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Research Article


New species of Asymphyloporinae Szidat, 1943 (Digenea: Lissorchiidae), fish parasites from the East Asian Region: morphological and molecular data

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We generated new morphological and molecular data on some trematodes of the family Lissorchiidae from fish from the south of the Russian Far East. Four new species, including two species previously considered *Parasymphyloporina japonica* and *P. markewitschi* in the Far East, were established on the basis of molecular data and morphological characteristics of adult worms and metacercariae. Molecular phylogenetic analysis based on partial 28S rDNA sequences showed that the subfamily Asymphyloporinae was monophyletic, while the subfamily Lissorchiinae was polyphyletic within the Lissorchiidae, confirming the results of previous studies on this family. All asymphyloporine trematodes from our phylogenetic analysis were sorted into several groups, which differ from each other at the intergeneric level by molecular data but could not be confidently delimited based on morphological characteristics. For this reason, we consider all trematodes from Asymphyloporinae within two existing genera, *Parasymphyloporina* and *Asymphyloporina*, both *sensu lato*. The first molecular data were generated for *Asymphyloporina macrocetabulum*, the type species of the genus.

<http://zoobank.org/urn:lsid:zoobank.org:E2800CDB-D2EF-4DA3-8912-07BFBE47E1E0>

Key words: *Asymphyloporina*, Asymphyloporinae, *Asymphyloporina*, East Asian Region, fish parasites, Lissorchiidae, *Parasymphyloporina*, 28S

Introduction

The genus *Asymphyloporina* Looss, 1899 includes species formally described in the genus and reassigned species from the genera *Parasymphyloporina* Szidat, 1943 and *Orientotrema* Tang, 1962 (Bray, 2008). At present, five species of *Asymphyloporina* are recognized in East Asia: *A. innominata* (Faust, 1924), *A. japonica* Yamaguti, 1938, *A. stenothyrae* Tang, 1980, *A. percotti* Besprozvannykh et al., 2012 and *A. markewitschi* Kulakowskaja, 1947 (Besprozvannykh, 2005; Besprozvannykh et al., 2012; Shimazu, 2016). The type locality of the former four species is located in East Asia, while *A. markewitschi* was first described in Europe (Kudlai, 2010; Kulakowskaja, 1947). *Asymphyloporina innominata* and *A. japonica* are

fish intestinal parasites in Japan (Shimazu, 2016) and China; in addition, *A. japonica* was also reported parasitizing fish from the south of the Russian Far East under the name *Parasymphyloporina japonica* (Besprozvannykh, 2005). *Asymphyloporina stenothyrae* has been reported only from China (Tang, 1980). *Asymphyloporina markewitschi* (under the name *Parasymphyloporina markewitschi*) and *A. percotti* were found in freshwater fish in the south of the Russian Far East (Besprozvannykh, 2005; Besprozvannykh et al., 2012).

Data from the first and second intermediate host species and morphological characteristics of different developmental stages are available for all *Asymphyloporina* species from East Asia (Besprozvannykh, 2005; Besprozvannykh et al., 2012; Dvoryadkin & Besprozvannykh, 1985; Tang, 1980). With the accumulation of molecular data on lissorchiids that can be united under the name of *Asymphyloporina sensu lato*, the generic affiliation of some species was

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reconsidered. The genus *Parasymphylodora* was restored for *P. parasquamosa* Kulakov, 1972 and *P. markewitschi* (Petkeviciūtė *et al.*, 2022).

In this study, we found a number of adult worms and metacercariae morphologically close to representatives of Asymphylodorinae Szidat, 1943 in freshwater fish and snail species. We also found mature worms of *Asymphylotrema macrocetabulum* Dvoryadkin, Besprozvannykh, 1985, the type species of this genus. We generated comprehensive data on these worms, including their morphological and molecular characterization, in order to clarify their taxonomic status and reconstruct the phylogenetic relationships of new trematode species within the Lissorchiidae Magath, 1917.

Material and methods

Material collection and morphological analysis

Adult worms and metacercariae of trematodes from the family Lissorchiidae Magath, 1917 were collected from several locations in the south of the Russian Far East (Fig. 1, Table 1) and identified as members of the family Lissorchiidae according to Bray (2008). Adult worms were isolated from freshly caught dead fish, the cyprinid *Carassius gibelio* (Bloch, 1782) from ponds of the Bolshaya Ussurka River basin (one pond connected with the river and one isolated from it), the ponds of the Arsenyevka and the Komissarovka River basins, and from the loach *Misgurnus anguillicaudatus* (Cantor, 1842) caught in the pond of the Komissarovka River basin (Fig. 1). Fish species were identified according to Antonov *et al.* (2019). Overall, 35 individuals of *C. gibelio* were 'prospected' for parasites. Infection with lissorchiid trematodes was found in 28 individuals, with infection intensities ranging from 4–75 specimens. Since mixed infections could not be ruled out, we performed primary species identification of the adult worms using a high-resolution light microscope. In the process, we underwent stereomicroscopy, revealed three morphologically different groups of worms, and obtained morphological and molecular data on them. Additional molecular data were obtained for individuals from the group not differentiated by morphological parameters. Adults and juvenile *Asymphylotrema macrocetabulum* were detected in three of five *M. anguillicaudatus* fish specimens, with the intensity of infection being 3, 5, and 6 worms per fish.

Lissorchiid metacercariae were extracted from bithyniid and stenothyrid snails collected in the water bodies of the basins of the Komissarovka River, the Razdolnaya River, the Bolotnaya River, and the Karasik River. From the stenothyrid snails collected in these water bodies, progenetic

worms were also collected. The water bodies differed in the composition of the fauna of the snails of these two families (Fig. 1). Overall, 780 and 220 individuals of the Bithyniidae (*Parafossarulus* spp. and *Boreoelona* spp.) and the Stenothyridae (*Stenothyra recognita*), respectively, were dissected. Snail species were identified according to Starobogatov *et al.* (2004). Metacercariae were detected in the snails collected in all locations. Snails *Parafossarulus* spp. and *Boreoelona* spp. are the first intermediate hosts of *Parasymphylodora japonica* and *P. markewitschi*, respectively (Besprozvannykh, 2005). We collected the snails from two ponds, one inhabited only by *Parafossarulus* spp. and the other only by *Boreoelona* spp. Then we extracted metacercariae from these snails and obtained morphological and molecular data on them (Fig. 3). These data were used as a reference for the identification of species of *Asymphylodora* s. lato from ponds inhabited by snails of both genera. Trematodes previously identified as belonging to the Asymphylodorinae were also extracted from the intestine of the cyprinid *Leuciscus idus* (Linnaeus, 1758) caught in the Rybinsk Reservoir, 58°5'N, 38°17'E. This material was used for the molecular analysis in the present study.

