Zoological Journal of the Linnean Society, 2022, **XX**, 1–13. With 4 figures.

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

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Received 10 October 2021; revised 23 November 2021; accepted for publication 28 November 2021

Alaria, *Didelphodiplostomum* and *Pharyngostomoides* are among genera of diplostomid digeneans known to parasitize mammalian definitive hosts. Despite numerous recent molecular phylogenetic studies of diplostomids, limited DNA sequence data is available from diplostomids parasitic in mammals. Herein, we provide the first 28S rDNA and *cox1* mtDNA sequences from morphologically identified, adult specimens of *Didelphodiplostomum* and *Pharyngostomoides*. Newly generated 28S sequences were used to infer the phylogenetic interrelationships of these two genera among other major lineages of diplostomoideans. The phylogeny based on 28S and a review of morphology clearly suggests that *Pharyngostomoides* should be considered a junior synonym of *Alaria*, while *Didelphodiplostomum* should be considered a junior synonym of *Tylodelphys*. *Pharyngostomoides procyonis* (type species), *Pharyngostomoides adenocephala* and *Pharyngostomoides dasyuri* were transferred into *Alaria* as ***Alaria procyonis* comb. nov.**, ***Alaria adenocephala* comb. nov.** and ***Alaria dasyuri* comb. nov.**; *Didelphodiplostomum variabile* (type species) and *Didelphodiplostomum nunezae* were transferred into *Tylodelphys* as ***Tylodelphys variabilis* comb. nov.** and ***Tylodelphys nunezae* comb. nov.** In addition, ***Alaria ovalis* comb. nov.** (formerly included in *Pharyngostomoides*) was restored and transferred into *Alaria* based on a morphological study of well-fixed, adult specimens and the comparison of *cox1* DNA sequences among *Alaria* spp. The diplostomid genus *Parallelorchis* was restored based on review of morphology.

ADDITIONAL KEYWORDS: *Alaria* – *Canis latrans* – *Didelphis virginiana* – digeneans – *Mustela frenata* – *Nyctereutes procyonoides* – parasites – *Puma concolor* – *Procyon lotor* – *Taxidea taxus*.

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 (e.g. avians and mammals). At present, members of 13 genera are known to utilize mammalian definitive hosts (Niewiadomska, 2002; Uhrig *et al.*, 2015; Achatz *et al.*, In press); however, DNA sequence data are only available for adult

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specimens of two of these genera: *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.*, In press). *Alaria* spp. are commonly studied, in part due to their association with a variety of diseases in their mammalian and some intermediate hosts. Furthermore, *Alaria* spp. are often reported in ecological and parasite survey studies (e.g. Fernandes *et al.*, 1976; Dyer *et al.*, 1997; Locke *et al.*, 2011; Uhrig *et al.*, 2015; Chinchilla-Carmona *et al.*, 2020; Bilska-Zając *et al.*, 2021).

Harkema (1942) erected the genus *Pharyngostomoides* Harkema, 1942 for *Pharyngostomoides procyonis* Harkema, 1942 collected from the common raccoon *Procyon lotor* (Linnaeus, 1758) in North Carolina and Texas, USA. Later, Harkema & Miller (1961) established *Parallelorchis* Harkema & Miller, 1961 for their new species *Parallelorchis diglossus* Harkema & Miller, 1961 collected from *Pr. lotor* in Florida, USA. Dubois (1966) synonymized *Parallelorchis* with *Pharyngostomoides*, but Beckerdite *et al.* (1971) rejected this synonymization. In addition, Beckerdite *et al.* (1971) redescribed *Ph. procyonis* and described *Pharyngostomoides adenocephala* Beckerdite *et al.*, 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 variabilis* Chandler, 1932 collected from a Virginia opossum, *Didelphis virginiana* (Kerr, 1792) in Texas, USA. 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 partial sequences of the nuclear large ribosomal subunit (28S) rDNA and mitochondrial cytochrome *c* oxidase (*cox1*) mtDNA genes for ten species of *Alaria*, *Didelphodiplostomum*, *Pharyngostomoides* and *Tylodelphys* Diesing, 1850. The 28S sequences were used to determine the phylogenetic position of *Pharyngostomoides* and *Didelphodiplostomum* among other major diplostomoidean lineages. Partial *cox1* sequences of *Alaria* spp. were used to study the interrelationships among members of the genus.

