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Neodiplostomum cf. seoulense (Seo, Rim, Lee, 1964) sensu Pyo *et al.*, 2014 (Trematoda: Diplostomidae Poirier, 1886): morphology, life cycle, and phylogenetic relationships

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Research Paper

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Abstract

Furcocercariae of the genus *Neodiplostomum* Railliet, 1919 (Diplostomidae Poirier, 1886) were found in freshwater snails *Helicorbis sujfunensis* Starobogatov, 1957 (Planorbidae Rafinesque, 1815) collected from three localities in the Russian southern Far East. For the trematodes from each locality, frogs played the role of the second intermediate host, and rats were the definitive host. Chickens were insusceptible to infection. The morphological and molecular data obtained for these trematodes indicated they were representatives of the same species. The experimentally-derived adult individuals were morphometrically similar to the East Asian *Neodiplostomum seoulense* (Seo, Rim, Lee, 1964), *Neodiplostomum oriolinum* Oschmarin, 1963, *Neodiplostomum leei* Chai and Shin, 2002, and *Neodiplostomum boryongense* Shin et al., 2008. Analysis of available data on the life cycle, developmental stage morphology, and molecular genetic characteristics of East Asian *Neodiplostomum* revealed a lack of information for objective assessment of the species status of neodiplostomula found in the East Asia region. Based on the considerations above and the data for the *cox1* marker, we named the trematode *Neodiplostomum cf. seoulense* (Seo, Rim, Lee, 1964) sensu Pyo *et al.*, 2014. In a phylogenetic reconstruction based on nuclear and mitochondrial markers, neodiplostomulas clustered into geographically related groups: South American, North American, European, and East Asian, with the former occupying an external position in the tree, which may indicate South America as a center of *Neodiplostomum* speciation.

Introduction

The genus *Neodiplostomum* Railliet, 1919 (Diplostomidae Poirier, 1886) comprises trematode species that parasitize birds and mammals, including humans. Of the six *Neodiplostomum* species known in the Russian southern Far East, three — *Neodiplostomum cohleare* (Kause, 1914), *Neodiplostomum spathoides* Dubois, 1937, and *Neodiplostomum spathula* (Creplin, 1960) — were recorded from various regions of the Western and Eastern Hemispheres. The other three species —*Neodiplostomum lanocolum* Oschmarin, 1963, *Neodiplostomum oriolinum* Oschmarin, 1963, and *Neodiplostomum paraoriolinum* Oschmarin, 1963 — were found in birds only in the Russian southern Far East (Skrjabin 1960; Oshmarin 1963). For *N. oriolinum*, data on the life cycle and morphology of developmental stages were obtained by Besprozvannykh (2009). In addition, based on the morphological characteristics of mature trematodes, Besprozvannykh suggested *N. oriolinum* and *N. paraoriolinum* belong to the same species. Until recently, molecular data for trematodes of the genus *Neodiplostomum*, discovered in the Russian southern Far East, have not been obtained.

From 2017–2021, we conducted studies to identify cercariae that emerged from pulmonate aquatic snails in the Russian southern Far East. As a result we found snails *Helicorbis sujfunensis* Starobogatov, 1957 infected by furcocercariae morphologically identical to representatives of *Neodiplostomum* in three localities of the study region. In order to identify the species of the found trematode, we collected data on the life cycle, developmental stage morphology, and molecular genetic characteristics from each locality.

Material and Methods

Life cycle and morphology of worms

The materials for this study were the naturally infected freshwater snails *H. sujfunensis* (Planorbidae Rafinesque, 1815) collected in the Russian southern Far East: from the drainage

basins of the Komissarovka and Arsenyevka Rivers and also from a reservoir in Russky Island (city of Vladivostok).

The infected snails, one from each locality, were placed in Petri dishes with water to obtain cercariae. Then the water containing cercariae from each Petri dish was transferred into three separate 1.5 L containers, where we had preliminarily placed snails *H. sujfunensis*, *Polypylis semiglobosa* Moskvicheva, 1980, and *Anisus centrifugops* Prozorova & Starobogatov, 1997 (five individuals of each species), juvenile fish *Phoxinus percnurus* (Pallas, 1814) (four individuals), and tadpoles *Rana dybowskii* Gunther, 1876 (seven individuals). All animals were caught in water bodies where no source of any trematodosis invasion was found. The control group consisted of 100% of the animals used in the experiment. The experimental animals were exposed to furcocercariae in the containers for 2 d. Upon completion of the exposure, the animals were kept in separate aquaria.

Subsequent necropsies showed that, of all the animals used in the experiment, only tadpoles were infected. Cercariae actively invaded the tadpole intermediate host, where they developed into metacercariae without cyst formation.

The tadpoles parasitized by 22 d old metacercariae were fed to incubated chickens and laboratory rats. We used two chickens and two rats to be infected by metacercaria from each locality. On day 8 post-infection, adult trematodes were found in the intestines of all rats. The intensity of invasion was from 3–12 mature trematodes. The chickens were not infected.

The morphology of sporocysts, cercariae, and metacercariae was examined on live specimens. The sporocyst and metacercariae were measured live, while cercariae were fixed in a hot 4% formalin solution before measurements. Adult trematodes were fixed in 70% ethanol and then transferred to 96% ethanol for storage. Whole mounts of adult specimens were prepared by staining with alum carmine, dehydration in a graded ethanol series, clearing in clove oil, and mounting in Canada balsam. All measurements were in micrometers (μm).