Adult worms extracted from the fish and progenetic worms extracted from *Stenothyra* snails were fixed in 70% ethanol and then transferred to 96% ethanol. Whole-mounts for the descriptions of adult worms were soaked in distilled water and stained with alum carmine, dehydrated in an ethanol series, cleared in clove oil, and mounted in Canada balsam on a slide under a coverslip. The morphology of metacercariae was studied on living specimens, while the metric parameters were estimated on specimens fixed in hot 4% formalin. Metacercariae for DNA extraction were fixed in 96% ethanol. All measurements are given in micrometres.

We also used the slides of *Parasymphylodora japonica* (Yamaguti, 1938) Bykhovskaya-Pavlovskaya, Kulakova, 1969 (accession no. Tr-35/1–38/1) and *P. markewitschi* (Kulakowskaja, 1947) Bykhovskaya-Pavlovskaya, Kulakova, 1969 (accession no. Tr-39/2–42/2) from (Besprozvannykh, 2005) and the slides of *Asymphylotrema macrocetabulum* (holotype no. Tr-4, paratype no. 4/1-Tr) from the parasitological collection of the Zoological Museum (Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia).

DNA extraction, amplification, and sequencing

Thirty-three specimens of worms fixed in 96% ethanol were used for the molecular analysis (Table 1). Total DNA was extracted from flukes using a hot shot

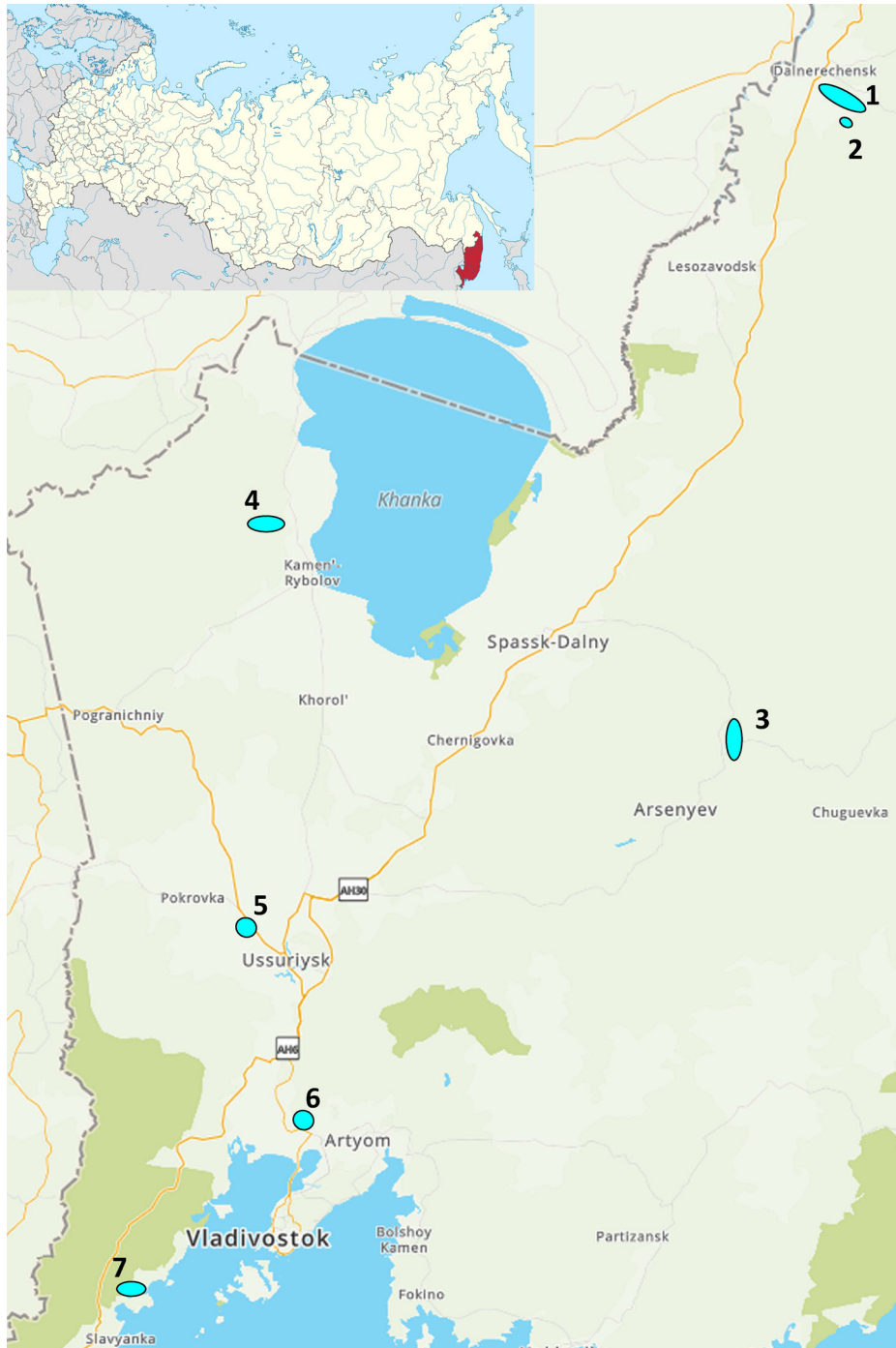


Fig. 1. Map of material collection localities of the south of Russian Far East (see details in Table 1): 1 – Magdykovo Lake, Bolshaya Ussurka River basin; inhabiting snails: *Parafossarulus* spp., *Boreoelona* spp.; 2 – Pond near Magdykovo Lake, Bolshaya Ussurka River basin; inhabiting snails: *Boreoelona* spp.; 3 – Arsenyevka River; inhabiting snails: *Parafossarulus* spp., *Boreoelona* spp.; 4 – Komissarovka River, inhabiting snails: *Boreoelona* spp., *Cincinna* spp.; 5 – Razdolnaya River; inhabiting snails: *Parafossarulus* spp.; 6 – Bolotnaya River; inhabiting snails: *Parafossarulus* spp.; 7 – Karasik River, inhabiting snails: *Stenothyra recognita*.

technique (Truett, 2006). The polymerase chain reaction (PCR) amplification volume amounted to 10 μ L containing 5 μ L GoTaq[®] Green Master Mix, 1 μ L each primer, 1 μ L DNA template, and 3 μ L sterile deionized water.

28S ribosomal DNA (rDNA) was amplified with the primers 28SA (5'-TCG ATT CGA GCG TGA WTA CCC GC-3') (Matejusova & Cunningham, 2004) and 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3')

Table 1. Taxa included in the 28S rRNA gene sequence analysis (sequences new to this study in bold).

