Parasitology

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

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Research Article

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Abstract

Fibricola and *Neodiplostomum* are diplostomid genera with very similar morphology that are currently separated based on their definitive hosts. *Fibricola* spp. are normally found in mammals, while *Neodiplostomum* spp. typically parasitize birds. Previously, no DNA sequence data was available for any member of *Fibricola*. We generated nuclear ribosomal and mtDNA sequences of *Fibricola cratera* (type-species), *Fibricola lucidum* and 6 species of *Neodiplostomum*. DNA sequences were used to examine phylogenetic interrelationships among *Fibricola* and *Neodiplostomum* and re-evaluate their systematics. Molecular phylogenies and morphological study suggest that *Fibricola* should be considered a junior synonym of *Neodiplostomum*. Therefore, we synonymize the two genera and transfer all members of *Fibricola* into *Neodiplostomum*. Specimens morphologically identified as *Neodiplostomum cratera* belonged to 3 distinct phylogenetic clades based on mitochondrial data. One of those clades also included sequences of specimens identified morphologically as *Neodiplostomum lucidum*. Further study is necessary to resolve the situation regarding the morphology of *N. cratera*. Our results demonstrated that some DNA sequences of *N. americanum* available in GenBank originate from misidentified *Neodiplostomum banghami*. Molecular phylogentic data revealed at least 2 independent host-switching events between avian and mammalian hosts in the evolutionary history of *Neodiplostomum*; however, the directionality of these host-switching events remains unclear.

Introduction

Fibricola Dubois, 1932 (Diplostomidae Poirier, 1886) 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 hosts (e.g. Ulmer, 1970; Premvati and Bair, 1979; Gillilland and Muzzall, 1999; Goldberg and Bursey, 2001; Goldberg *et al*., 2001; Bolek and Coggins, 2003; Richardson, 2013; Weinstein *et al*., 2019). Although most members of *Fibricola* are known to parasitize intestines of mammals, some *Fibricola* species have also been reported from crocodilians (Bisseru, 1957; Dubois, 1982).

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 and Lee, 1964) and *Neodiplostomum minor* (Dubois, 1936)), and later transferred to *Neodiplostomum* (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). Noteworthily, *N. 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 morphologically similar. In the most recent detailed 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.

Currently, DNA sequences are available for six species of *Neodiplostomum* (Woodyard *et al.*, 2017; Heneberg *et al.*, 2020; Lee *et al.*, unpublished results), but DNA sequence data from morphologically identified adult *Fibricola* specimens are lacking. Herein, we provide partial sequences of the nuclear small ribosomal subunit (18S), internal transcribed spacer region (ITS1, 5.8S, ITS2) and large ribosomal subunit (28S) rRNA genes as well as a fragment of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) mtDNA gene for *Fibricola cratera* (Barker and Noll, 1915) and *Fibricola lucidum* (La Rue and 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.

Materials and 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 1). 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 are deposited in the collection of the Harold W. Manter Laboratory (HWML), University of Nebraska State Museum, Lincoln, NE, USA and the Museo de Zoología, Pontificia Universidad Católica del Ecuador (QCAZI), Quito, Ecuador. Due to the inconsistent reporting of morphological characteristics in the descriptions of *Neodiplostomum* spp., we re-measured type and voucher specimens of *Neodiplostomum americanum* Chandler and Rausch, 1947, *Neodiplostomum banghami* Penrod, 1947 and *Neodiplostomum reflexum* Chandler and Rausch, 1947 (syn. *Neodiplostomum delicatum* Chandler and Rausch, 1947) for comparison with specimens collected in the current 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. We use the terms prosoma and opisthosoma as explained by Achatz *et al.* (2019*a*) and Tkach *et al.* (2020).

For comparative purposes, specimens of the 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) and *Neodiplostomum spathula* (Creplin, 1829) from Minnesota (vouchers: NHM 1975.1.7.35-42).

Genomic DNA was extracted from either fragments (in case of larger specimens) or whole individuals of each species according to the methods described by Tkach and Pawlowski (1999). An approximately 1800 bp long fragment at the 5′ end of the 18S rRNA gene and a 1300 bp long fragment at the 5′ end of the 28S rRNA gene were amplified by polymerase chain reactions (PCRs) in a T100™ thermal cycler (Bio-Rad, Hercules, CA, USA). The 18S fragment was amplified using the forward primer WormA (5′–GCG AAT GGC TCA TTA AAT CAG–3′) and the reverse primer WormB (5′–ACG GAA ACC TTG TTA CGA CT–3′), whereas 28S was amplified using the forward primer digL2 (5′–AAG CAT ATC ACT AAG CGG–3′) and the reverse primer 1500R (5′–GCT ATC CTG AGG GAA ACT TCG–3′) (Littlewood and Olson, 2001; Tkach *et al.*, 2003). In addition, fragments of the ribosomal internal transcribed spacer region (ITS1 + 5.8S + ITS2) were amplified for some of the studied taxa using the forward primer ITSf (5′–CGC CCG TCG CTA CTA CCG ATT G–3′) and the reverse primer 300R (5′–CAA CTT TCC CTC ACG GTA CTT G–3′) (Littlewood and Olson, 2001; Snyder and Tkach, 2007). A fragment of the mitochondrial *cox*1 gene was amplified using the previously published forward primer Dipl_Cox_5′ (5′-ACK TTR GAW CAT AAG CG-3′) and the reverse primer Dipl_Cox_3′ (5′-WAR TGC ATN GGA AAA AAA CA–3′) (Achatz *et al.*, 2021*b*). For a subset of taxa collected in Mississippi and Arkansas (USA), the molecular methods described by Woodyard *et al.* (2017) were used.