MATERIAL AND METHODS

Collection and morphological study

Several species belonging to *Alaria*, *Didelphodiplostomum* and *Pharyngostomoides* (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 collected from a frog in Europe (Table 1). Live adult diplostomids were removed from the intestines of recently euthanized mammals, briefly rinsed with saline, killed with hot water and stored in 70% ethanol. In some cases, dead diplostomids were removed from the intestines of frozen mammal carcasses and immediately stored in 70% ethanol. Diplostomids for microscopical study were stained with aqueous alum carmine and permanently mounted following the protocol of Lutz *et al.* (2017). Stained specimens were studied with light microscopy using an Olympus BX53 microscope (Olympus America, Center Valley, Pennsylvania, USA) equipped with a digital imaging system. The morphology of specimens that were readily identifiable and conformed to original descriptions is not discussed in the text. 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, USA. We use the terms prosoma and opisthosoma as discussed and justified by Achatz *et al.* (2019a) and Tkach *et al.* (2020).

Molecular study

Genomic DNA was extracted following the protocol described by Tkach & Pawlowski (1999). Fragments of the 28S and *cox1* genes were amplified by polymerase chain reactions (PCR). Amplifications of 28S used the forward primer digL2 (5'-AAG CAT ATC ACT AAG CGG-3') and reverse primer 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3') (Tkach *et al.*, 2003). Fragments of *cox1* were amplified using the forward primer Dipl_Cox_5' (5'-ACK TTR GAW CAT AAG CG-3') and reverse primers Dipl_Cox_3' (5'-WAR TGC ATN GGA AAA AAA CA-3') and Dipl650R (5'-CCA AAR AAY CAR AAY AWR TGY TG-3') (Achatz *et al.*, 2021b). The ribosomal internal transcribed spacer region (ITS1+5.8S+ITS2) was amplified for *Alaria mustelae* Bosma, 1931 using the forward primer ITSf (5'-CGC CCG TCG CTA CTA CCG ATT G-3') and reverse primer 300R (5'-CAA CTT TCC CTC ACG GTA CTT G-3') (Littlewood & Olson, 2001; Snyder & Tkach, 2007). In addition, the ribosomal 18S and ITS region were amplified for *Didelphodiplostomum variabile* (Chandler, 1932)

Table 1. Hosts, geographic origin, Harold W. Manter Laboratory (HWML) museum and GenBank accession numbers of diplostomids collected in our study

Diplostomid taxa	Host species	Stage	Geographical origin	HWML numbers	GenBank accession numbers	
					28S	*cox1*
Alaria alata	*Nyctereutes procyonoides*	Adult	Ukraine	HWML 216724	OL435536, OL435537	OL439156
Alaria arisaemoides	*Canis latrans*	Adult	Oregon, USA	HWML 216725	OL435538	OL439157
Alaria marcianae	*Taxidea taxus*	Adult	North Dakota, USA	HWML 216726	OL435539, OL435540	OL439158–OL439160
A. marcianae	*Procyon lotor*	Adult	California, USA	HWML 216727	OL435541	OL439161
Alaria mustelae	*Mustela frenata*	Adult	North Dakota, USA	–	OL435542§	OL439162
A. mustelae	*Neogale vison*	Adult	North Dakota, USA	–	OL435543, OL435544	OL439163–OL439166
A. mustelae	*Neogale vison*	Adult	Minnesota, USA	HWML 216728	–	OL439167
A. mustelae	*Taxidea taxus*	Adult	North Dakota, USA	HWML 216729	OL435545	OL439168–OL439170
A. mustelae	*Thamnophis sirtalis parietalis*	Mesocercaria	Manitoba, Canada	–	–	OL439171
*Alaria ovalis**	*Procyon lotor*	Adult	Mississippi, USA	HWML 216730	OL435546	OL439172
*Alaria procyonis**	*Procyon lotor*	Adult	Minnesota, USA	HWML 216731	OL435547	OL439173
Alaria sp. 1	*Thamnophis sirtalis parietalis*	Mesocercaria	Manitoba, Canada	–	OL435548, OL435549	OL439174, OL439175
Alaria sp. 3	*Puma concolor*	Adult	Florida, USA	–	OL435550	OL439176
Tylodelphys excavata	*Pelophylax ridibundus*	Metacercaria	Ukraine	–	OL435551	OL439177
Tylodelphys variabilis†	*Didelphis virginiana*	Adult	Arkansas, USA	HWML 216733	OL435552‡, OL435553‡	OL439178, OL439179
T. variabilis†	*Didelphis virginiana*	Adult	North Carolina, USA	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.

following the protocol and primers of Woodyard *et al.* (2017). The PCR amplifications were carried out with a total volume of 25 µL using GoTaq G2 DNA Polymerase from Promega (Madison, Wisconsin, USA) using an annealing temperature of 53 °C for rDNA amplifications and 45 °C for *cox1* amplifications.

The PCR products were purified using an ExoSAP-IT PCR clean-up enzymatic kit from Affymetrix (Santa Clara, California, USA) and cycle-sequenced directly using a BrightDye Terminator Cycle Sequencing kit (MCLAB, California, USA); PCR primers were used for sequencing reactions. Sequencing reactions were purified using a BigDye Sequencing Clean-Up kit from MCLAB and subsequently run on an ABI 3130 automated capillary sequencer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Newly generated sequences were assembled using Sequencher v.4.2 software (GeneCodes Corp., Ann Arbor, Michigan, USA) and deposited in the GenBank database (Table 1).