Species	n	Host	Location	Author	GenBank reg. number
Lissorchiidae					
Asymphylogorinae					
<i>Parasymphylogora s. lato</i>					
<i>Parasymphylogora khankensis</i> sp. nov.	6	<i>Boreolona</i> spp.	Komissarovka River Basin, Primorsky Region, Russia, (44°56'47''N, 131°39'32''E)	This study	OQ914409–OQ914414
<i>Parasymphylogora khankensis</i> sp. nov.	1	<i>Carassius gibelio</i>	Pond near Magdykovoe Lake, Bolshaya Ussurka River basin, Primorsky Region, Russia, (45°57'34''N, 133°56'07''E)	This study	OQ914408
<i>Parasymphylogora ussuriensis</i> sp. nov.	3	<i>Parafossarulus</i> spp.	Razdolnaya River Basin, Primorsky Region, Russia, (43°46'40''N, 131°56'03''E)	This study	OQ914394–OQ914396
<i>Parasymphylogora ussuriensis</i> sp. nov.	7	<i>C. gibelio</i>	Magdykovoe Lake, Bolshaya Ussurka River basin, Primorsky Region, Russia, (45°57'45''N, 133°56'06''E)	This study	OQ914397–OQ914403
<i>Parasymphylogora ussuriensis</i> sp. nov.	2	<i>C. gibelio</i>	Arsenyevka River basin, Primorsky Region, Russia, (45°43'05''N, 133°33'34''E)	This study	OQ914405
<i>Parasymphylogora ussuriensis</i> sp. nov.	1	<i>Cyprinus carpio</i>	Arsenyevka River basin, Primorsky Region, Russia, (45°43'05''N, 133°33'34''E)	This study	OQ914404
<i>Parasymphylogora ussuriensis</i> sp. nov.	1	<i>Parafossarulus</i> spp.	Bolotnaya River Basin, Primorsky Region, Russia, (43°23'10''N, 132°03'10''E)	This study	OQ914407
<i>Parasymphylogora</i> sp. 1	3	<i>C. gibelio</i>	Magdykovoe Lake, Bolshaya Ussurka River basin, Primorsky Region, Russia, (45°57'45''N, 133°56'06''E)	This study	OQ914415–OQ914417
<i>Parasymphylogora</i> sp. 1	1	<i>C. gibelio</i>	Arsenyevka River basin, Primorsky Region, Russia, (45°43'05''N, 133°33'34''E)	This study	OQ914406
<i>Parasymphylogora parasquamosa</i>	1	<i>Scardinius erythrophthalmus</i>	Ilmėdas Lake, Lithuania	Petkevičiūtė et al., 2022	OP106441
<i>Parasymphylogora parasquamosa</i>	4	<i>Bithynia tentaculata</i>	Ilmėdas Lake, Lithuania	Petkevičiūtė et al., 2022	OP106439, OP106443 – 45
<i>Parasymphylogora markewitschi</i>	1	<i>S. erythrophthalmus</i>	Curonian Lagoon, Lithuania	Petkevičiūtė et al., 2022	OP106444
<i>Parasymphylogora markewitschii</i>	1	<i>B. tentaculata</i>	Stirniai Lake, Lithuania	Petkevičiūtė et al., 2022	OP106447
<i>Parasymphylogora</i> sp. 2	2	<i>Leuciscus idus</i>	Rybinsk Reservoir, Russia, (58°5'N, 38°17'E)	This study	OQ914422–OQ914423

(continued)

Table 1. Continued.

Species	n	Host	Location	Author	GenBank reg. number
<i>Parasymphylodora lacustris</i> sp. nov.	2	<i>C. gibelio</i>	Magdykovoe Lake, Bolshaya Ussurka River basin, Primorsky Region, Russia, (45°57'45''N, 133°56'06''E)	This study	OQ914418–OQ914419
<i>Asymphylodora s. lato</i>					
<i>Asymphylodora progenetica</i>	3	<i>Bithynia tentaculata</i>	Jeruzalė pond, Vilnius, Lithuania	Petkevičiūtė et al., 2020	MT103401 – 03
<i>Asymphylodora progenetica</i>	1	<i>B. tentaculata</i>	Verkiai pond, Vilnius, Lithuania	Petkevičiūtė et al., 2020	MT103400
<i>Asymphylodora perccotti</i>	2	<i>Perccottus glenii</i>	Bolshaya Ussurka River basin, Primorsky Region, Russia	Besprozvannykh et al., 2012	FR822715–FR822731
<i>Asymphylodora khasanensis</i> sp. nov.	2	<i>Stenothyra recognite</i>	Karasik River Basin, Primorsky Region, Russia, (42°34'38''N, 130°41'16''E)	This study	OQ914420–OQ914421
<i>Asymphylodora s. str.</i>					
<i>Asymphylodora tincae</i>	1	<i>Anisus vortex</i>	Verkiai pond, Vilnius, Lithuania	Petkevičiūtė et al., 2022	OP106446
<i>Asymphylodora tincae</i>	1	<i>Tinca tinca</i>	Ilmedas Lake, Lithuania	Petkevičiūtė et al., 2022	OP106440
<i>Asymphylodora</i> sp.	2	<i>Lithoglyphus naticoides</i>	Danube River, Hungary	Petkevičiūtė et al., 2020	MT153916 – 17
Lissorchiinae					
<i>Lissorchis</i>					
<i>Lissorchis kritskyi</i>	1	<i>Minytrema melanops</i>	USA	Curran et al., 2006;	EF032689
<i>L. kritskyi</i>	1	<i>Carpiodes cyprinus</i>	USA	Olson et al., 2003	AY222250
<i>L. kritskyi</i>	1	<i>Carpiodes velifer</i>	USA	Truong et al., 2021	MT928329
<i>Lissorchis</i> cf. <i>nelsoni</i>	1	<i>Minytrema melanops</i>	USA	Truong et al., 2021	MT928354
<i>Asymphylotrema macrocetabulum</i>	3	<i>Misgurnus anguilicaudatus</i>	Komissarovka River Basin, Primorsky Region, Russia, (44°56'47''N, 131°39'32''E)	This study	OQ914424–OQ914426
<i>Palaeorchis</i>					
<i>Palaeorchis incognitus</i>	1	<i>Lithoglyphus naticoides</i>	Elektrėnai water reservoir, Lithuania	Petkevičiūtė et al., 2020	MT103407
<i>P. incognitus</i>	1	<i>Rutilus rutilus</i>	Kaunas water reservoir, Lithuania	Petkevičiūtė et al., 2020	MT103408
<i>P. incognitus</i>	1	<i>L. naticoides</i>	Kaunas water reservoir, Lithuania	Petkevičiūtė et al., 2020	MT103409
<i>P. incognitus</i>	1	<i>L. naticoides</i>	Balaton Lake, Hungary	Petkevičiūtė et al., 2020	MT103410
<i>Posthovitellinum psilotermim</i>	2	<i>Cyclocheilos enoplos</i>	Vietnam	<i>Posthovitellinum</i> Truong et al., 2021	MT928351 – 52
<i>Asaccotrema vietnamiense</i>	1	<i>Rasbora paviana</i>	Vietnam	Sokolov & Gordeev, 2019	MK863409
<i>Anarhichotrema ochotense</i>	3	<i>Anarhichas orientalis</i>	Sea of Okhotsk, Russia	Sokolov et al., 2022	OM108706–OM108708
Monorchidae (outgroup)					
<i>Diplomonorchis leiostomi</i>	1	<i>Leiostomus xanthurus</i>	USA	Olson et al., 2003	AY222252
<i>Monorchis monorchis</i>	1	<i>Diplodus vulgaris</i>	Near Corsica	Tkach et al., 2001	AF184257
<i>Ancylocoelium typicum</i>	1	<i>Trachurus trachurus</i>	UK	Olson et al., 2003	AY222254