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), alcohol precipitated 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 *cox*1 genes as well as the ribosomal ITS region. In addition, internal forward primer 18S-8 (5′-GCA GCC GCG GTA ATT CCA GC-3′) and internal reverse primer WB1 (5′-CTT GTT ACG ACT TTT ACT TCC-3′) were used for sequencing of the 18S fragment; internal forward primer DPL600F (5′-CGG AGT GGT CAC CAC GAC CG-3′) and internal reverse primer DPL700R (5′-CAG CTG ATT ACA CCC AAA G-3′) were used for sequencing of 28S PCR reactions; internal forward primer d58F (5′-GCG GTG GAT CAC TCG GCT CGT G-3′ was used to sequence the ITS region (Littlewood and Olson, 2001; Kudlai *et al.*, 2015; Achatz *et al.*, 2019*d*). Contiguous sequences were assembled using Sequencher version 4.2 software (GeneCodes Corp., Ann Arbor, Michigan, USA). Our newly generated sequences are deposited in GenBank (Table 1).

Phylogenetic interrelationships among the members of *Fibricola* and *Neodiplostomum* and other members of the Diplostomoidea Poirier, 1886 were analysed using the 18S, 28S and *cox*1 sequence data, in part, to match the data published by Heneberg *et al.* (2020). Interrelationships among the 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 observed from the results of our analyses of 18S and 28S as well as the suprageneric analysis of *cox*1 data. Sequences were aligned with the assistance of ClustalW as implemented in MEGA7 (Kumar *et al.*, 2016); alignments were trimmed to the length of the shortest respective sequence. *Cyathocotyle prussica* Mühling, 1896 was used as the outgroup for the analysis of 18S and *Strigea strigis* (Schrank, 1788) was used as the outgroup for the suprageneric analysis of *cox*1 to be comparable with the analysis of Heneberg *et al.* (2020). *Suchocyathocotyle crocodili* (Yamaguti, 1954) was selected as the outgroup for the 28S analysis based on the topology presented by Achatz *et al.* (2019*d*). On the basis of the results of the broader phylogenetic analyses (see 'Results' section), we

Table 1. Hosts, geographic origin, GenBank and museum accession numbers of *Neodiplostomum* (syn. *Fibricola*) spp. used in this study

Digenean taxa	Host species	Geographic origin	Museum number	Accession numbers	
				Ribosomal	*cox*1
Neodiplostomum cf. *cratera* 1 n. comb.*	*Didelphis virginiana*	Arkansas, USA	HWML-216754	—	OL770020
Neodiplostomum cf. *cratera* 2 n. comb.*	*Didelphis virginiana*	California, USA	HWML-216765	OL799069, OL799070 (ITS1), OL770124, OL770125 (ITS2)	OL770021–OL770023
Neodiplostomum cf. *cratera* 2 n. comb.*	*Procyon lotor*	California, USA	—	OL799097 (28S)	OL770024
Neodiplostomum cf. *cratera* 3 n. comb.*	*Didelphis virginiana*	Mississippi, USA	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, USA	HWML-216766	OL799072, OL799073 (ITS2–28S)	OL770027, OL770028
Neodiplostomum cf. *cratera* 3 n. comb.*	*Neogale vison*	Minnesota, USA	HWML-216767	OL799074 (18S–ITS2)	OL770029
Neodiplostomum cf. *cratera* 3 n. comb.*	*Taxidea taxus*	North Dakota, USA	—	OL799099 (28S)	OL770030
Neodiplostomum cf. *lucidum**	*Didelphis virginiana*	Arkansas, USA	HWML-216752, HWML-216753	OL799075 (18S–28S)	OL770031, OL770032
Neodiplostomum cf. *lucidum**	*Didelphis virginiana*	Mississippi, USA	—	—	OL770033–OL770037
Neodiplostomum cf. *lucidum**	*Didelphis virginiana*	Nebraska, USA	HWML-216768	OL799076 (18S–28S)	OL770038, OL764381
Neodiplostomum cf. *lucidum**	*Didelphis virginiana*	North Carolina, USA	HWML-216769	OL799100, OL799101 (28S)	OL770039, OL770040
Neodiplostomum cf. *lucidum**	*Lithobates catesbeianus*	Mississippi, USA	—	OL799077, OL799078 (ITS1–28S)	OL770041, OL770042
Neodiplostomum cf. *lucidum**	*Procyon lotor*	California, USA	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, USA	HWML-216773	OL799080 (18S), OL770126 (ITS1–28S)	OL770046
N. americanum	*Bubo virginianus*	Arkansas, USA	HWML-216774	OL799103 (28S)	OL770047
N. americanum	*Bubo virginianus*	Mississippi, USA	HWML-216756, HWML-216757, HWML-216760	OL799081–OL799083 (ITS region)	OL770048–OL770050
N. americanum	*Nerodia fasciata*	Mississippi, USA	—	OL799084 (ITS1–28S)	OL770051
N. americanum	*Strix varia*	Mississippi, USA	—	OL799085 (ITS region)	OL770052
N. americanum	*Thalasseus maximus*	Mississippi, USA	—	OL799086 (ITS1–28S)	OL770053
Neodiplostomum banghami	*Falco columbarius*	North Dakota, USA	—	OL799087 (18S–28S)	OL770054
N. banghami	*Lithobates sylvatica*	North Dakota, USA	—	OL799104 (28S)	OL770055
N. banghami	*Thamnophis sirtalis*	North Dakota, USA	—	OL799105 (28S)	OL770056
Neodiplostomum reflexum	*Bubo virginianus*	North Dakota, USA	HWML-216775	OL799106 (28S)	OL770057
N. reflexum	*Bubo virginianus*	Mississippi, USA	HWML-216759	OL799088 (ITS region)	OL770058
N. reflexum	*Buteo jamaicensis*	North Dakota, USA	—	OL799089 (18S–28S)	OL770059
N. reflexum	*Buteo jamaicensis*	Mississippi, USA	—	OL799090 (ITS region)	OL770060
N. reflexum	*Strix varia*	Mississippi, USA	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, USA	HWML-216776	OL799096 (18S–28S)	OL770068