Phylogenetic analyses

ClustalW as implemented in MEGA7 software was used to initially align DNA sequences of each locus separately (Kumar *et al.*, 2016). Alignments were trimmed to the length of the shortest sequence. All sites with ambiguous homology were excluded from analyses. Phylogenetic positions of *Didelphodiplostomum* and *Pharyngostomoides* spp. (as currently recognized) within the Diplostomoidea Poirier, 1886 were determined using a 28S alignment (1135 bp long; 28 sites excluded) with *Suchocyathocotyle crocodili* (Yamaguti, 1954) (Cyathocotylidae Mühling, 1896) as the outgroup. 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 Diplostomidae, 12 representatives of Strigeidae Railliet, 1919 and two representatives of 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 *cox1* sequences with *Sphincterodiplostomum musculosum* Dubois, 1936 (see Dubois, 1936b) as the outgroup. The 28S alignment limited to only *Alaria* and *Pharyngostomoides* spp. (1132 bp long; no sites were excluded) included eight newly generated sequences. The *cox1* alignment (470 bp long; no sites excluded) included 14 newly generated sequences and 16 previously published sequences.

Bayesian inference (BI), as implemented in MrBayes v.3.2.6 software, was used for the phylogenetic analyses (Ronquist & Huelsenbeck, 2003). 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 three alignments using MEGA7 (Kumar *et al.*, 2016). The BI analyses were performed with MrBayes software as follows: Markov chain Monte Carlo (MCMC) chains were run for 3 000 000 generations with sample frequency set at 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 as the standard deviation stabilized below 0.01. Pairwise comparisons of 28S and *cox1* alignments were carried out using MEGA7.

RESULTS

Molecular phylogenies

The initial phylogenetic analysis based on 28S convincingly demonstrated non-monophyly of Diplostomidae and Strigeidae, while Proterodiplostomidae appeared monophyletic (Fig. 1). Considering the similarity of the results of our analysis compared to numerous recent molecular phylogenetic studies of diplostomoideans (e.g. Achatz *et al.*, 2021a, b, c), we opt to only discuss the clades which contained our newly generated DNA sequences. *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. 1).

Surprisingly, *Did. variabile* [= *Tylodelphys variabilis* (Chandler, 1932) comb. nov.; see discussion below] was positioned in a 100% supported cluster of *Tylodelphys*+*Austrodiplostomum* Szidat & Nani, 1951 species (Fig. 1). *Tylodelphys* was non-monophyletic, in part, due to the inclusion of *Austrodiplostomum* spp., as recently demonstrated and discussed by Achatz *et al.* (In press). *Tylodelphys excavata* was positioned as a sister branch to the larger *Tylodelphys*+*Austrodiplostomum* clade (100% supported). Within the remaining members of the *Tylodelphys*+*Austrodiplostomum* clade, *Did. variabile* was positioned in an 85% supported clade, which contained most other members of *Tylodelphys* (Fig. 1). Considering that details of the interrelationships in the *Tylodelphys*+*Austrodiplostomum* clade were recently discussed by Achatz *et al.* (In press), we do not discuss this clade in detail here.