(Tkach *et al.*, 2003) with an annealing temperature of 55°C. Products were sequenced using the internal primers 300 F (5'-CAA GTA CCG TGA GGG AAA GTT G-3'), ECD2 (5'-CTT GGT CCG TGT TTC AAG ACG GG-3'), 1200 R (5'-GGG CAT CAC AGA CCT G-3') and 900 F (5'-CCG TCT TGA AAC ACG GAC CAA G-3') from Tkach *et al.* (2003).

Negative and positive controls using both primer pairs were included. Polymerase chain reaction (PCR) products were directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA) following the manufacturer's recommendations with the internal sequencing primers described by Tkach *et al.* (2003) for 28S rDNA. PCR product sequences were analysed using an ABI 3500 genetic analyser at the Federal Scientific Center of the East Asia Terrestrial Biodiversity FEB RAS. The sequences were submitted to the GenBank database (NCBI) (Table 1).

Alignments and phylogenetic analysis

Ribosomal DNA sequences were assembled with SeqScapev.2.6 software provided by Applied Biosystems. Alignment and estimations of the number of variable sites and sequence differences were performed using MEGA 7.0 software (Kumar *et al.*, 2016). The values of genetic p-distances were calculated for the 28S rDNA fragment.

Phylogenetic analysis of the Lissorchiidae was performed using the 28S dataset, 1115 bp in length. The best-fit model of sequence evolution for the phylogenetic analysis was estimated using jModeltest version 2.1.5 software (Darriba *et al.*, 2012). The model of best fit was (TIM3 + G). The phylogenetic analysis was performed for the 28S rDNA dataset using the Bayesian algorithm with MrBayes v.3.2.6 software (Huelsenbeck *et al.*, 2001). Bayesian analysis was used with the following parameters: nst = 6, rates = gamma, Revmatpr = estimate, statefreqpr = estimate, shapepr = estimate, ngen = 10,000,000 via four simultaneous Markov chain Monte Carlo (MCMC) chains (nchains = 4) with every 100th tree saved (samplefreq = 100) with two independent runs with the standard deviation of split frequencies at 0.0099. Summary parameters and the phylogenetic tree were calculated with a burn-in of 250,000 generations. Nodal support was estimated as posterior probabilities in the Bayesian inference analyses (Huelsenbeck *et al.*, 2001). Accession numbers, authority, and supporting information about rDNA sequences from GenBank used for the phylogenetic analyses are provided in Table 1 (Besprozvannykh *et al.*, 2012; Curran *et al.*, 2006; Olson *et al.*, 2003; Petkevičiūtė

et al., 2020, 2022; Sokolov & Gordeev, 2019; Tkach *et al.*, 2001; Truong *et al.*, 2021). *Diplomonorchis leios-tomi*, *Ancylocoelium typicum*, *Monorchis monorchis* from the family Monorchidae were used as an outgroup (the authors of these data and other information are given in Table 1).

Results

Molecular characteristics of Lissorchiidae members

Based on the newly generated sequences and the 28S rDNA sequence data on the Lissorchiidae available in GenBank (Table 1), phylogenetic relationships between the species within this family were reconstructed (Fig. 2), and the genetic divergence between the members of this family was estimated. Because the new trematode species from different clades reported here have no morphological differences at the intergeneric level but differ from each other based only on molecular data, we will consider these new trematodes within two recognized genera, *Asymphylogora* and *Parasymphylogora*, both with status *sensu lato*. All members of the Lissorchiidae were subdivided into six clades (Fig. 2), and the genetic divergence between the members of this family was estimated. Because the new trematode species from different clades reported here have no morphological differences at the intergeneric level but differ from each other based only on molecular data, we will consider these new trematodes within two recognized genera, *Asymphylogora* and *Parasymphylogora*, both with status *sensu lato*. All members of the Lissorchiidae were subdivided into six clades (Fig. 2). The subfamily Asymphylogorinae was monophyletic and subdivided into three clades. The clade I represented was *Parasymphylogora (s. lato)*. Within this clade, two new far eastern trematode species, *Parasymphylogora ussuriensis* sp. nov., *Parasymphylogora khankensis* sp. nov., plus one unidentified *Parasymphylogora* sp.1 grouped together. Genetic differentiation between species within this group ranged from 1.59 ± 0.41% to 2.34 ± 0.52%. Four other species, *Parasymphylogora parasquamosa*, *Parasymphylogora markewitschi*, new specimens of *Parasymphylogora* sp.2 ex *Leuciscus idus* from the Rybinsk Reservoir with unclear taxonomic status, and the new species *Parasymphylogora lacustris* sp. nov. formed another group within clade I. These two groups of *Parasymphylogora* differed from each other by 7.67 ± 0.78%. *Parasymphylogora parasquamosa* and *Parasymphylogora markewitschi* were closely related to each other with 3.35 ± 0.46% of differentiation. The clade II consisted of two Vietnamese species, *Posthovitellinum psiloterminae* and *Asaccotrema vietnamense*, the genetic difference between them was 9.9 ± 0.9%. The clade III included trematodes, which can be considered members of the genus *Asymphylogora sensu lato*, including new specimens from the south of the Russian Far East, *Asymphylogora khasanensis* sp. nov. and two existing species, *Asymphylogora progenetica* and *Asymphylogora percottii*. Additionally, within this clade, a type species of the