HWML, Harold W. Manter Laboratory; QCAZI, Museo de Zoología, Pontificia Universidad Católica del Ecuador.
*Species previously considered to be within *Fibricola*.

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 the members of this clade and other diplostomoidean taxa.

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 the members of the *Fibricola*/*Neodiplostomum* clade (clade I) included 21 newly generated sequences. The second *cox*1 alignment limited to the second clade of *Neodiplostomum* species (clade II) included nine newly generated sequences and nine previously published sequences.

Independent phylogenetic analyses were conducted using Bayesian inference (BI) as implemented in MrBayes Ver. 3.2.6 software (Ronquist and 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 datasets using MEGA7 (Kumar *et al.*, 2016). BI analyses of 18S, 28S and *cox*1 of the Diplostomidae were carried out with the following settings: 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 *cox*1 analyses of clades I and II were carried out with identical settings, but sequence data were analysed as codons. The number of generations was considered sufficient when the s.d. value reduced well below 0.01. Due to limited representation, the ITS region sequences were not used for phylogenetic inference; however, we provided a pairwise sequence comparison for all isolates that have a complete ITS1 + 5.8S + ITS2 fragment sequenced.

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 18S, 28S and suprageneric *cox*1 analyses and discussion below). We refer to *F. cratera* and *F. lucidum* as *Neodiplostomum cratera* n. comb. (Barker and Noll, 1915) and *Neodiplostomum lucidum* La Rue and Bosma, 1927, respectively, throughout the remainder of the text. Justification for the synonymization is provided in the discussion.

The 18S alignment was 1619 bp long; 22 bases were excluded from the analysis due to ambiguous homology. The phylogenetic tree resulting from the BI analysis of 18S (Fig. 1) demonstrated a 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 and Noll, 1915) (former type-species of *Fibricola*; see discussion below), *Neodiplostomum* cf. *lucidum* La Rue and 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 1135 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. 2), 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*, 2019*c*, 2022, 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. 2).

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 the two clades (Fig. 3). Clade I (86% supported) consisted of a large polytomy with a poorly resolved internal topology (Fig. 3). The polytomy consisted of *Neodiplostomum spathulaeforme* (Brandes, 1888) (type-species of *Neodiplostomum*) + *Neodiplostomum seoulense* + 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. 3).

On the basis of 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*1 analysis together with former *Fibricola* spp. (clade I in Figs 1–3). This alignment was 456 bp long; three bases (one codon) were excluded from the analysis as an indel. The internal branch topology of the resulting tree (Fig. 4) 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. *Neodiplostomum microcotyle* was positioned as a sister group to a weakly supported clade of *Neodiplostomum* sp. VVT1 + *N. vaucheri*. 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. 4). 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. 4). The other was a 100% supported clade of *N.* cf. *cratera* 2 (formerly *F.* cf. *lucidum*).

The *cox*1 alignment of the second *Neodiplostomum* clade (clade II in Figs 1–3) was 366 bp long. The sequences of *N. americanum* and *N. banghami* formed separate 100% supported clades (Fig. 5).

Genetic variation

Taxa included in clade I demonstrated a low interspecific divergence in 18S sequences (0–1.1%). No differences were detected

