Metacerca	Nearctic	Locke et al. (2010)
					Lepomis gulosus (heart, kidney, liver)	Metacerca	Nearctic	Boone et al. (2018)
					Lepomis humilis (heart)	Metacerca	Nearctic	Boone et al. (2018)
					Lepomis macrochirus (head, heart, kidney,	Metacerca	Nearctic	Boone et al. (2018)
					Lepomis microlophus (head, heart, kidney,	Metacerca	Nearctic	Boone et al. (2018)
					Lepomis punctulatus (heart)	Metacerca	Nearctic	Boone et al. (2018)
Po. cuticola	*Nycticorax nycticorax*	Adult	Palaearctic	*Posthodiplostomum cuticola*	*Planorbis planorbis*	Cercaria	Palearctic	Stoyanov et al. (2017)
					Abramis brama (fin)	Metacerca	Palearctic	Cech et al. (2020)
					Scardinius erythrophthalmus (muscle)	Metacerca	Palearctic	Cech et al. (2020)
					Squalius cephalus (gills)	Metacerca	Palearctic	Simsek et al. (unpublished)
Po. erickgreenei n. sp.	*Pandion haliaetus*	Adult	Nearctic	No Match				
Po. eurypygae n. sp.	*Eurypyga helias*	Adult	Neotropic	No Match				
Po. macrocotyle	*Busarellus nigricollis*	Adult	Neotropic	No Match				
Po. microsicya	*Tigrisoma* sp.	Adult	Neotropic	No Match				
Po. minimum	*Ardea herodias*	Adult	Nearctic	*Posthodiplostomum* sp. 4	*Pimephales notatus*	Metacerca	Nearctic	Moszczynska et al. (2009)
	Nycticorax nycticorax	Adult	Nearctic		*Pimephales notatus* (viscera)	Metacerca	Nearctic	Locke et al. (2010)
					Ardea herodias	Adult	Nearctic	Locke et al. (2010)
				Posthodiplostomum sp. UG1	*Tribolodon hakonensis* (abdominal cavity)	Metacerca	Palearctic	Komatsu et al. (2020)
Po. nanum	*Ardea alba*	Adult	Nearctic	*Posthodiplostomum nanum*	*Gundlachia ticaga*	Cercaria	Neotropic	López-Hernández et al. (2018)
					Poecilia reticulate (body cavity)*	Metacerca	Neotropic	López-Hernández et al. (2018)
					Mus musculus *	Adult	Neotropic	López-Hernández et al. (2018)
Po. orchilongum	*Ardea alba*,	Adult	Nearctic	No Match				
	Egretta caerulea	Adult	Nearctic					
Po. pacificus	*Larus californicus*	Adult	Nearctic	No Match				
Po. cf. *podicipitis*	*Catostomus commersonii* (skin)	Metacerca	Nearctic	No Match				
	Pimephales promelas (brain)	Metacerca	Nearctic	No Match				
	Lophodytes cucullatus	Adult	Nearctic					
Po. pricei n. comb.	*Larus delawarensis*	Adult	Nearctic	*Mesocoelodiplostomum*	*Fundulus diaphanus*	Metacerca	Nearctic	Blasco-Costa & Locke (2017)
					Lepomis gibbosus	Metacerca	Nearctic	Moszczynska et al. (2009)
					Lepomis gibbosus	Metacerca	Nearctic	Locke et al. (2010)
					Morone americana	Metacerca	Nearctic	Locke et al. (2010)
					Larus argentatus	Adult	Nearctic	Locke et al. (2010)
					Larus delawarensis	Adult	Nearctic	Locke et al. (2010)
					Larus delawarensis *	Adult	Nearctic	Locke et al. (2010)
Po. ptychocheilus n. comb.	*Mergus merganser*	Adult	Nearctic	No Match				
Po. recurvirostrae n. sp.	*Recurvirostra americana*	Adult	Nearctic	Diplostomidae gen. sp. O	*Physella gyrina*	Cercaria	Nearctic	Gordy et al. (2016)
Posthodiplostomum sp. 11	*Chrosomus eos*	Metacerca	Nearctic	*Ornithodiplostomum* sp. 2	*Physella gyrina*	Cercaria	Nearctic	Gordy et al. (2016)
	Unidentified fish (eyes)	Metacerca	Nearctic		*Notemigonus crysoleucas*	Metacerca	Nearctic	Moszczynska et al. (2009)
					Notemigonus crysoleucas	Metacerca	Nearctic	Locke et al. (2010)
Posthodiplostomum sp. 17	*Lophodytes cucullatus*	Adult	Nearctic	No Match				
Posthodiplostomum sp. 18	*Physa gyrina*	Cercaria	Nearctic	No Match				
	Pelecanus erythrorhynchos	Adult	Nearctic					
Posthodiplostomum sp. 19	*Physa* sp.	Cercaria	Nearctic	No Match				
Posthodiplostomum sp. 20	*Physa gyrina*	Cercaria	Nearctic	No Match				
Posthodiplostomum sp. 21	*Jabiru jabiru*	Adult	Neotropic	No Match				
	Tigrisoma lineatum	Adult	Neotropic					
Posthodiplostomum sp. 22	*Ardea alba*	Adult	Neotropic	No Match				
	Ardea cocoi	Adult	Neotropic	No Match				
	Tigrisoma lineatum	Adult	Neotropic	No Match				
Posthodiplostomum sp. 23	*Ardea herodias*	Adult	Nearctic	No Match				

* Obtained through experimental infection

Supplementary Table S2 Morphological comparison of specimens of *Diplostomum huronense* La Rue, 1927 and *Diplostomum indistinctum* Guberlet, 1922. All measurements are provided in micrometers.