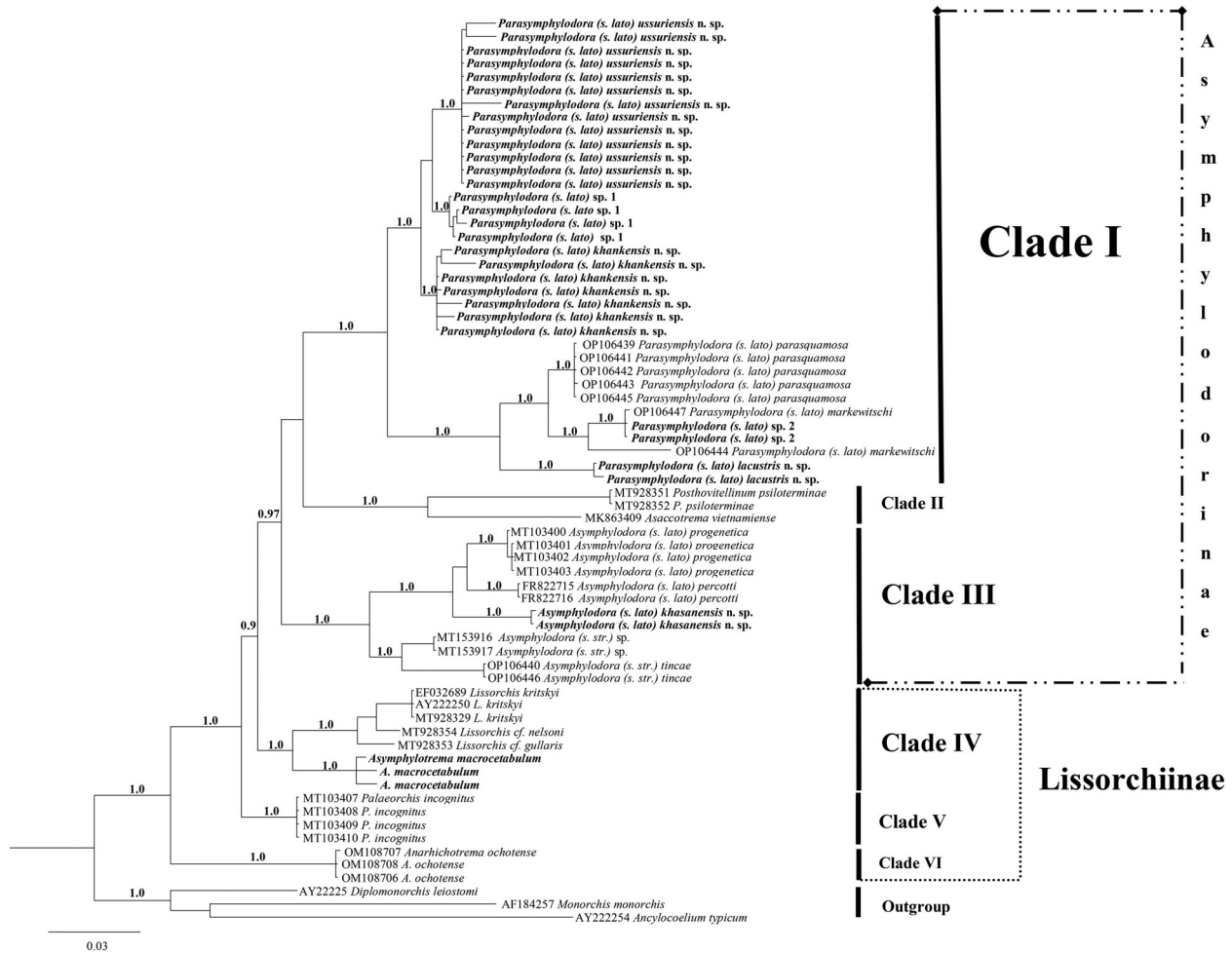


Fig. 2. Phylogenetic relationships of the family Lissorchiidae obtained with Bayesian algorithm based on partial 28S rDNA gene (alignment length 1115 bp). Nodal numbers – posterior probabilities that indicate statistical support of phylogenetic relationships, only significant values (0.9–1.0) are showed.

genus *Asymphyllodora*–*A. tincae* and another undescribed species, *Asymphyllodora* sp. formed a separate group. These two species can be considered members of *Asymphyllodora sensu stricto*. Genetic differentiation between these two species was $3.38 \pm 0.56\%$ and between them and the other three species of *Asymphyllodora s. lato* ranged from 2.91 ± 0.49 to $4.26 \pm 0.58\%$. Mean value of genetic differentiation between these two ‘Species groups’ was $6.55 \pm 0.72\%$.

The subfamily Lissorchiinae was found to be polyphyletic. Species of the genus *Lissorchis* and *Asymphyllotrema macrocetabulum* were closely related and formed Clade IV. Genetic differentiation between representatives of these two genera was 5.47% , which can be accepted as the minimal intergeneric differentiation within the Lissorchiidae. The species *Palaeorchis incognitus* and *Anarhichotrema ochotense* formed clades V and VI, respectively.

Lissorchiidae Magath, 1917

Asymphyllodorinae Szidat, 1943

Parasympylodora

Parasympylodora khankensis sp. nov.

(Syn. *Parasympylodora markewitschi* of Besprozvannykh (2005))

ZooBank registration LSID: urn:lsid:zoobank.org:act:E2800CDB-D2EF-4DA3-8912-07BFBE47E1E0.

Host: *Carassius gibelio* (Cyprinidae).

Other hosts: *Cyprinus carpio haematopterus* Temminck & Schlegel, 1846, *Phoxinus phoxinus manchouricus* Berg, 1907 (Besprozvannykh, 2005) (Cyprinidae).

Site: Intestine.

Type locality: Lake in the Komissarovka River basin, Primorsky Region, south of the Russian Far East; $44^{\circ}56'47''N$, $131^{\circ}39'32''E$.

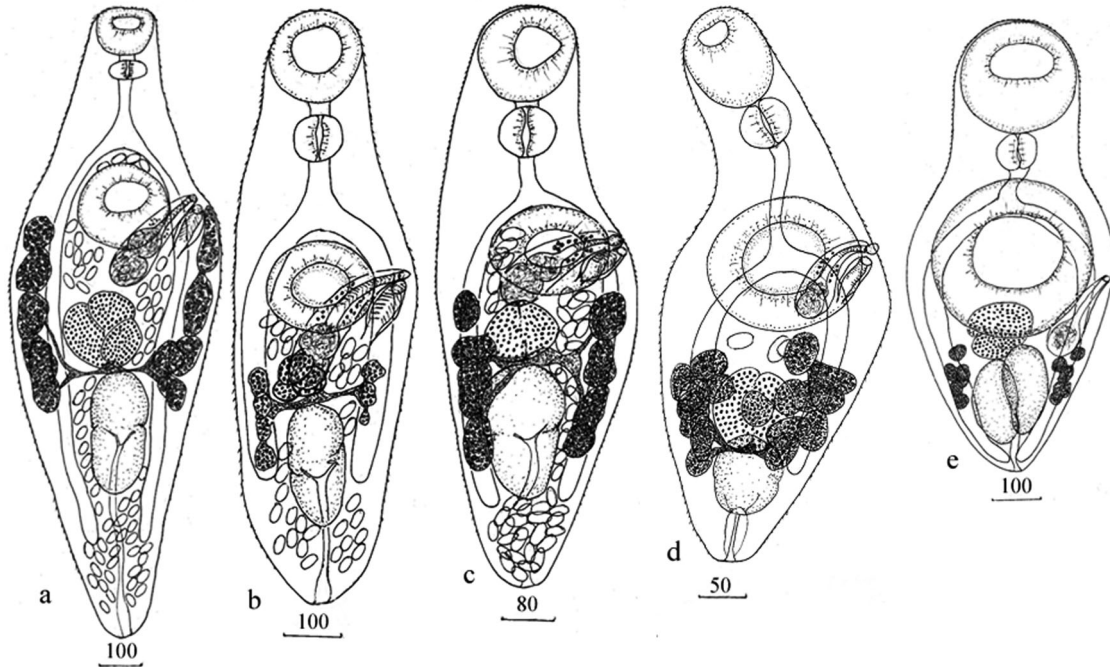


Fig. 3. Adult worms Lissorchiidae: a. *Parasympylodora khankensis* sp. nov.; b. *Parasympylodora ussuriensis* sp. nov.; c. *Parasympylodora lacustris* sp. nov.; d. *Asympylodora khasanensis* sp. nov.; e. *Asympylotrema macrocetabulum*. Scale bars: μm .