Euthanasia of laboratory animals was carried out in accordance with the Committee on the Ethics of Animal Experiments, Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch, Russian Academy of Sciences, Russia (Permit Number: 1 of 25.04.2022).

Molecular data

DNA extraction, amplification and sequencing

To obtain molecular data, two mature trematodes from each locality were used. Genomic DNA was extracted from these individual parasites using the HotSHOT technique (Truett *et al.* 2000). Partial sequences of the 28S rRNA gene (*28S*) nuclear DNA and partial sequences of the *cox1* gene of mitochondrial DNA were amplified by polymerase chain reaction (PCR) using specific primers (Table 1). The annealing temperature was 55°C for the *28S* marker and 50°C for the *cox1* marker. The efficiency and contamination of PCR were tested by setting positive and negative controls, respectively. The PCR products were sequenced by the Sanger method, using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, CA USA) (Sanger *et al.* 1977). Nucleotide sequences were determined on an ABI 3500 genetic analyzer (Applied Biosystems, USA) at the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch, Russian Academy of Sciences, Russia. Both external and internal primers were used for sequencing (Table 1).

Analysis of genetic data

Processing and alignment of consensus sequences were carried out using the FinchTV 1.4 and MEGA 5.0 programs (Tamura *et al.* 2011). The *p*-distances between and within species were analyzed in the MEGA software without including indels. To construct a phylogenetic reconstruction based on the 28S marker, only sequences longer than 1000 bp were taken at the National Center for Biotechnology Information (NCBI).

Phylogenetic relationships within the family Diplostomidae were assessed independently for both markers using newly obtained sequences and data for all species deposited in GenBank. Members of the trematode families located left (basal) of the Diplostomidae in the phylogenetic tree constructed by Olson *et al.* (2003) using nucleotide sequences of the *28S* and 18S rRNA genes were selected as the outgroup in our study (*Postharmostomum commutatum* (Diesing, 1858) (Brachylaimoidea (Joyeux & Foley, 1930)) was used for reconstruction based on *28S* marker and *cox1*).

Phylogenetic relationships were reconstructed using the Bayesian algorithm in the MrBayes 3.1.2. program (Ronquist & Huelsenbeck 2003) by applying a model selected as optimal on the basis of the Akaike information criterion (AIC) in the jModeltest 2.1.7 program (Darriba *et al.* 2012): TVM+I+G for *28S* and GTR+I+G for *cox1*. The method of posterior probabilities was used for the trees constructed by the Bayesian algorithm. In the Bayesian analysis, 4,700,000 and 6,000,000 generations of the Markov chain Monte Carlo posterior third (MCMC) were simulated for *28S* and *cox1*, respectively. The number of generations was determined to be sufficient, since the SD value calculated was < 0.01. The chain

Table 1. List of primers for amplification and sequencing

DNA region	Primer	Sequence 5′→3′	Direction	Reference
28S	digl2	AAGCATATCACTAAGCGG	forward, external	Tkach *et al.* (2003)
	1500R	GCTATCCTGAGGGAAACTTCG	reverse, external	Tkach *et al.* (2003)
	900F	CCGTCTTGAAACACGGACCAAG	forward, internal	Tkach *et al.* (2003)
	1200R	CTTGGTCCGTGTTTCAAGACGGG	reverse, internal	Tkach *et al.* (2003)
cox1	JB3	TTTTTTGGGCATCCTGAGGTTTA	forward, external	Morgan & Blair (1998)
	JB4.5	TAAAGAAAGAACATAATGAAAATG	reverse, external	Bowles & McManus (1994)

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was sampled every 100 generations. Of the samples assessed, 25% were excluded to permit construction of consensus trees.

For the reconstruction of phylogenetic trees, aligned sequences of 1,133 bp and 318 bp were used for *28S* and *cox1*, respectively.

Results

Morphology data

Neodiplostomum cf. seoulense (Seo, Rim, Lee, 1964) sensu Pyo *et al.*, 2014.

Definitive host: Rattus norvegicus Berkenhout, 1769 (experimentally).

Site: small intestine.

First intermediate host: Helicorbis sujfunensis.

Second intermediate host: Rana dybowskii (experimentally).

Site: visceral muscle tissue.

Locality: backwater in basin of the Komissarovka River, 44°49′N, 131°33′E, Arsenyevka River, 44°38′N, 133°46′E and lake on the territory of the Russian Island (Vladivostok city), 43°01′N, 131°90′E, Primorsky Region, the Russian southern Far East.

Adult worm (6 experimentally-derived specimens) (Figure 1A, Table 2). Body distinctly bipartite. Prosoma spatulate, with well-developed ventral concavity, covered with numerous spines. Opisthosoma cylindrical. Pseudosuckers absent. Oral sucker subterminal, round. Prepharynx short. Pharynx well-developed, round. Oesophagus extremely short. Intestinal bifurcation in middle between oral sucker and ventral sucker. Caeca extend to near posterior end of body. Ventral sucker round, equal to the oral sucker, located just anterior to holdfast organ or partially covered by its front edge, or in front of it at short distance. Holdfast organ large, circular, with median longitudinal slit. Testes of similar size, dumbbell-shaped, tandem lay in opisthosoma. Seminal vesicle curved, lay posterior testis. Ovary oval, pretesticular, median, situated close to borderline between prosoma and opisthosoma. Mehlis' gland lay at level of middle of anterior testis. Seminal receptacle located to left of ovary. Uterus occupying middle field of posterior segment. Eggs oval, with a thin transparent shell. Vitelline fields contain small irregular follicles, begin at level of intestinal bifurcation, or at middle of distance between ventral sucker and intestinal bifurcation, or at level of anterior edge of ventral sucker. In opisthosoma vitelline follicles are aggregated in six longitudinal ribbon fields (three on each side of median line). In prosoma vitellarium formed by two merged median fields. Copulatory bursa opened dorsally, located near posterior end of body.