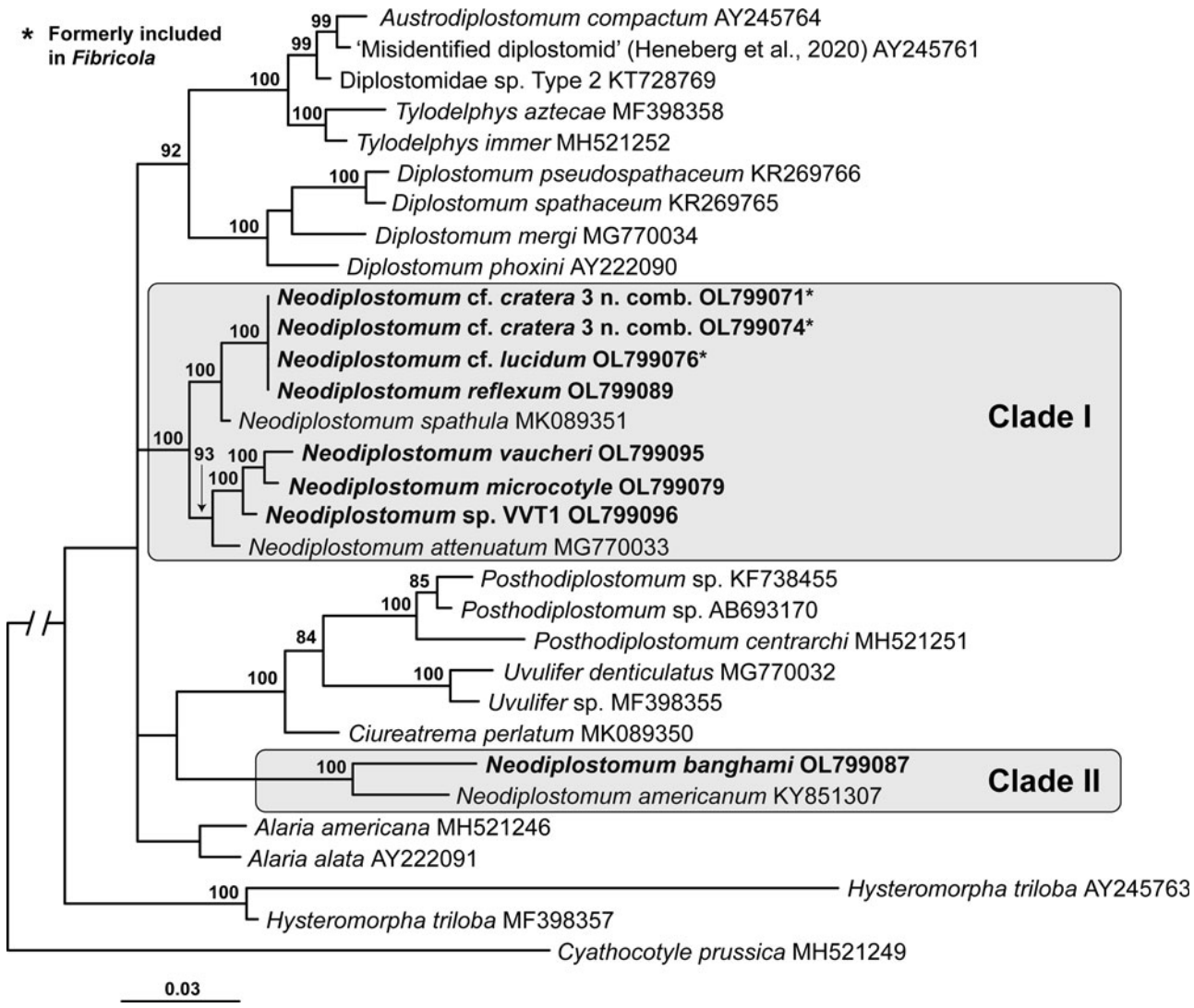

Fig. 1. Phylogenetic interrelationships among the Diplostomidae including *Neodiplostomum* (syn. *Fibricola*) based on BI analysis of partial 18S rRNA gene sequences. Members of *Neodiplostomum* are indicated by the shaded rectangles. BI posterior probability values lower than 80% are not shown. The 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.

among the 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 clade II, differed by 1.6% between their 18S sequences. No intraspecific variation was detected among the 18S sequences of *N.* cf. *cratera* 3 n. comb., *N. reflexum* and *N. americanum*. Complete pairwise comparisons of 18S sequences are provided in Table 2.

The interspecific divergence in 28S sequences of *Neodiplostomum* spp. (clade I) was similar to differences among the 18S sequences (0–1.2%). No differences were detected among the 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* had the greatest level of interspecific divergence in 28S (1.2%) among *Neodiplostomum* spp. in clade I. In contrast, the overall interspecific variability among the members of *Neodiplostomum* spp. in clade II was greater; *N. 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 3.

The interspecific divergence in ITS1 + 5.8S + ITS2 sequences of *Neodiplostomum* spp. (clade I) was greater than among the 18S and 28S sequences (0.2–6.6%). Up to 0.1% variation was detected among the ITS region sequences of *N.* cf. *lucidum* and *N. reflexum*. *Neodiplostomum reflexum* and *N. vaucheri* were the most divergent pairs of sequences among *Neodiplostomum* spp. in clade I. Up to 0.2% variation was detected among the *N.* cf. *lucidum*/*cratera* lineages. At the same time, the interspecific variability among the ITS region sequences from the two members of *Neodiplostomum* spp. in clade II was even greater (9.3–9.4%). Intraspecific variation in sequences of the ITS region was detected in *N. americanum* (up to 0.6%). The divergence between the members of clades I and II was much greater (13–16.7%). Complete pairwise comparisons of ITS region (ITS1 + 5.8S + ITS2) sequences are provided in Table 4.