Other localities: Pond in the Bolshaya Ussurka River basin, Primorsky Region, south of the Russian Far East; 45°57'34"N, 133°56'07"E.

Type deposition: Holotype no. 200-Tr, paratype No 201-208-Tr.

This material is deposited in the parasitological collection of the Zoological Museum (Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia); e-mail: petrova@ibss.dvo.ru. Deposited 22.11.2021.

Etymology: The species name refers to Lake Khanka, in close proximity to which the parasite was detected.

Adult worm (material examined: 9 specimens) (Fig. 3a; Table 2). Body elongated, with tapered or rounded anterior and tapered posterior end. Tegument with small fine spines. Oral sucker subterminal, transversally oval. Ventral sucker round, almost twice as large as oral sucker, pre-equatorial or equatorial. Prepharynx short. Pharynx transversally oval. Oesophagus long, bifurcating anteriorly of ventral sucker or at level of anterior half thereof. Caeca extending to level of middle post-testicular field. Testis single elongated oval, consisting of two large lobes in middle of hindbody. Cirrus sac slightly curved, expanded proximally, reaching to middle of distance between ventral sucker and ovary or slightly closer to ovary, partially covered by ventral sucker. Internal seminal vesicle bipartite. Prostatic cells

few. Ejaculatory duct with no clearly visible spines. Genital pore sinistrally lateral, at level of posterior border or middle of ventral sucker. Ovary consisting of three subglobular lobes, pre-testicular, dextrally of sub-midline or on midline. Laurer's canal present. Seminal receptacle not clearly visible. Uterus occupying all post-testicular space, sometimes extending to level of anterior border of ventral sucker. Metraterm short, elliptical, with spines. Eggs oval, yellow, operculated, embryonated. Vitelline follicles large, 5–6 on either side of body. Vitelline fields extending from level of middle of ventral sucker to level of middle of testis. Excretory vesicle I-shaped, reaching to middle or anterior border of testis.

Metacercaria (material examined: 10 specimens) (Fig. 4a, b; Table 3). Cyst round, thin-walled. Body elongate oval. Tegument with small spines, sensory papillae indistinguishable. Numerous glandular cells in fore-body. Oral sucker round subterminal. Ventral sucker round, internal wall without spines. Prepharynx short. Pharynx rounded. Oesophagus long, bifurcating immediately anteriorly to ventral sucker. Caeca extending to level of middle or posterior end of testis. Testis oval, in middle of hindbody. Ovary oval, adjoining anterior border of testis. Cirrus sac and metraterm with spines, sinistral at level of ventral sucker. Vitelline follicles not found. Excretory vesicle I-shaped, curved, reaching anterior border of testis.

Table 2. Morphometric comparison of adults (in micrometres).

Features	<i>Parasymphylodora khaankensis</i> sp. nov.			<i>Parasymphylodora markewitschi</i>			<i>Parasymphylodora assuriensis</i> sp. nov.			<i>Asymphylodora japonica</i>			<i>Parasymphylodora lacustris</i> sp. nov.		
	Holotype	Range (n=9)	Mean	(Kudlai, 2010)	(Lambert, 1976)	(Besprozvannykh, 2005)	Holotype	Range (n=6)	Mean	(Besprozvannykh, 2005)	(Shimazu, 2016)	Holotype	Range (n=8)	Mean	
Body length	1.540	662-1540	1.037	702-1700	940-1700	1000-2000	1.063	862-1,063	973	1.050-1.170	750-1310	893	708-1.109	932	
Body width	508	231-508	340	288-630	375-555	300-660	323	262-323	305	300-360	260-480	292	277-554	295	
Oral sucker length	116	108-169	138	120-170	184	130-200	166	135-166	152	160-170	120-180	146	127-154	146	
Oral sucker width	131	108-200	143	140-170	157	130-230	162	131-162	143	160-170	130-190	146	135-166	150	
Forebody	373	154-416	293	-	290-810	290-810	412	343-447	397	290-430	300-480	308	250-347	293	
Ventral sucker length	212	116-212	164	140-190	198	150-290	173	142-173	159	160-200	160-220	169	135-181	164	
Ventral sucker width	216	108-243	181	160-210	176	160-300	196	158-196	175	160-200	170-250	193	154-193	185	
Prepharynx length	23	7-62	23	-	-	-	19	0-27	15	22	-	8	0-19	5	
Pharynx x length	46	31-77	56	60-110	103	62-100	77	58-77	65	56-78	60-90	77	42-77	69	
Pharynx x width	85	58-96	79	60-110	82	78-130	81	62-84	75	62-84	60-70	81	69-92	72	
Oesophagus length	96	42-96	72	-	45-160	160-270	81	81-146	114	100-150	-	85	62-104	81	
Ovary length	85	77-185	119	130-195	164	160-270	96	52-96	82	28-95	90-140	100	77-131	97	
Ovary width	189	89-189	130	90-140	115	160-240	89	58-89	76	22-110	60-160	116	100-154	118	
Testis length	308	158-320	212	290-630	352	200-390	216	173-216	192	150-220	110-220	212	162-274	217	
Testis width	139	86-193	138	130-250	173	170-230	100	96-123	112	100-120	90-160	119	119-193	148	
Cirrus sac length	270	123-270	179	130-255	169	160-270	289	193-289	222	240-310	190-270	193	131-193	156	
Cirrus sac width	77	54-77	64	60-100	92	61-99	69	58-73	63	67-78	70-90	62	50-77	62	
Metaterm length	116	77-116	97	-	-	67-110	104	77-108	69	150	40-60	81	58-81	71	
Metaterm width	54	42-65	48	-	-	-	54	42-81	55	-	30-50	42	31-50	42	
Spines cirrus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Spines metaterm	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Posttesticular fields length	370	108-370	216	-	-	-	146	98-173	139	-	-	135	85-181	156	
Eggs length	28-33	28-33	-	20-24	24	28-33	31-34	31-34	-	31-34	32-40	35-39	35-39	-	
Eggs width	17-20	17-20	-	14-16	15	17-20	17-20	17-20	-	17-20	16-22	19-23	19-23	-	
Length/width %	33.0	28.7-44.5	32.8	-	-	-	30.4	26.2-35.7	31.3	-	-	32.7	27.3-39.1	31.7	
Forebody/body length ratio %	24.2	23.3-36.0	28.3	-	-	-	38.8	38.8-45.3	40.8	-	35-42%	34.5	26.2-35.3	31.4	
Suckers length ration	1.8	1.1-1.8	1.2	1.14-1.16	-	-	1.0	0.97-1.17	1.05	-	-	1.16	1.0-1.2	1.12	
Suckers width ration	1.6	1.2-1.6	1.3	-	-	-	1.2	1.08-1.30	1.22	-	1 : 1.2-1.6	1.32	1.1-1.3	1.23	
Vitellarium fields length	443/450	266-443/273-450	-	-	-	270-570	135/192	123-204/142-208	1.22	-	-	270/250	193-270/193-250	-	
smistral/ dextral															
Vitellarium follicles number	10	10-12	-	-	-	10-12	7-8	4-8	-	-	14-16	6-8	6-8	-	
Follicles length	104-189	96-189	-	-	-	-	54-62	31-92	-	-	-	-	58-116	-	
Follicles width	62-81	62-81	-	-	-	-	23-27	23-54	-	-	-	-	39-73	-	