Sporocyst. Body threadlike. Genital pore terminal. Sporocysts contain cercariae of different stages of development.

Cercaria (based on 30 specimens) (Figure 1D–F, Table 3). Body in shape of an elongated oval, covered with small spines, filled with numerous granular formations. Oral organ round. Prepharynx short. Pharynx round. Oesophagus long, reaches border anterior and middle thirds body. Intestinal bifurcation anterior to ventral sucker. Intestinal branches short, end at a short distance from posterior edge of the ventral sucker. Glands present by two pairs of cells located at level of ventral sucker. Ducts glands open at anterior end of body. Excretory system includes small excretory

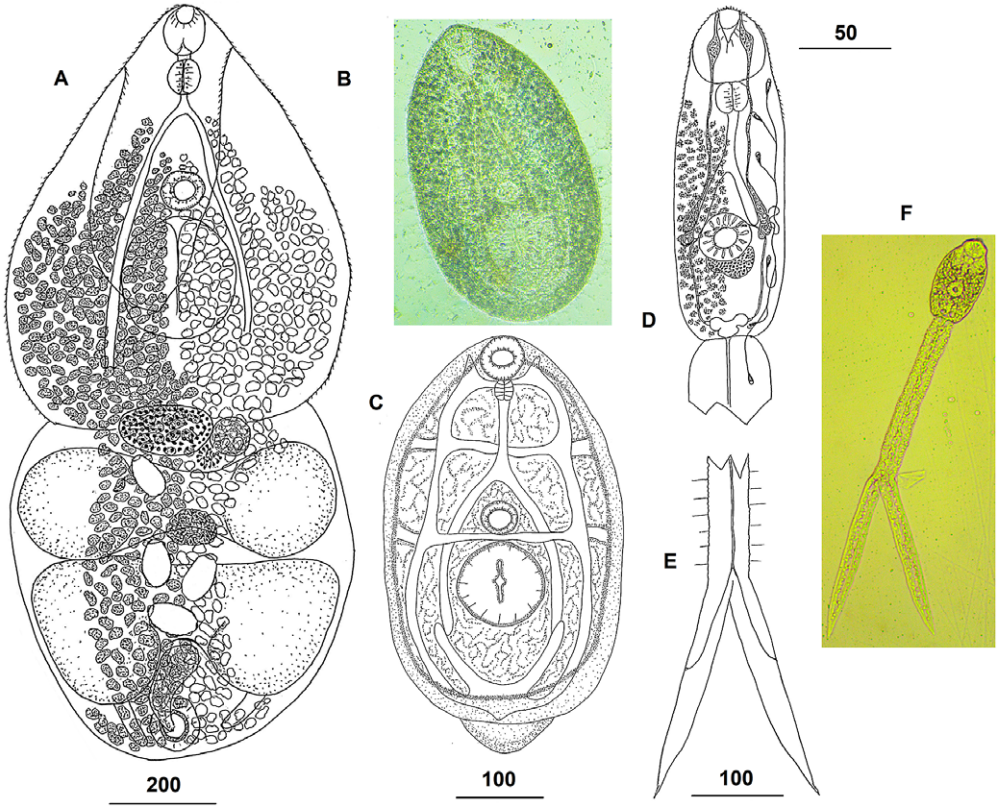

Figure 1. *Neodiplostomum cf. seoulense* (Seo, Rim, Lee, 1964) sensu Pyo *et al.*, 2014: A, adult worm; B–C, metacercaria; D, F, cercaria (body); E, F, cercaria (tail). Scale-bars: μm.

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Table 2. Measurements of adult *Neodiplostomum* flukes (μm)