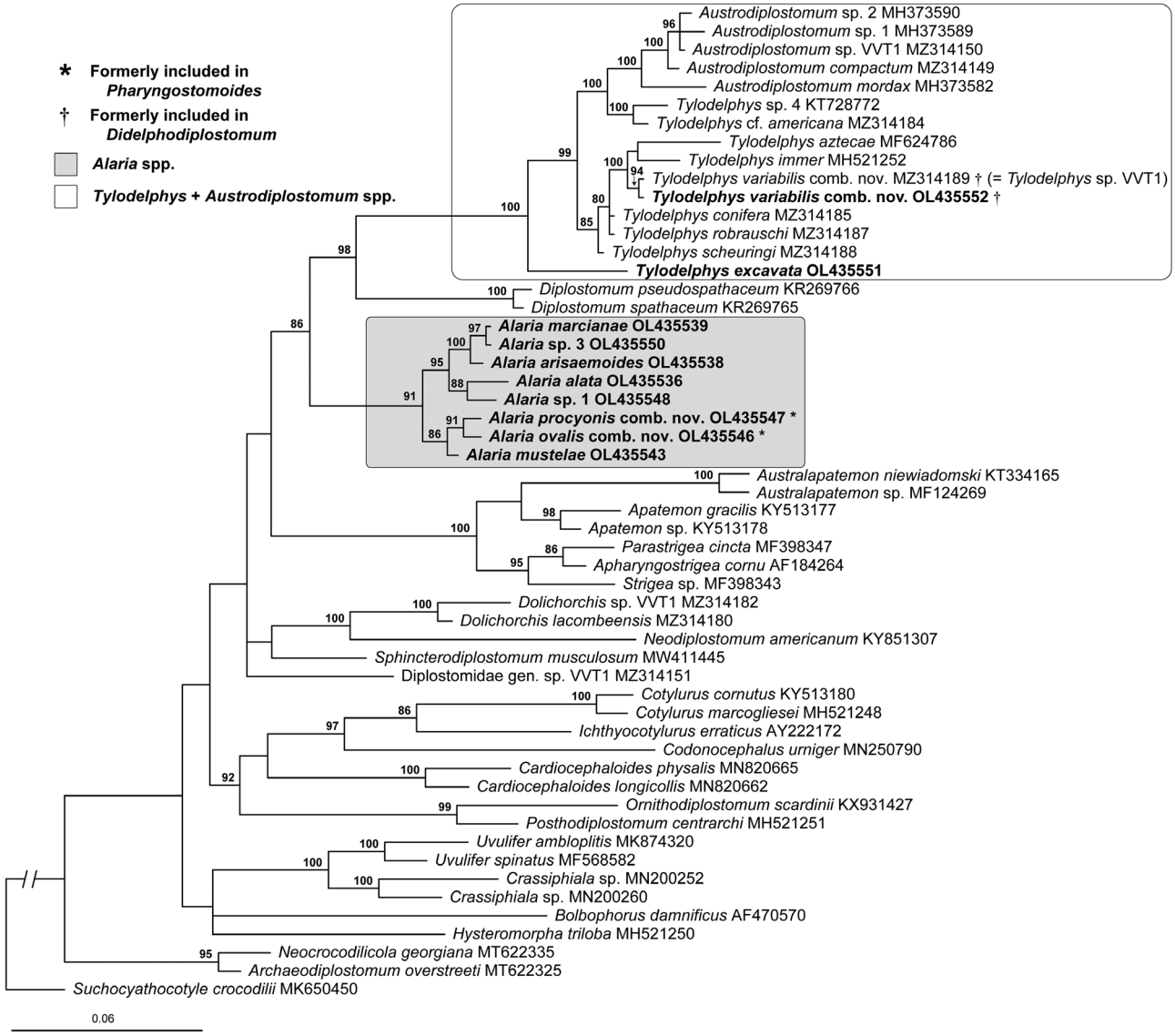

Figure 1. Phylogenetic interrelationships among 54 diplostomoidean taxa based on BI analysis of partial 28S rDNA gene sequences including *Didelphodiplostomum* and *Pharyngostomoides* spp. BI posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale bar indicates the number of substitutions per site.

The analysis of 28S limited to *Alaria* spp. (Fig. 2) had somewhat different topology and lower branch support compared to the initial analysis (Fig. 1). *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 of *A. arisaemoides*+a clade of [*A. marcianae*+*Alaria* sp. 3 (99% supported)] (Fig. 2).

The phylogeny of *Alaria* spp. based on partial *cox1* sequences had substantially different topology than both 28S phylogenies (Fig. 3). The two former *Pharyngostomoides* spp. (see discussion below) were positioned in an unsupported clade that was placed 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 that contained two additional subclades. The first subclade (87% supported)

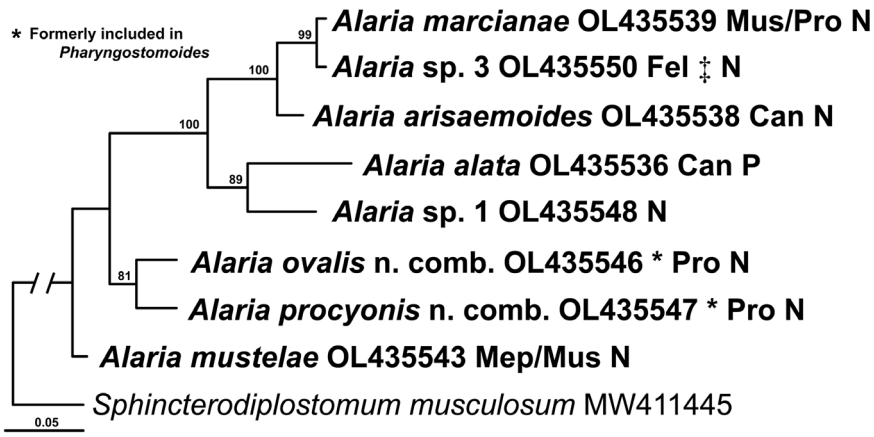

Figure 2. Phylogenetic interrelationships among eight species of *Alaria* (syn. *Pharyngostomoides*) based on BI analysis of partial 28S rDNA gene sequences. BI posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. The scale bar indicates 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, Palaearctic. Abbreviations of family of definitive host: Can, Canidae; Fel, Felidae; Mep, Mephitidae; Mus, Mustelidae; Pro, Procyonidae. ‡ All collected specimens are immature.