Features	<i>Asymphylodora khasanensis</i> sp. nov.			<i>Parasymphylodora progenetica</i>			<i>Asymphylodora stenothyrae</i>			<i>Asymphylodora amnicola</i>			
	Holotype	Range (n=5)	Mean	(Kudlai, 2010)	Sercowa & Bykhovskii, 1940)	(Tang, 1980)	Holotype	Range	Mean	(Tang, 1980)	Holotype	Range	Mean
Body length	601	597-678	0.615	480-810	610-820	210-300	601	597-678	0.615	715-963	280-710	601	597-678
Body width	235	235-270	0.251	210-380	210-300	210-300	235	235-270	0.251	360-457	140-250	235	235-270
Oral sucker length	112	112	0.112	80-120	80-120	80-120	112	112	0.112	72-132	60-95	112	112
Oral sucker width	85	85-119	0.109	80-130	80-130	80-130	85	85-119	0.109	72-132	60-95	85	85-119
Forebody length	212	193-223	0.205	-	305-455	-	212	193-223	0.205	-	-	212	193-223
Ventral sucker length	142	142-169	0.161	100-170	110-200	110-200	142	142-169	0.161	137-192	90-130	142	142-169
Ventral sucker width	173	169-173	0.172	110-180	110-200	110-200	173	169-173	0.172	137-192	90-130	173	169-173
Prepharynx length	0	0	0	19-30	-	-	0	0	0	-	-	0	0
Pharynx length	54	54-58	0.055	30-50	40-55	40-55	54	54-58	0.055	42-57	36-44	54	54-58
Pharynx width	58	50-58	0.054	40-50	40-55	40-55	58	50-58	0.054	42-57	36-44	58	50-58
Oesophagus length	116	62-116	0.067	57	60-170	-	116	62-116	0.067	-	-	116	62-116
Ovary length	100	65-100	0.085	-	60-90	-	100	65-100	0.085	88-155	60-110	100	65-100
Ovary width	85	77-89	0.085	-	45-60	-	85	77-89	0.085	64-100	50-90	85	77-89
Testis length	85	85-154	0.101	40-100	55-110	-	85	85-154	0.101	90-154	80-130	85	85-154
Testis width	77	77-96	0.091	40-90	50-80	-	77	77-96	0.091	64-94	60-100	77	77-96

(continued)

Table 2. Continued.

Features	<i>Asymphylodora khasanensis</i> sp. nov.			<i>Parasymphylodora progenetica</i>			<i>Asymphylodora stenothyrae</i> (Tang, 1980)	<i>Asymphylodora amnicola</i> (Stunkard, 1959)
	Holotype	Range (n=5)	Mean	(Kudlai, 2010)	Serecova & Bykhovskii, 1940)	(Tang, 1980)		
Cirrus sac length	108	108–123	0.116	–	80–160	–	–	–
Cirrus sac width	31	31–39	0.036	–	–	–	–	–
Metraterm length	54	50–77	0.060	–	–	–	–	–
Metraterm width	22	22–35	0.029	–	–	–	–	–
Spines cirrus	+	–	–	–	–	–	–	–
Spines metraterm	+	–	–	–	–	–	–	–
Post-testicular fields length	54	42–69	0.055	–	–	–	–	–
Eggs length	31–35	31–35	–	20–33	25–33	34–43	–	25–29
Eggs width	19–23	19–23	–	13–18	13–15	15–17	–	13–16
Length/width ratio %	39.1	39.1–41.9	40.8	–	–	–	–	–
Forebody/body length ratio %	35.3	31.3–35.3	33.3	–	–	–	–	–
Suckers length ration	1.3	1.3–1.5	1.4	–	–	–	–	–
Suckers width ration	2.0	1.5–2.0	1.6	–	–	–	–	–
Vitellarium follicles number	16	16–18	16	11–17	18–22	14–20	–	18

Molecular data. The nucleotide sequences of 28S rRNA gene fragment 1097 bp in length were generated for seven specimens of *Parasymphylodora khankensis* sp. nov. Twenty-nine singleton and two parsimony informative variable sites were found.

Parasymphylodora ussuriensis sp. nov.

(Syn. *Parasymphylodora japonica* of Besprozvannykh (2005))

ZooBank registration LSID:
urn:lsid:zoobank.org:act:FE5E2C2F-A746-4EC1-94FB-6652330F42B0

Host: *Carassius gibelio* (Cyprinidae).

Other host: *Cyprinus carpio haematopterus* (Besprozvannykh, 2005) (Cyprinidae).

Site: intestine.

Type locality: Lake Magdykovoye in the Bolshaya Ussurka River basin, Primorsky Region, south of the Russian Far East; 45°57'45"N, 133°56'06"E.

Other localities: Lakes in basins of Arsenyevka River 45°43'05"N, 133°33'34"E, Razdolnaya River 43°46'40"N, 131°56'03"E, Bolotnaya River 43°23'10"N, 132°03'10"E.

Type deposition: Holotype no. 194-Tr, paratype no. 195-199-Tr.

This material is deposited in the parasitological collection of the Zoological Museum (Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia); e-mail: petrova@ibss.dvo.ru. Deposited 22.11.2021.

Etymology: The species name refers to the Ussuri River, in close proximity to which the parasite was detected.