Species	Neodiplostomum seoulense					Neodiplostomum oriolinum		Neodiplostomum boryongense	Neodiplostomum leei
Source	Present study	Seo *et al.* (1964)	Seo *et al.* (1982)	Hong *et al.* (1982)	Seo *et al.* (1988)	Besprozvannykh (2009)	Oshmarin (1963)	Shin *et al.* (2008)	Chai & Shin (2002)
Host	Albino rats	House rats	Man	Albino rats	Chicken	Chicken	*Oriolus chinensis* Linnaeus, 1766	Chicken	Chicken
Body length	1460–1490	788–1660	756–1277	1100–1920	2182–2835	1080–1680	1980–2610	1725–2075	1121–1629
Prosoma length	800–910	749–788	391–630	481–962	910–1374	670–1030	1134–1260	730–875	645–1030
Prosoma width	640–650	630–798	356–497	511–696	738–1116	640–820	900	725–1100	491–790
Opisthosoma length	560–660	670–867	356–597	570–962	979–1288	630–750	846–900	950–1200	551–918
Opisthosoma width	620–670	532–658	292–497	398–583	601–979	560–680	650–684	625–725	468–795
Oral sucker length	80–100	73–110	30–55	44–81	85–118	80–100	140–155	68–88	52–106
Oral sucker width	90–100	69–79	32–71	57–95	77–103	80–100	155–160	53–88	45–97
Pharynx length	62–81	73–99	33–71	55–71	75–85	61–67	95–102	55–73	46–63
Pharynx width	58–81	46–53	27–47	35–55	42–68	67–72	70–95	30–63	26–80
Ventral sucker length	70–80	69–70	38–63	47–92	89–104	90–100	140–164	60–88	61–112
Ventral sucker width	70–80	89–99	46–65	87–98	96–135	90–100	170–178	88–113	85–150
Holdfast organ length	240–310	250–294	146–232	–	306–477	280–310	400–415	310–400	149–299
Holdfast organ width	250	238	179–298	–	238–340	240–310	280–360	300–380	186–387
Anterior testis length	150–220	210–252	86–186	126–219	257–377	230–250	225–235	250–420	58–316
Anterior testis width	510–560	420–658	239–424	298–497	579–807	470–500	450–590	610–720	380–650
Posterior testis length	160–219	210–546	93–166	159–279	313–481	220–290	225–235	250–430	78–438
Posterior testis width	500–540	378–532	219–378	272–431	511–755	380–560	450–590	560–660	189–336
Ovary length	100–260	42–140	66–197	66–100	115–170	110–160	160–170	100–168	81–167
Ovary width	160–260	210–280	119–239	133–252	272–347	200–260	250–270	250–330	140–358
Eggs length	90–100	72–86	86–99	–	55–92	100–110	70–94	88–90	80–93
Eggs width	50–58	50–56	55–63	–	49–54	56–61	50–56	53–60	49–58
Ventral/oral sucker length ratio	0.80–0.87	–	–	–	–	–	–	–	–
Ventral/oral sucker width ratio	0.77–0.80	–	–	–	–	–	–	–	–

Table 3. Measurements of larval stages of *Neodiplostomum* (μm)

Species	Neodiplostomum seoulense		Neodiplostomum oriolinum
Source	Present study	Seo *et al.* (1988)	Besprozvannykh (2009)
Cercaria			
Body length	175–200	80–121	110–130
Body width	45–95	34–57	42–60
Oral organ length	30–45	27–34	25–27 diameter
Oral organ width	25–45	25–30	
Pharynx length	15–17	–	11
Pharynx width	15–18	–	13
Ventral sucker length	25–30	20–25	17–20 diameter
Ventral sucker width	25–30	20–27	
Tail stem length	250–325	138–175	220–260
Tail stem width	45–50	25–42	35–45
Furcae length	205–265	139–185	200
Furcae width	20–40	–	20–30
Metacercaria			
Body length	450–750	–	390–410
Body width	250–600	–	320–410
Oral sucker length	50–65	46	56–67
Oral sucker width	50–60	41	45–67
Pharynx length	25–35	28	28–34
Pharynx width	25–30	20	22–39
Ventral sucker length	30–50	39	39–50 diameter
Ventral sucker width	35–50	44	
Holdfast organ length	98	–	56–78
Holdfast organ width	101	–	45–84

bladder, first-order ducts connected by transverse commissure at level ventral sucker, second-order ducts and caudal duct. Caudal duct splits up in front of furcae into two canals reaching middle of furcae, where it opens with pores. Tail stem with sensitive hair. Furcae equal to tail stem. Excretory formula 2[(1 + 1 + 1) + (1 + 1 + [1])] = 12.

Metacercaria (based on 30 specimens) (Figure 1B–C, Table 3). Body consists of large prosoma and small opisthosoma. Opisthosoma is not expressed in all individuals. Prosoma is leaf-shaped, gray due to pigmentation. Oral sucker round. Prepharynx absent. Pharynx small round or oval. Oesophagus long, reaches border anterior and middle thirds body. Intestinal bifurcation on the border of the anterior and middle thirds of the body. Intestinal branches reach anterior edge of excretory bladder. From excretory bladder, four ducts diverge, two of them blind and reach holdfast organ, and the other two form longitudinal channels connected transverse commissures form network of channels.

Molecular data

For all the trematodes obtained in this study, the lengths of the obtained nucleotide sequences were as follows: 1,187 bp for *28S* and 446 bp for *cox1*. The nucleotide sequences of the *28S* marker for the samples from Russky Island and the Arsenyevka River basin had a double peak (C and T) at position 176 bp. The nucleotide sequences of the *28S* marker for the sample from the Komissarovka River basin had a bp indel of one base pair at a position of 560 bp, and *p*-distances between these sequences were 0%.

The nucleotide sequences of the mtDNA *cox1* gene for four samples from the Komissarovka and Arsenyevka rivers were 100% identical, while both samples from Russky Island differed from them by one G/T nucleotide substitution at position 115 bp (0.3%). The samples from the latter locality were identical to each other.

In the phylogenetic reconstruction based on the nuclear marker *28S*, representatives of the family Diplostomidae are divided into 16 genera (Figure 2). As for the phylogenetic reconstruction of Diplostomidae relationships using the mitochondrial marker *cox1*, the distribution of species over genera is consistent to that based on the *28S* marker (Figure 2, 3).