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.

Pairwise comparisons of *Alaria* spp.

Interspecific divergence of partial 28S sequences among *Alaria* spp. was 0.0–1.4% (Table 2). *Alaria marcianae* and *Alaria* sp. 3 were the least divergent pair of species (0%), whereas *A. alata* and *Alaria ovalis* (Chandler & Rausch, 1946) comb. nov. (= *Pharyngostomoides ovalis* Chandler & Rausch, 1946; see discussion below) were the most divergent pair of species (1.4%). No intraspecific variation of 28S sequences was detected within *Alaria* spp. with multiple sequences.

Interspecific divergence of partial *cox1* sequences among *Alaria* spp. was 6.8–13.8% (Supporting Information, Table S1). Similar to comparisons of 28S sequences, *A. marcianae* and *Alaria* sp. 3 were the least divergent pair of species (6.8–7.7%), whereas *A. ovalis* and *Alaria* sp. 1 (GenBank FJ477181) were the most divergent pair of species (13.6–13.8%). The intraspecific variation of *cox1* sequences included in our analyses varied among *Alaria* spp. (*A. arisaemoides*: up to 2.6%; *A. marcianae*: up to 2.1%; *A. mustelae*: up to 2.3%; *Alaria* sp. 1: 0.2%) (Supporting Information, Table S1).

DISCUSSION

Status of *Pharyngostomoides*

The morphological characteristics of *Pharyngostomoides* spp. in our material conform to the original descriptions of *Ph. procyonis* and *Ph. ovalis* (Fig. 4E, 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 that appears remarkably similar to *Ph. ovalis*. Our material of *Ph. procyonis* and *Ph. ovalis* differ by 0.4% and 10% in partial sequences of 28S and *cox1*, respectively (Table 2; Supporting Information, Table S1). 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 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 µm in *Ph. procyonis* vs. egg length 100–115 µm in *Ph. ovalis*). Considering the genetic and morphological differences listed above, we restore *Ph. ovalis*.

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. 4E, F vs. Fig. 4B-D, G, H). However, our molecular phylogeny based on 28S (Fig. 1) positioned *Ph. procyonis* (type species) and *Ph. ovalis* among *Alaria* spp., including the type species *A. alata*.