	Diplostomum huronense			*Diplostomum indistinctum*		
Reference	La Rue (1927)	Galazzo et al. (2002)	Present study	Guberlet (1922)	Galazzo et al. (2002)	Present study
Number of specimens	n = 11	n = 14	n = 4	n = Unknown	n = 10	n = 5
Host	*Larus argentatus*	*Larus delawarensis*	*Larus dominicanus*	*La. delawarensis*	*La. delawarensis*	*La. delawarensis*
Locality	Lake Huron, Canada & U.S.A.	Montreal, Canada	Chile	Oklahoma, U.S.A.	Montreal, Canada	North Dakota, U.S.A.
Overall body length	1,070–2,480	1,630–2,990	2097–2239	1,000–2,000	1,790–2,380	2409–2655
Prosoma length	518–1,330	810–1,280	840–1007	820–880	740–890	1067–1253
Prosoma width	503–873	370–710	540–613	270–330	380–620	511–650
Opisthosoma length	518–1,332	770–1,970	1105–1289	880–1,000	950–1,580	1327–1447
Opisthosoma width	407–592	130–620	408–424	250–330	210–340	414–471
Prosoma:opisthosoma length ratio	1.04	0.49–1.41	0.67–0.91	0.89–1.21	0.55–0.93	0.78–0.94
Opisthosoma 'neck':maxiumum	–	–	–	0.43–0.5	0.96[a]	0.44–0.56
Oral sucker length	90–112	48–72	64–66	55–70	55–84	68–81
Oral sucker width	51–105	50–100	65–81	49–55	64–88	76–90
Ventral sucker length	60–120	50–86	77–89	60–80	62–91	87–108
Ventral sucker width	97–138	48–108	96–101	60–80	72–84	89–118
Oral sucker:ventral sucker width ratio	0.64	0.9	0.68–0.8	0.56–0.68	0.81	0.68–0.93
Anterior margin of ventral sucker	Approx. 50%	42–54%	49–62%	Approx. 50%	30–52%	46–53%
Holdfast organ length	180–257	120–312	228–251	110–190	192–283	226–289
Holdfast organ width	150–325	96–288	186–240	120–165	192–261	226–345
Anterior margin of holdfast positioned at	59%[a]	53–69%	50–62%	53–60%	51–61%	57–63%
Pharynx length	75–98	45–74	53–80	50–60	57–72	61–78
Pharynx width	36–61	48–84	39–50	30–50	52–64	48–67
Oral sucker:pharnyx length	1.51	1.15	0.8–1.21	0.78–1.06	1.66	1–1.13
Anterior testis length	259–333	148–384	179–234	170–220	168–240	231–285
Anterior testis width	407–518	252–484	319–323	220–260	204–280	326–418
Posterior testis length	318–444	204–564	245–324	220–260	228–268	272–320
Posterior testis width	407–518	206–540	331–353	240–280	204–288	307–412
Ovary length	69–148	87–177	88–124	80–100	86–124	104–153
Ovary width	123–181	96–172	129–130	90–100	96–124	126–188
Anterior margin of ovary positioned at	7%	12–34%	14–28%	Approx. 33%	27–38%	22–39%
Egg length	95–105	96–108	91–103	99–110	96–108	86–105
Egg width	53–60	52–69	51–79	60–66	69–72	45–65
Anterior vitellarium-free zone:prosoma	0.43	0.65	0.51–0.55	0.57–0.59	0.64	0.46–0.55

Supplementary Table S3 Pairwise comparisons of partial sequences of the cox1 mtDNA gene among *Tylodelphys podicipina* , *Tylodelphys immer* and *Tylodelphys robrauschi* n. comb. based on a 317 bp long alignment. Percentage differences are given above diagonal and the number of variable nucleotide positions is given below the diagonal together with GenBank access numbers.

	1. MG972694	**2.** MH536513	**3.** MZ323303	**4.** MZ323304
1. *Tylodelphys podicipina* MG972694	–	8.8%	8.8%	10.1%
2. *Tylodelphys immer* MH536513	28	–	0.6%	10.1%
3. *Tylodelphys immer* MZ323303	28	2	–	10.7%
4. *Tylodelphys robrauschi* n. comb. MZ323304	32	32	34	–