The genus *Neodiplostomum* in the reconstructions based both on the *28S* marker and on the *cox1* marker is represented by two groups with high statistical support (Group 3 and Group 4) (Figure 2, 3). The genetic distances between these groups in the reconstructions correspond to the intergeneric level (5.56% for *28S*; 16.38% for *cox1*).

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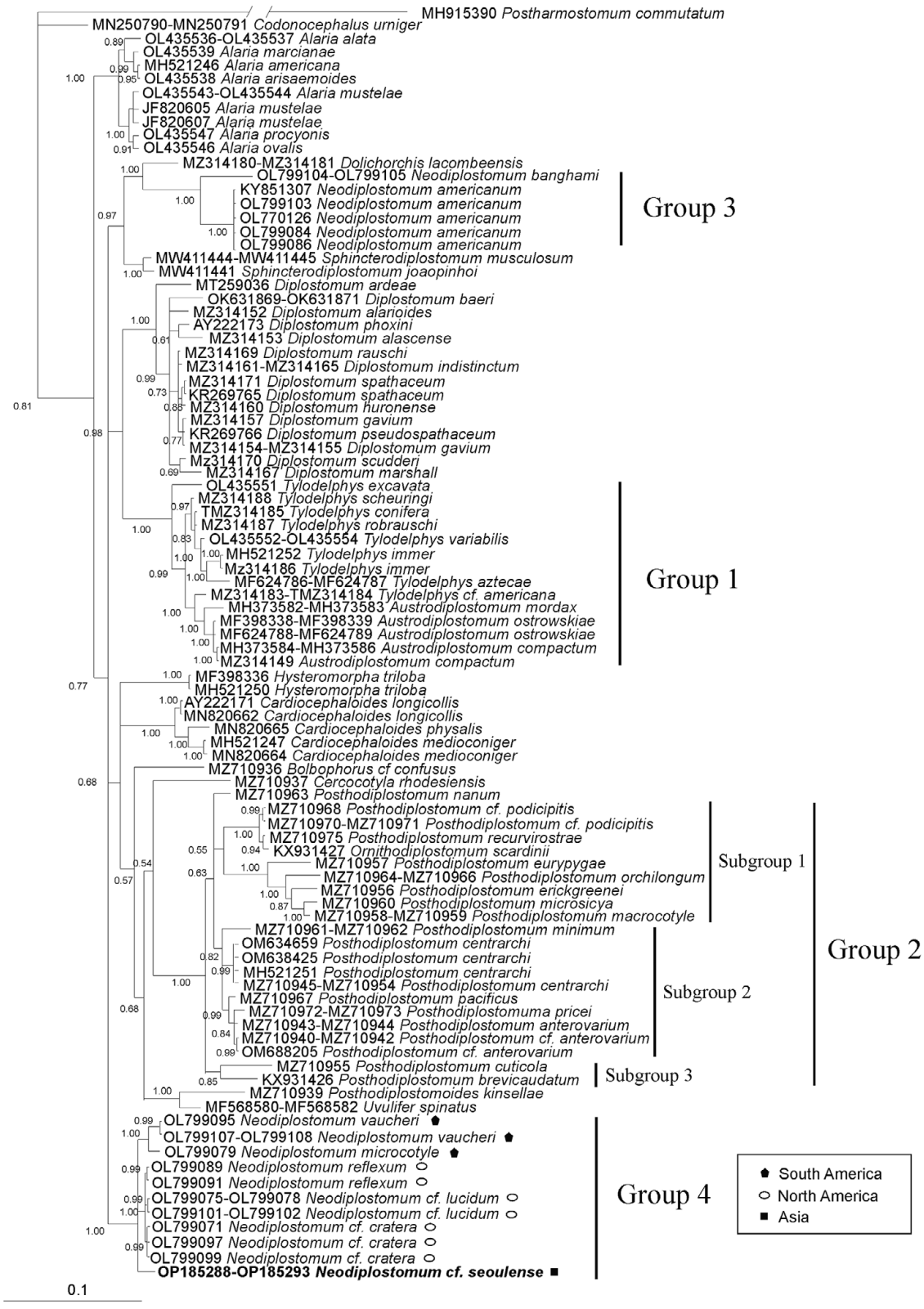

Figure 2. The phylogeny reconstruction based on *28S* sequences using the Bayesian algorithm. The outgroup is *Postharmostomum commutatum.*

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Figure 3. The phylogeny reconstruction based on *cox1* sequences using the Bayesian algorithm. The outgroup is *Postharmostomum commutatum.*

Discussion

Life cycles, morphology and taxonomy

Based on the morphological features of mature trematodes, cercariae, and metacercariae, and also data on the life cycle, the trematodes that we found correspond to representatives of the genus *Neodiplostomum.* For six species from this genus, the type localities are in the East Asia region: *N. lanocolum*, *N. oriolinum*, *N. paraoriolinum* (discovered in the Russian southern Far East), *N. seoulense* (Seo, Rim, Lee, 1964), *N. leei* Chai & Shin, 2002, and

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N. boryongense Shin *et al.*, 2008 (discovered in South Korea) (Skrjabin 1960; Oshmarin 1963; Seo *et al.* 1964; Chai *et al.* 2002; Shin *et al.* 2008).