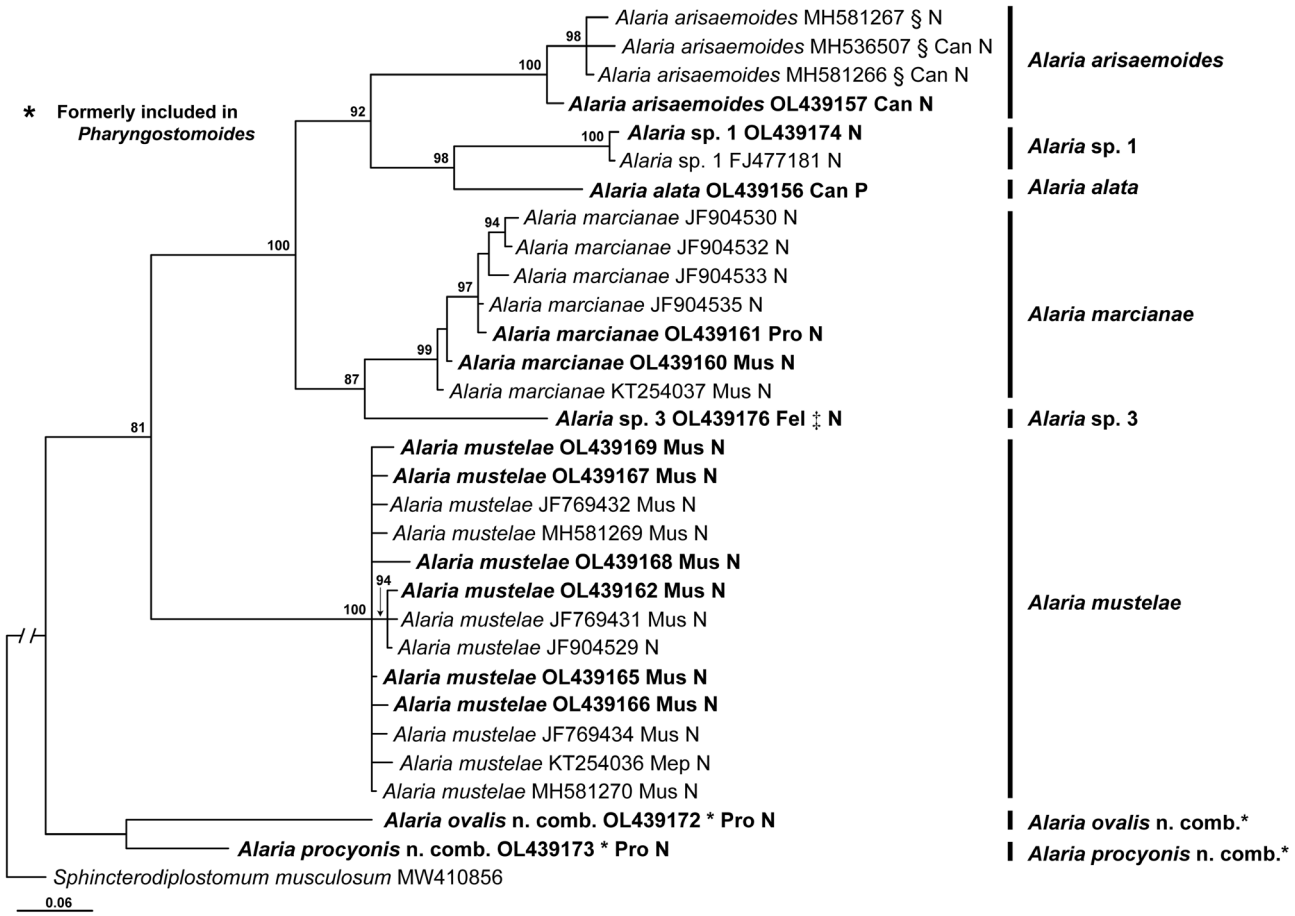

Figure 3. Phylogenetic interrelationships among 31 sequences from members of *Alaria* (syn. *Pharyngostomoides*) based on BI analysis of partial *cox1* mtDNA gene sequences. BI posterior probability values lower than 80% are not shown. The new sequences generated in this study are indicated in bold. 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, Palaearctic. 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).

Table 2. Pairwise comparisons of 28S sequences among *Alaria* spp. (syn. *Pharyngostomoides*) based on an 1132 bp long alignment. Percentage difference given above diagonal. Number of nucleotide differences provided below diagonal

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
	OL435539	OL435550	OL435538	OL435536	OL435548	OL435546	OL435547	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* OL435546*	15	15	14	16	15	–	0.4%	0.4%
7 *Alaria procyonis* OL435547*	13	13	14	15	15	4	–	0.4%
8 *Alaria mustelae* OL435543	12	12	13	15	14	5	5	–

*Previously included in *Pharyngostomoides*.