Interspecific differences of *cox*1 sequences among *Neodiplostomum* spp. of clade I, excluding the *N.* cf. *cratera*/*N.* cf. *lucidum* cluster, were in the range of 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*

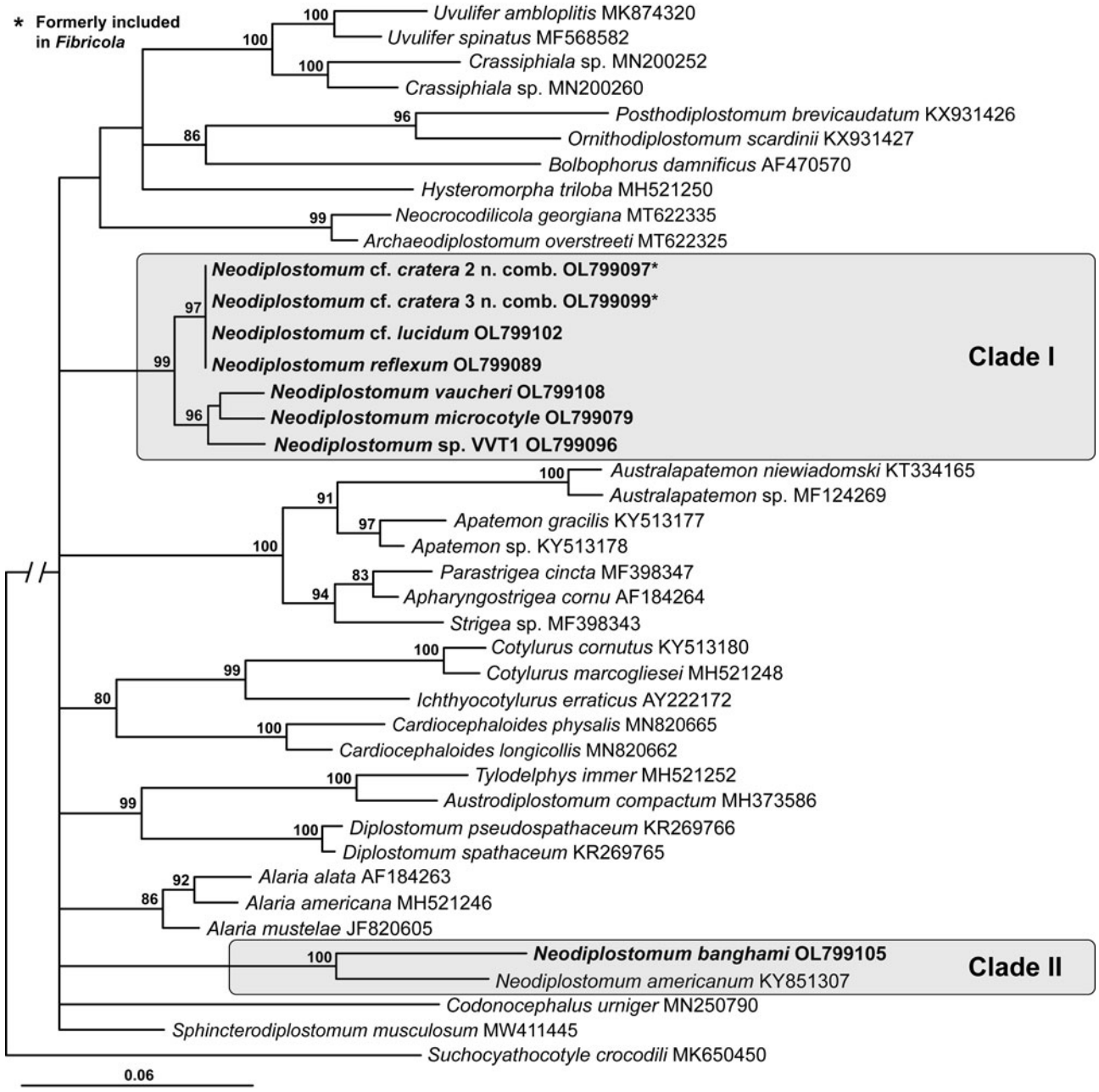

Fig. 2. Phylogenetic interrelationships among the diplostomoidean taxa including *Neodiplostomum* (syn. *Fibricola*) based on BI analysis of partial 28S rRNA gene sequences. Members of *Neodiplostomum* are indicated by the shaded rectangles. BI posterior probability values lower than 80% are not shown. 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.

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 clades I and II with more than a single sequence available showed no more than 1.6% intraspecific variation in *cox*1 sequences (Supplementary Tables S1 and S2). Complete pairwise comparisons of *cox*1 sequences of clade I *Neodiplostomum* taxa are provided in Supplementary Table S1 and clade II *Neodiplostomum* taxa in Supplementary Table S2.

Discussion

The systematic histories of *Fibricola* and *Neodiplostomum* are complex and tightly interwoven. Dubois (1932) originally established genus *Fibricola* for *F. cratera* described from muskrat *Ondatra zibethicus* (Linnaeus) by Barker (1915). Subsequently, Dubois (1937) added *Fibricola minor* Dubois, 1936 to the genus and noted an error in the topography of 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 that 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.