The adult trematodes that we analyzed were morphologically similar both to the *N. oriolinum* and to *N. seoulense, N. boryongense,* and *N. leei.* The worms of the first three species listed above differ from *N. leei* in the extension of the vitelline fields in the prosoma of the body: the fields reach the level of the pharynx (*N. leei*) vs. may reach the level of intestinal bifurcation (*N. oriolinum, N. seoulense,* and *N. boryongense*). At the same time, the trematodes in our material were similar in most metric parameters to *N. seoulense* described by Seo *et al.* (1964), Seo *et al.* (1982), Hong *et al.* (1982), *N. oriolinum* by Besprozvannykh (2009), and *N. leei* by Chai & Shin (2002), but differ from *Neodiplostomum oriolinum* specimens described by Oshmarin (1963) *N. seoulense* by Seo *et al.* (1988), and *Neodiplostomum boryongense* by Shin *et al.* (2008) (Table 2, 3). For *N. oriolinum* and *N. seoulense*, in addition to data on the morphology of mature individuals, information was obtained on the morphometry of cercariae and metacercariae (Table 3) and the life cycle of these species. The first intermediate host for these trematode species is snails of the family Planorbidae, in particular snails of the genera *Helicorbis* Benson, 1855 and *Polypylis* Pilsbry, 1906 for *N. oriolinum* (*Helicorbis* for trematodes that we found) and *Hippeutis* Charpentier, 1837, *Segmentina* Fleming, 1818 for *N. seoulense.* The second intermediate host of *N. oriolinum* it is frogs (as the trematodes that we found); for the three *Neodiplostomum* species from South Korea the second intermediate hosts are frogs and snakes. Birds and mammals are the definitive hosts for *N. oriolinum*, mammals for *N. seoulense* (as the trematodes that we found), and birds for *N. leei* and *N. boryongense* (Seo *et al.* 1988; Chai *et al.* 2002; Shin *et al.* 2008; Besprozvannykh 2009). When studying the life cycle of *N. oriolinum* in one experiment, we identified the stages of trematode development from cercaria to the sexually mature stage and obtained morphometric data for cercariae, metacercariae, and mature individuals (Besprozvannykh 2009). Information about the life cycle of *N. seoulense* is based on summarized data from studies that provided descriptions of certain life cycle stages of this species. (Seo *et al.* 1964; Seo *et al.* 1982; Lee *et al.* 1986; Seo *et al.* 1988; Seo *et al.* 1989; Seo 1990; Chung *et al.* 1996; Chung *et al.* 2002; Chai *et al.* 2002; Chai *et al.* 2007; Shin *et al.* 2008; Pyo *et al.* 2014). In all of these studies, metacercariae from naturally infected snakes were the initial source for life cycle reconstruction. As the authors point out, a mixed invasion of metacercariae by neodiplostomula was revealed in snakes. Among the found metacercariae, small ones were selected and fed to rats, mice, and chickens. Based on the results of these experiments, it was concluded that the sexually mature individuals in the intestines of rats and mice belonged to *N. seoulense.* The experimentally obtained trematodes showed morphological similarity to *N. seoulense* discovered Seo *et al.* (1964) in naturally infected rats. Other trematodes in these experiments penetrated into the liver of rats or localized in the intestines of mice, where both in the former and latter cases they remained juvenile and reached sexual maturity only after being fed to chickens. These worms, morphologically similar to *N. seoulense*, were referred to as *N. leei.* Mature trematodes that grew in the intestines of chickens from metacercariae extracted from snakes and did not have significant morphological differences from *N. seoulense* were referred to as *N. boryongense.* The main morphological criterion used by Pyo *et al.* (2014) for distinguishing South Korean *Neodiplostomum* is the level of extent of vitelline fields in the prosoma of the body and the structure of the testes. However, Seo *et al.* (1988) and Besprozvannykh (2009) showed significant variability in these characters for *N. seoulense* and *N. oriolinum*, respectively. Thus, the main criterion for identifying Korean neodiplostomula species is the feature of their development from metacercaria to the mature stage in infected mammals and birds. During the experimental growing of mature worms from metacercariae from naturally infected intermediate hosts, it is impossible to simultaneously infect a rat and a chicken with the same metacercariae and, therefore, it is not legitimate to claim that trematodes that reached sexual maturity in rats could not have developed before this stage in chickens. For example, it was reported for *N. oriolinum* that metacercariae obtained from cercariae from a single snail were able to develop to the sexually mature stage both in chickens and in rats (Besprozvannykh 2009). There is also information on mature *N. seoulense* grown in chickens that had been fed metacercariae from naturally infected snakes (Seo *et al.* 1988).