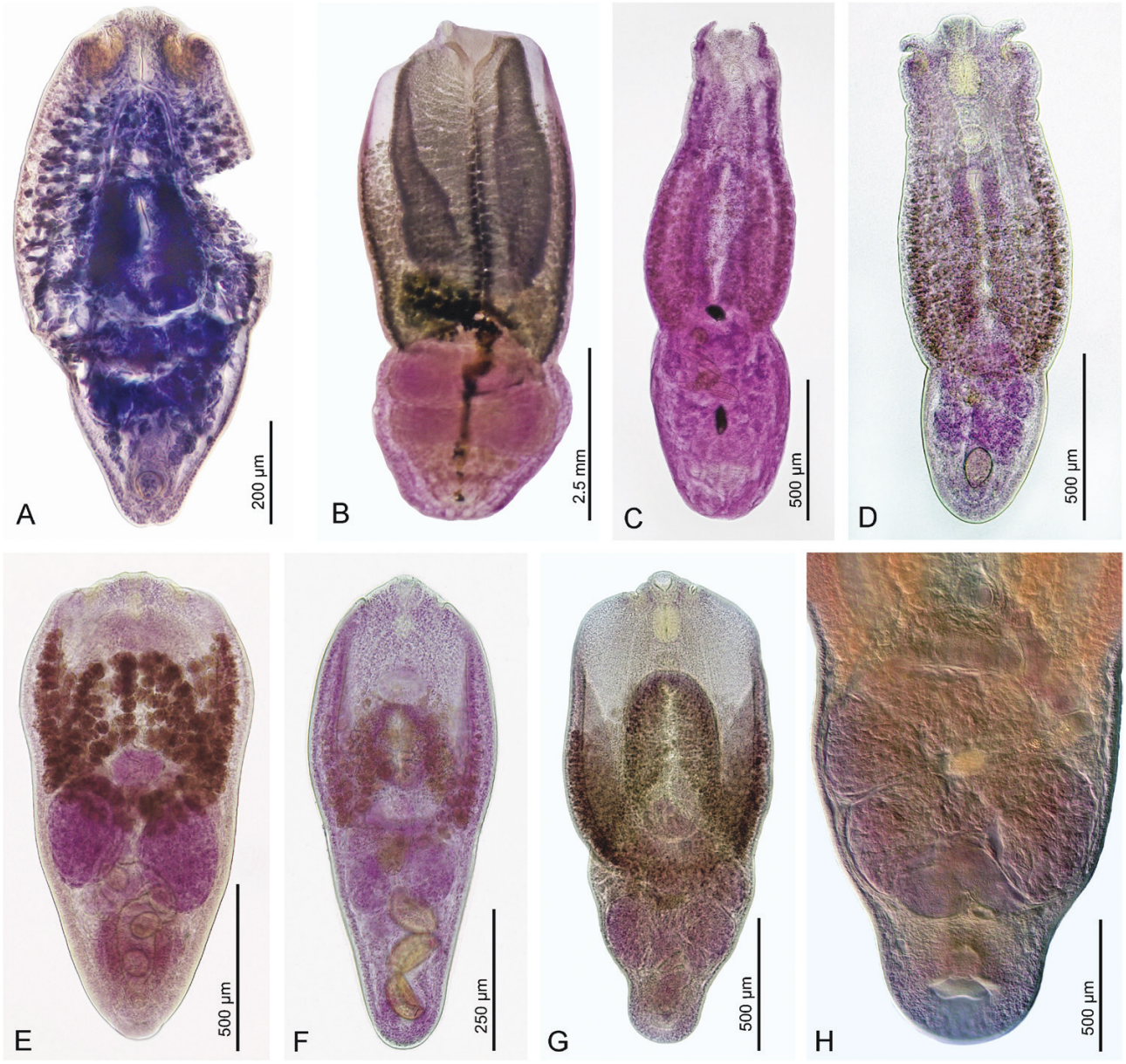

Figure 4. Photographs of: A, *Tylodelphys variabilis* comb. nov. from *Didelphis virginiana*, Arkansas; B, *Alaria arisaemoides* from *Canis latrans*, Oregon; C, *Alaria alata* from *Nyctereutes procyonoides*, Ukraine; D, *Alaria marcianae* from *Taxidea taxus*, North Dakota; E, *Alaria ovalis* comb. nov. from *Procyon lotor*, Mississippi; F, *Alaria procyonis* comb. nov. from *Procyon lotor*, Minnesota; G, H, *Alaria mustelae* from *Mephitis mephitis*, North Dakota.

Interestingly, the two *Pharyngostomoides* spp. were placed 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. 1) 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. 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-enteropulmonary 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 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 of two lateral tongue-like lobes (see description and illustrations provided by Harkema & Miller, 1961). In contrast, the holdfast organ of *Alaria* spp. is distinct and usually sucker-like as shown in multiple descriptions and seen on some of the photographs in Figure 4F, 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 in topology (Figs 1, 2). At the same time, the phylogenies of 28S and *cox1* limited to members of *Alaria* showed more pronounced differences in branch topology (Figs 2, 3). *Alaria mustelae* was positioned as a sister taxon to the other *Alaria* spp. in the second 28S analysis (Fig. 2), while in the *cox1* phylogeny, *A. ovalis* and *A. procyonis* formed an unsupported clade that was placed as a sister group to the other members of *Alaria* (Fig. 3). The positions of *A. alata*+*Alaria* sp. 1 and *A. marcianae*+*Alaria* sp. 3 varied between the two analyses as well (Figs 2, 3). 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.*, In press). Faster mutating genes, such as *cox1*, are more reliable for distinguishing between closely related diplostomoidean species/species-level lineages (Table 2; Supporting Information, Table S1), but 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 Palaearctic (Figs 1–3).

It is difficult to address questions related to host switching of *Alaria* spp., considering that many species have been historically reported in a diversity of mammalian hosts (e.g. see Dubois, 1968 and references therein). The accuracy of *Alaria* spp. identifications in previous reports is questionable considering that most publications lack DNA sequence data and many *Alaria* spp. are morphologically similar. Some *Alaria* spp., such as *A. arisaemoides*, are also known to have substantial morphological variation (e.g. Hall & Wigdor, 1918; Dubois, 1968). The topology of our molecular phylogeny based on the 28S of *Alaria* spp. (Fig. 2) is not well enough supported to confidently infer evolutionary patterns of definitive host associations; the discordance between topologies of 28S (Fig. 2) and *cox1* (Fig. 3) further complicates the situation. Our specimen of *Alaria* sp. 3 from the cougar *Puma concolor* (Linnaeus, 1758) is immature; hence, additional collection of well-fixed, mature specimens of *Alaria* sp. 3 is crucial for accurate species identification and confirmation of its definitive host.