A third member of the genus from North America, *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

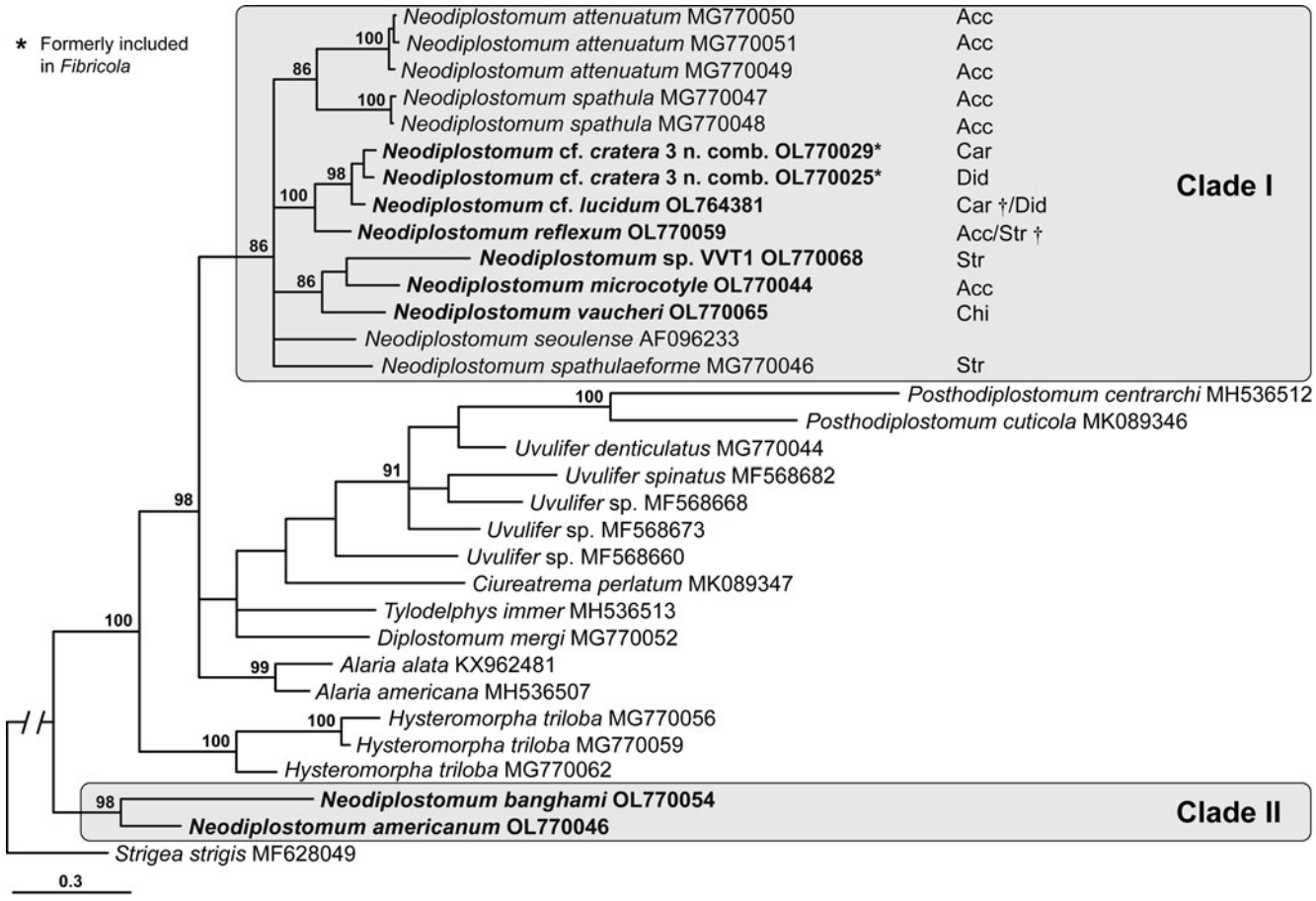

Fig. 3. Phylogenetic interrelationships among the Diplostomidae including 16 members of *Neodiplostomum* (syn. *Fibricola*) based on BI analysis of partial *cox*1 mtDNA gene sequences. Members of *Neodiplostomum* are indicated by the shaded rectangles. BI posterior probability values lower than 80% are 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. The orders of definitive hosts are provided after GenBank accession numbers for *Neodiplostomum* spp. in clade I. 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.

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.

Later, Dubois (1944) erected *Theriodiplostomum* Dubois, 1944 for *F. texensis* and *N. lucidum* from Virginia opossum *Didelphis virginiana* (Kerr) 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 and 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 the 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 and 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. Noteworthily, this species has been reported from humans in Korea (Huh *et al*., 1994). Hong and Shoop (1994) transferred this species into *Neodiplostomum* based on the morphology of adults and metacercariae. Similarly, 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 the placement of *Fibricola* as a subgenus of *Neodiplostomum* and insisted that the distribution of vitellarium and specificity to mammals were sufficient for separation between the two genera. In spite of his own statement, Dubois (1983) placed *N. vaucheri* collected from a chiropteran host into *Neodiplostomum*.

Although specificity to either mammalian or avian hosts has often been used as a distinguishing characteristic 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

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Fig. 4. Phylogenetic interrelationships among the 20 members of *Neodiplostomum* clade I based on BI analysis of partial *cox*1 mtDNA gene sequences. BI 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.