The work of Seo *et al.* (1988) is the only study available to date that has attempted a complete description of the life history of *N. seoulense.* However, this study, as well as other life cycle data obtained in South Korea for neodiplostomula, do not answer the question of accurate species identification of *Neodiplostomum* individuals. The authors divided the study of the cycle into two stages. At one of the stages, eggs were obtained from sexually mature individuals grown in rats from metacercariae from naturally infected snakes. Snails were infected with miracidia from these eggs, and cercariae were obtained. At the other stage, second intermediate hosts, the amphibians, were infected with cercariae from naturally infected snails, after which metacercariae from amphibians were fed to rats, and mature trematodes were obtained. With this approach to studying the life cycle, there is no link between the first and second stages of the study, which, as a consequence, does not exclude the probability that the trematodes obtained at each stage belong to different species. Along with this, Seo *et al.* (1988) point out that the emission of cercariae from snails began on the 13th day after the infection of the latter with miracidia, which is impossible and may indicate errors in the experiment setup. In view of the above, one can state that there is no objective information that would allow species identification of South Korean neodiplostomulas obtained through experimental studies, in particular, individuals classified as *N. seoulense.* Thus, based on the results of the studies, not only *N. seoulense* but also other species capable of infecting mammals such as *N. oriolinum* may be present in the fauna of neodiplostomula of South Korea. This may also be evidenced by a number of differences in metrics of the body, oral and ventral suckers, pharynx, and eggs between trematode samples indicated as *N. seoulense* (Table 2). As mentioned above, the marites in our material that were morphologically similar to *N. seoulense* reached sexual maturity in rats and did not infect chickens. At the same time, there were significant differences in the size of cercariae between our samples and *N. seoulense.* The cercariae that we used to obtain sexually mature trematodes differed in size of tail, furcae, and body length, and were also distinguished by large maximum sizes of oral and ventral suckers and body width compared to both Korean *N. seoulense* cercariae and Far Eastern *N. oriolinum.* Such significant metric differences may indicate that either, based on the above-mentioned method of life cycle reproduction, the cercariae of Korean neodiplostomula do not belong to *N. seoulense*, or the trematodes in our material belong to another *Neodiplostomum* species.

Nucleotide sequences for the *cox1* marker of the samples from the localities of the Kommisarovka and Arsenievka rivers were identical to those of the specimen indicated as *N. seoulense*

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(AF096233) in the NCBI. At the same time, there is no publication for AF096233 that confirms the species identification of this specimen. A publication by Pyo *et al.* (2014) provides the nucleotide sequence of the *cox*1 region for three Korean mature individuals identified as *N. seoulense*, *N. leei*, and *N. boryongense* (absent in the NCBI). However, the data published by Pyo *et al.* (2014) are questionable. Since the mitochondrial genome is homozygous, the presence of polymorphic sites in the sequence for the *N. boryongense* specimen may be associated with the presence of heteroplasmy in it, which could be detected by cloning. However, in this case, it is not correct to combine them into a consensus sequence. All variants of the nucleotide sequences should be presented at cloning. Regardless, it is impossible to interpret the presented data unambiguously. In addition, in the sequences of the samples designated as *N. leei* and *N. seoulense*, there are indels that are not multiples of three, and, since these are protein-coding sequences, such insertions also indicate the incorrectness of the presented results. Thus, the data described in this study, in our opinion, cannot be reliable evidence of genetic differences between the three indicated species, and additional molecular data are required to finally resolve this issue. Nevertheless, due to the fact that partial sequences of the *cox1* that we obtained have a 249-bp region identical to the sample designated in the study by Pyo et al. (2014) as *N. seoulense*, and the adult worms are similar morphologically, we suggest that the trematodes found in the south of the Russian Far East should be considered as *Neodiplostomum cf. seoulense* (Seo, Rim, Lee, 1964) sensu Pyo et al., 2014.

However, in our opinion, to objectively validate the species identification of the representatives of *Neodiplostomum* circulating in the East Asian region, additional studies are needed, including data about morphology and experiments growing metacercariae from cercariae and sexually mature trematodes from metacercariae in the same experiment. In this case, all morphological characteristics should be supported by nuclear and mitochondrial DNA markers of these trematodes.

Phylogenetic relationships

In constructing the phylogenetic reconstruction based on the nuclear marker 28S, a number of groups of trematodes uniting worms belonging to different genera are distinguished (Figure 2). One of these groups (Group 1) includes *Tylodelphys* spp. and *Austrodiplostomum* spp. At the same time, samples MZ314183–MZ314184 designated as *Tylodelphys cf. americana* (Dubois, 1936) are clustered separately from other *Tylodelphys* Diesing, 1850 sequences and occupy a basal position relative to *Austrodiplostomum* Szidat & Nani, 1951. Genetic distances constitute 1.32% between *Tylodelphys cf. americana* and *Austrodiplostomum* spp. and 1.36% between *Tylodelphys cf. americana* and *Tylodelphys* spp., corresponding to interspecific distances in the reconstruction. Differentiation between the species included in this group is not greater than 2.8%, whereas the smallest intergeneric distance among other representatives of Diplostomidae reaches 3.35% between *Alaria* spp. and *Neodiplostomum* spp., which are well distinguished at the morphological level (Group 4). The presence of specimens belonging to *Tylodelphys* spp. and *Austrodiplostomum* spp. in a single group agrees with the results obtained by Achatz *et al.* (2022a). Based on the reconstruction using the *cox1* marker, the authors also found an intrageneric level of differences between *Austrodiplostomum* spp. and *Tylodelphys* spp. However, in view of the existing morphological differences between members of these genera (Niewiadomska 2002)—reduced ventral sucker or no ventral sucker at all and the lack of a genital cone vs. small, but well developed ventral sucker and a small genital cone respectively—Achatz *et al.* (2022a) left the question of the level of relationship to *Austrodiplostomum* and *Tylodelphys* pending additional data on the life cycles, developmental stage morphology, and molecular characteristics of these trematodes. Furthermore, it is necessary to take into account the known discrepancies between morphological and molecular characteristics in trematodes. For example, among Haplosplanchninae Poche, 1926, *Pseudohaplosplanchnus catbaensis* Atopkin et al., 2020 are similar to *Haplosplanchnus* Looss, 1902 in morphological characteristics, but by molecular characteristics this trematode is a representative of a different genera (Atopkin *et al.* 2020). The situation with *Unisaccus* Martin, 1973 (Haploporinae Nicoll, 1914) representatives is opposite (Atopkin *et al.* 2022). Based on the above, we suggest that, in resolving the issue of relationships of between *Austrodiplostomum* spp. and *Tylodelphys* spp., priority should be given to molecular indicators that confirm belonging of these trematodes to the same genus rather than to morphological differences.