It is worth noting that our specimens of *A. arisaemoides* (Fig. 4B) conform closely to the original description of the species and subsequent descriptions of the species (e.g. Augustine & Uribe, 1927; Dubois, 1968). However, the *cox1* 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) (Supporting Information, Table S1). The material described by Locke *et al.* (2018) is somewhat different to 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. 1) places *Did. variabile* (shown as *Tylodelphis variabilis* comb. nov. in the figure) in the cluster of *Tylodelphys* and *Austrodiplostomum* species. The morphology of adult *Didelphodiplostomum* and *Tylodelphys* spp. is remarkably similar (Fig. 4A; 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. 1) clearly demonstrates that *Did. variabile* belongs to one of the two major clades of *Tylodelphys*. Taking into account the results of our phylogenetic analysis (Fig. 1) and minor morphological differences between *Didelphodiplostomum* and *Tylodelphys*, we consider *Didelphodiplostomum* to be a junior synonym of *Tylodelphys*. As such, we transfer *Did. variabile* and *Did. nunezae* into *Tylodelphys* as *T. variabilis* (Chandler, 1932) comb. nov. and *Tylodelphys nunezae* (Dubois, 1976) comb. nov., respectively. The partial 28S and *cox1* sequences of *T. variabilis* and *Tylodelphys* sp. VVT1 of Achatz *et al.* (In press) are identical. It is clear that the larval specimens of *Tylodelphys* sp. VVT1 from the mole salamander *Ambystoma talpoideum* Holbrook, 1838 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 caecal 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. 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 *Tylodelphys clavata* (von Nordmann, 1832).

Remarks on *Tylodelphys*

Based on our analysis, *Tylodelphys* spp. belong to at least three distinct clades (Fig. 1). Achatz *et al.* (In press) recently suggested that *Tylodelphys americana* (Dubois, 1936) (see Dubois, 1936b) and *Tylodelphys* sp. 4 may need to be placed in 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 requires the establishment of at least two new genera. DNA sequences from adult specimens of *T. 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.*, In press). Achatz *et al.* (In press) recently revealed the presence of two *Diplostomum* spp. parasitizing North American river otters *Lontra canadensis* (Schreber, 1777) in the USA. Based on the results of the present study, *T. variabilis* represents the first species of *Tylodelphys* that 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.

CONCLUSION

Our results clearly demonstrate that *Pharyngostomoides* and *Didelphodiplostomum* should be considered junior synonyms of *Alaria* and *Tylodelphys*, respectively. Our study has demonstrated 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* (Achatz *et al.*, In press). Despite recent progress in the understanding of the phylogenetic interrelationships of Diplostomoidea, and the diversity and distribution of its members (e.g. Hernández-Mena *et al.*, 2014; Rosser *et al.*, 2016; Achatz *et al.*, 2019a, b, 2021a, b, c, In press; Lopez-Hernandez *et al.*, 2019; Sereno-Uribe *et al.*, 2019; Locke *et al.*, 2020, 2021; Tkach *et al.*, 2020; López-Jiménez *et al.*, 2022), DNA sequences from adult diplostomoideans parasitic in mammals remain scarce. Future studies should strive to include DNA sequence data from adults of the other diplostomoidean genera that parasitize mammal definitive hosts to further improve the system of this large digenean group.

ACKNOWLEDGEMENTS

We are grateful to Yuriy Kuzmin, Ivanna Dmytrieva and Roman Svitin (Institute of Zoology, Kyiv, Ukraine) for their help with collecting specimens in Ukraine and Andrew Janik (Oregon State University, USA) for providing specimens of *Alaria arisaemoides*. C.T.M. thanks the Arkansas Game and Fish Commission and the Oklahoma Department of Wildlife Conservation for providing Scientific Collecting Permits. We thank the anonymous referees and editor for constructive comments on this manuscript. There are no conflicts of interest.

FUNDING

This study was supported by the National Science Foundation (grant DEB-1120734), the National Institutes of Health (grant R15AI092622) and the University of North Dakota (Joe K. Neel Memorial Award, Esther Wadsworth Hall Wheeler Award, Student Research Stipend and Summer Doctoral Fellowship). In addition, T.P.C. and J.R.M. were supported by the National Science Foundation (REU Site award number 1852459) and the National Institute of General Medical Sciences of the National Institutes of Health [Institutional Development Award (IDeA) grant number P20GM103442] to the University of North Dakota School of Medicine & Health.

DATA AVAILABILITY

The data underlying this article are available in the article and in its online supplementary material. The newly generated DNA sequences are deposited in the GenBank with accession numbers provided in the article. The voucher specimens of digeneans are deposited in the collection of the Harold W. Manter Laboratory (HWML), University of Nebraska State Museum, Lincoln, Nebraska, U.S.A., with accession numbers provided in the article.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Pairwise comparisons among *cox1* sequences of *Alaria* spp. (syn. *Pharyngostomoides*) based on a 470 bp long alignment. Percentage difference given above diagonal. Number of nucleotide differences provided below diagonal.