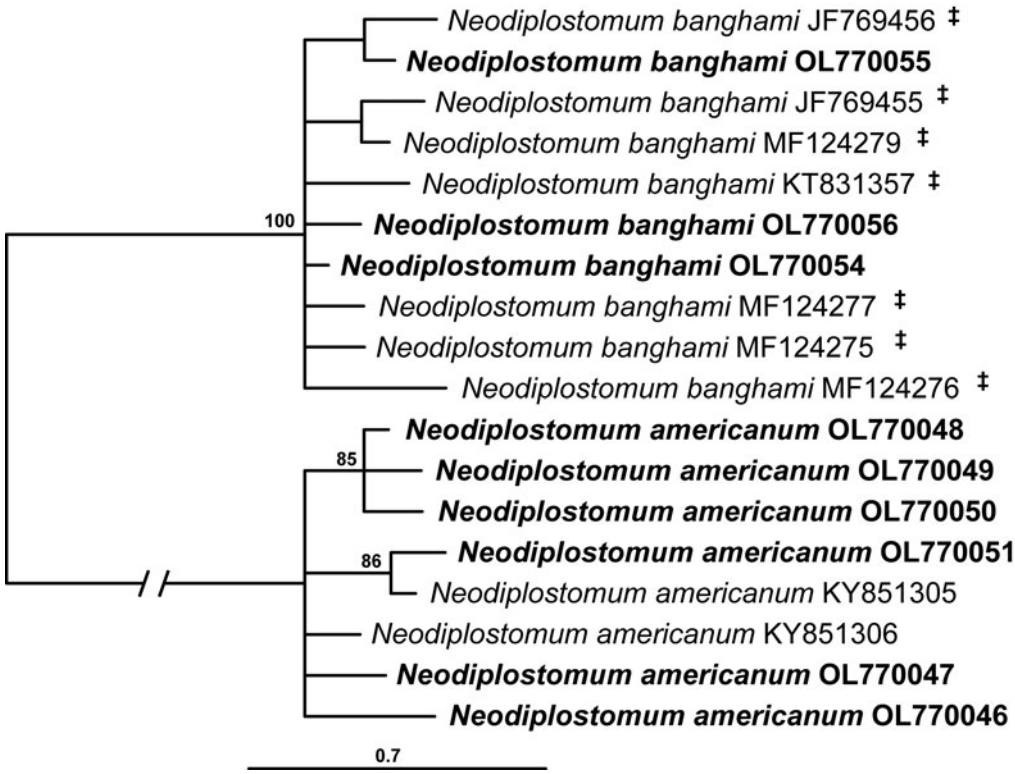

Fig. 5. Phylogenetic interrelationships among the two species of *Neodiplostomum* clade II based on BI analysis of partial *cox*1 mtDNA gene sequences. BI posterior probability values lower than 80% are not shown. The new sequences generated are indicated in bold. The scale bar indicates the number of substitutions per site. GenBank accession numbers are provided after the names of species. ‡Isolates previously identified as *Neodiplostomum americanum* in GenBank.

Table 2. Pairwise comparisons of partial sequences of the 18S rDNA among *Neodiplostomum* (syn. *Fibricola*) species included in this study based on a 1602 bp long alignment

	(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	—

Percentage differences are given above the diagonal and the number of variable nucleotide positions is given below the diagonal. Taxa previously included in *Fibricola* are denoted by *.

Table 3. Pairwise comparisons of partial sequences of the 28S rDNA among *Neodiplostomum* (syn. *Fibricola*) species included in this study based on a 1176 bp long alignment

	(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	—

Percentage differences are given above the diagonal and the number of variable nucleotide positions is given below the diagonal. Taxa previously included in *Fibricola* are denoted by *.

Table 4. Pairwise comparisons of ITS1 + 5.8S + ITS2 rDNA region among *Neodiplostomum* (syn. *Fibricola*) species included in this study based on a 1073 bp long alignment

	(1) OL799074	(2) OL799076	(3) OL799077	(4) OL799089	(5) OL799091	(6) OL799079	(7) OL799095	(8) OL799096	(9) OL799080	(10) OL799086	(11) OL799084	(12) OL799087
(1) *Neodiplostomum* cf. *cratera* 3 n. comb. VT OL799074*	—	0.2%	0.1%	0.4%	0.3%	5.9%	6.3%	4.8%	13%	13.3%	13.1%	14.9%
(2) *Neodiplostomum* cf. *lucidum* OL799076*	2	—	0.1%	0.4%	0.3%	6%	6.5%	4.9%	13.1%	13.4%	13.2%	15%
(3) *Neodiplostomum* cf. *lucidum* OL799077*	1	1	—	0.5%	0.4%	6%	6.4%	4.9%	13.1%	13.4%	13.2%	15%
(4) *Neodiplostomum reflexum* OL799089	4	4	5	—	0.1%	6%	6.5%	4.9%	13.1%	13.4%	13.2%	15%
(5) *Neodiplostomum reflexum* OL799091	3	3	4	1	—	6.1%	6.6%	5%	13.2%	13.5%	13.3%	15.1%
(6) *Neodiplostomum microcotyle* OL799079	63	64	64	64	65	—	5.3%	4.8%	14.1%	14.4%	14.2%	15.8%
(7) *Neodiplostomum vaucheri* OL799095	68	70	69	70	71	57	—	4.4%	14.4%	14.7%	14.5%	16.7%
(8) *Neodiplostomum* sp. VVT1 OL799096	52	53	53	53	54	51	47	—	13.2%	13.5%	13.3%	15.5%
(9) *Neodiplostomum americanum* OL799080	140	141	141	141	142	151	155	142	—	0.6%	0.1%	9.4%
(10) *Neodiplostomum americanum* OL799086	143	144	144	144	145	154	158	145	6	—	0.5%	9.4%
(11) *Neodiplostomum americanum* OL799084	141	142	142	142	143	152	156	143	1	5	—	9.3%
(12) *Neodiplostomum banghami* OL799087	160	161	161	161	162	169	179	166	101	101	100	—

Percentage differences are given above the diagonal and the number of variable nucleotide positions is given below the diagonal. Taxa previously included in *Fibricola* are denoted by *.