In another group (Group 2) with representatives of *Posthodiplostomum* Dubois, 1936, specimen KX931427 indicated as *Ornithodiplostomum scardinii* (Shulman, 1952) is clustered. This specimen is differentiated from *Posthodiplostomum* spp. at an interspecific level (Figure 2). Considering that the molecular data for KX931427 were obtained from metacercariae, the species identification of this specimen, due to the morphological similarity of closely related trematodes at this stage, may be incorrect. Therefore, we assume that the trematodes indicated as *O. scardinii* belong to the genus *Posthodiplostomum.* Achatz *et al.* (2021) discussed this earlier in their work. Thus, our data confirm the results published by Achatz *et al.* (2021). In general, Group 2, which includes worms attributed to the genus *Posthodiplostomum*, can be considered a group with complex phylogenetic relationships. In Group 2, several subgroups are distinguished (Subgroup 1, Subgroup 2, Subgroup 3) (Figure 2). Genetic distances between subgroups vary within 3.10–3.52%, comparable to the above-indicated minimum intergeneric distance for the *28S* marker (3.35%). In addition, if we consider the mitochondrial marker (*cox1*), then the variability among the *Posthodiplostomum* specimens reaches 13.43%. Furthermore, intergeneric distances within Diplostomidae for this marker range from 13.13 (*Alaria* Schrank, 1788 vs. *Neodiplostomum* (Group 4) to 23.3% (*Posthodiplostomum* vs *Hysteromorpha* Lutz, 1931). Thus, based on the presented molecular data, we assume that the trematodes from Subgroups 1, 2, and 3 may be representatives of different genera within the Diplostomidae.

In the phylogenetic reconstruction of Diplostomidae relationships using the mitochondrial marker *cox1*, the distribution of species over genera is consistent to that based on the *28S* marker (Figure 2, 3). In our opinion, this may indicate the objectivity of molecular data on *28S* to determine the level of relationship between representatives of Diplostomidae, despite some unresolved taxonomic issues.

As for the division of the genus *Neodiplostomum* two groups (Group 3 and Group 4) on both reconstructions (Figure 2, 3), this separation was previously noted by Achatz *et al.* (2022b). Since the type species of *Neodiplostomum* (*Neodiplostomum spathulaeforme* (Brandes, 1888)) is included in Group 4 in the *cox1*-based reconstruction, we consider specimens from this group as true *Neodiplostomum*, while *Neodiplostomum americanum* Chandler and Rausch, 1947 and *Neodiplostomum banghami* Penrod, 1947 should be attributed to a different genus. In this case, as mentioned above, there is a discrepancy between morphological and molecular

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characteristics. Mature *N. americanum*, as well as *N. banghami*, are morphologically similar to *Neodiplostomum*, but molecular data indicate that they belong to a separate genus, which, according to the currently available data, is differentiated from Group 4 *Neodiplostomum* only at the molecular level. It is possible that some non-molecular criteria for differentiating the specimens indicated as *N. americanum* and *N. banghami* from the true *Neodiplostomum* can also be identified based on the morphology of their cercariae or the characteristics of their life cycles. The belonging of *N. americanum* and *N. banghami* to a separate genus is also evidenced by the indicators of genetic distances (4.5–7.89% for *28S*; 15.4–21.5% for *cox1*) between them and representatives that are not included in *Neodiplostomum*.

Along with this, an analysis of the phylogenetic relationships of *Neodiplostomum* (Group 4) based on data on the *28S* and *cox1* markers has shown that both reconstructions combine into separate subgroups the *Neodiplostomum* species circulating in geographically remote regions: South America (*Neodiplostomum vaucheri* Dubois, 1983 and *microcotyle* Dubois, 1937), North America (*Neodiplostomum cf. cratera* (Barker & Noll, 1915), *Neodiplostomum reflexum* Chandler and Rausch, 1947, and *Neodiplostomum cf. lucidum* (La Rue & Bosma, 1927)), Europe (*Neodiplostomum spathulaeforme*, *Neodiplostomum attenuatum* (Linstow, 1906)), and *Conodiplostomum spathula* (*Neodiplostomum spathula* according to Heneberg *et al.* (2020) (Creplin, 1829)), and the East Asian region (*N. seoulense* and *Neodiplostomum cf. seoulense* sensu Pyo *et al.* 2014). Taking into account the external position of South American neodiplostomula relative to North American, European, and East Asian species in the reconstruction based on a mitochondrial marker and relying on the less conserved *cox1* gene compared to *28S*, it can be assumed that the speciation center of *Neodiplostomum* was in South America. However, to finally resolve this issue, more molecular data for neodiplostomula from different regions are required.

Data availability. The data supporting the findings of this study are available within the article. All newly generated sequences were deposited in the GenBank database under the following accession numbers: OP185288–OP185293, OP185212–OP185217.

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Competing interest. The authors declare no conflict of interest.

Ethical standard. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals including birds and mammals.

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