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. Shoop (1989) provided an alternative hypothesis for the subfamily structure of the Diplostomidae; in his system, *Fibricola* was placed together with *Neodiplostomum* within the Neodiplostominae Shoop, 1989 based on morphology. Recently, Achatz *et al.* (2021*c*) rejected the use of subfamilies of the Diplostomidae based on morphological and molecular data. The molecular phylogeny presented by Achatz *et al.* (2021*c*) and other recent molecular phylogenetic studies (e.g. Hernández-Mena *et al.*, 2017; Achatz *et al.*, 2019*c*, 2022, in press; Queiroz *et al.*, 2020) clearly do not support the system provided by Shoop (1989).

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 1–3). Currently, 18S and 28S sequences of *N. spathulaeforme* (type-species) are not available. The suprageneric analysis of shorter fragment of *cox*1 (Fig. 3) 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. 3) and contained only *N. americanum* + *N. banghami*. Similar patterns related to the constituents of the two *Neodiplostomum* clades (e.g. the position of *Fibricola* within clade I) were strongly supported in 18S and 28S analyses (Figs 1 and 2). The position of the type-species of *Neodiplostomum* (*N. spathulaeforme*) in the suprageneric analysis of *cox*1 (Fig. 3) clearly indicates that taxa within clade I should be considered true *Neodiplostomum*.

On the basis of 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 the 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. Notably, *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 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 usually 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 and Rausch, 1948, *N. attenuatum*, *Neodiplostomum centuri* Dubois and Macko, 1972, *Neodiplostomum isomegalocotyle* Dubois and Macko, 1972, *Neodiplostomum pearsoni* Dubois, 1962, *N. reflexum*, *N. americanum*, *N. banghami* from birds (e.g. Dubois, 1968, 1982; Dubois and Macko, 1972; current 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. 4). 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 USA, morphologically conform to the original description of *F. cratera* by Barker (1915) from *O. zibethicus* collected in Nebraska and redescribed by Dubois (1937). Because 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*. Although 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 (Supplementary Table S1). 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, it is clear that distribution of vitellarium cannot be used to distinguish between these species.

Interestingly, the morphology of samples in the cluster of *N.* cf. *cratera* 3 somewhat varied. Specimens collected in the northern USA (HWML-216766, 216767) were distinctly smaller than the sequenced specimen from Mississippi (HWML-216755). These morphologically distinct forms from geographically distant regions differed by 2.6–2.9% of nucleotide positions in *cox*1. It should be mentioned that the vertebrate hosts of these species have broad, overlapping distributions. Notably, *F. laruei*, a species synonymized with *F. cratera* (=*N. cratera*) by Read (1948), was originally described from Canada, relatively close to the area where we collected our specimens. The main characters differentiating *F. laruei* from *F. cratera* were the smaller body size and elliptical shape of the holdfast organ in the former species. The somewhat significant level of genetic divergence between the larger form from the south and smaller form from the north suggests that the validity of *F. laruei* may need to be re-visited. A definitive answer can be obtained only when DNA sequence data from the type territory of *F. laruei* (Quebec) become available and the question of morphological identity of *N. cratera* is resolved.

Our results clearly demonstrate that the sequences of *N. americanum* available in GenBank represent two distinct species (Tables 2 and 3, Fig. 3; Supplementary Table S2). 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. The morphological characteristics of *N. americanum* sequenced and deposited by Blasco-Costa and Locke (2017) closely conform to those of *N. banghami* (Supplementary Table S3). Additionally, *cox*1 sequences of *N. americanum* published by Blasco-Costa and Locke (2017) are clearly conspecific with our sequences of *N. banghami* (Supplementary Table S2; Fig. 5).

Our *cox*1 phylogeny (Fig. 3) 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*] suggest 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 placement 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 observed 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 1–3) (e.g. Achatz *et al.*, 2019*b*, 2021*a*, 2021*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; Sereno-Uribe *et al.*, 2019; Achatz *et al.*, 2020, 2021*a*, 2021*b*, 2021*c*, 2022, in press; 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 study and Achatz *et al.* (in press) further corroborate the decision by Achatz *et al.* (2021*c*). Re-evaluation of the systematics of the superfamily Diplostomoidea remains necessary, but is beyond the scope of the current study.

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Data availability. The data supporting the findings of this study are available within the article and in the Supplementary materials. All newly generated sequences were deposited in the GenBank database under the following accession numbers: OL764381, OL770020–OL770068, OL770124–OL770126 and OL799069–OL799108.

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Author contributions. TJA, EEP, ETW, TGR and VVT conceived and supervised the study. All authors involved in the collection of digeneans or their hosts. TJA, EEP, ETW, TGR, JRM and VVT conducted the morphological and molecular studies of the digeneans. TJA, JRM and VVT were responsible for writing and revising the manuscript. All authors read and approved the final manuscript.

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Ethical standards. All applicable institutional, national and international guidelines for the collecting, care and use of animals were followed.

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