Adult worm (material examined: 6 specimens) (Fig. 3b; Table 2). Body elongated with rounded anterior and posterior ends. Tegument with fine spines. Oral sucker subterminal, round. Ventral sucker round, with minute, barely visible spines on internal wall, larger than oral sucker, pre-equatorial or equatorial. Prepharynx short. Pharynx rounded or transversally oval. Oesophagus equal to or longer than pharynx. Oesophagus bifurcating usually anteriorly or at level of anterior half of ventral sucker. Caeca extending to level of middle or posterior border of testis. Testis single elongated oval, consisting of two large lobes in middle of hindbody. Cirrus sac curved, expanded proximally and reaching to level of posterior end of ovary or anterior end of testis, partially covered by ventral sucker. Internal seminal vesicle bipartite. Prostatic cells few. Ejaculatory duct with no clearly visible spines. Genital pore sinistrally lateral, in range from level of posterior border of ventral sucker to

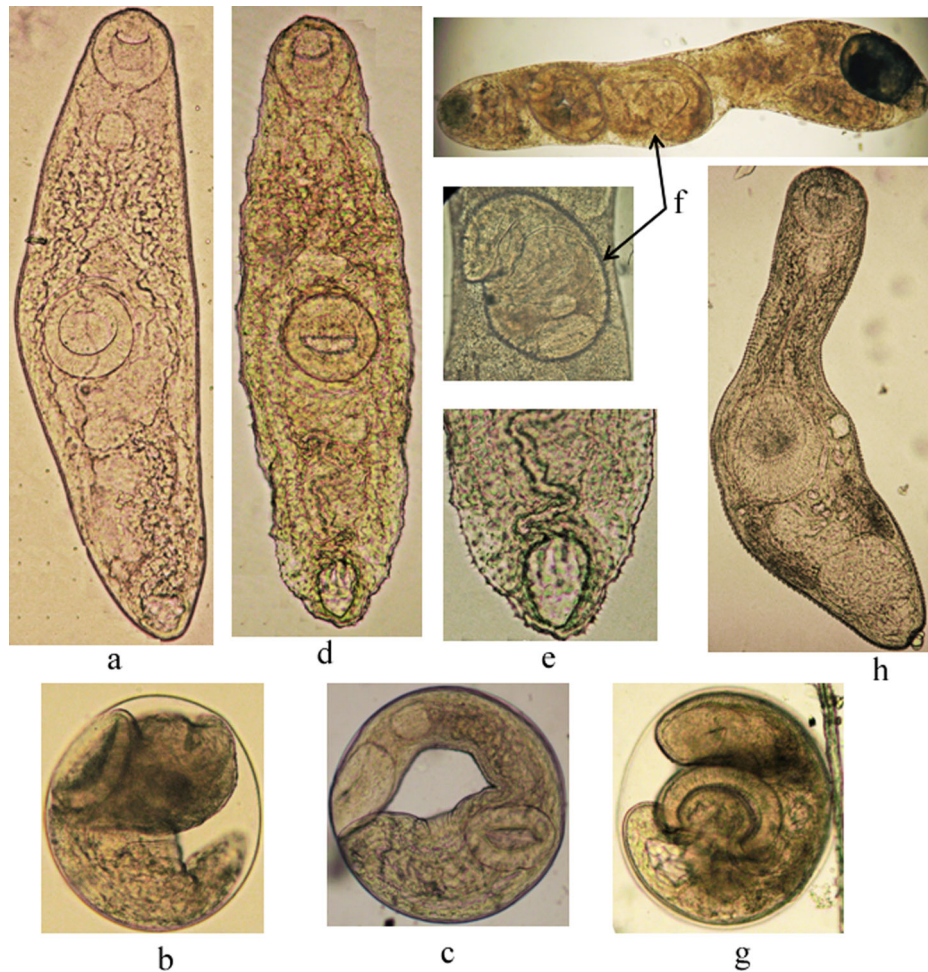


Fig. 4. Metacercaria Lissorchiidae. *Parasympylodora khankensis* sp. nov.: a, in cyst; b, without cyst; *Parasympylodora ussuriensis* sp. nov.: c, in cyst; d, without cyst; e, posterior end of body f, encysted within redia; *Asympylodora khasanensis* sp. nov.: g, in cyst; h, without cyst.

its middle. Ovary consisting of three subglobular lobes, pre-testicular, dextrally below midline or on midline. Laurer's canal present. Seminal receptacle not clearly visible. Uterus occupying all post-testicular space, sometimes extending to level of ventral sucker. Metraterm short, elliptical, with spines. Eggs oval, yellow, operculated, embryonated. Vitelline follicles small, 2–4 on either side of body. Vitelline fields extending from level of ovary or posterior end of ventral sucker to middle of testes. Excretory vesicle I-shaped, reaching to middle or anterior border of testis.

Metacercaria (material examined: 6 specimens) (Figs 4c–f; Table 3). Cyst round, thick-walled. Body elongate oval. Tegument with clearly visible numerous sensory papillae, anterior two-thirds of body covered with small spines, posterior third of body covered with large needle-shaped spines. Numerous glandular cells in fore-body. Oral sucker round subterminal. Ventral sucker

round, internal wall with spines. Prepharynx, oesophagus short. Pharynx round. Oesophagus bifurcating immediately anteriorly to ventral sucker. Caeca extending to level of middle of testis. Testis oval, in middle of hindbody. Ovary oval, adjoining anterior border of testis. Cirrus sac and metraterm with spines, sinistral at level of ventral sucker. Vitellarium at level of ovary and anterior half of testis. Excretory vesicle I-shaped, curved, reaching anterior border of testis.

Remarks

In an isolated population of *Parafossarulus* snails from the Bolotnaya River (Fig. 1, location no. 6), the snails producing cercariae of *Parasympylodora ussuriensis* sp. nov. were infected with rediae that contained metacercariae of this trematode species (Fig. 4f). This is unusual for representatives of the *Asympylodorinae*.

Table 3. Morphometric comparison of metacercariae (in micrometres).

Features	<i>Parasymphylodora</i> (<i>s. lato</i>) <i>khanakensis</i> sp. nov. <i>n</i> = 5		<i>Parasymphylodora</i> (<i>s. lato</i>) <i>khankensis</i> sp. nov. <i>n</i> = 5		<i>Parasymphylodora</i> (<i>s. lato</i>) <i>ussuriensis</i> sp. nov. <i>n</i> = 6		<i>Parasymphylodora</i> <i>markewitschi</i> (Lambert, 1976)		<i>Parasymphylodora</i> <i>japonica</i> (Besprozvannykh, 2005)		<i>Asymphylodora</i> <i>klasaniensis</i> sp. nov. <i>n</i> = 6		<i>Asymphylodora</i> <i>stenothyra</i> (Tang, 1980)		<i>Parasymphylodora</i> <i>progenetica</i> (Kudlai, 2010)	
	Host: <i>Boreoelona</i> Komissarovka	Location of basin r. Bolshaya Ussurka	Host: <i>Boreoelona</i> Razdolnaya	Location of basin r. Razdolnaya	Host: <i>Parafossarulus</i>	Location of basin r.	Host: <i>Bithynia</i> France	Location of basin r. Komissarovka	Host: <i>Boreoelona</i> Komissarovka	Location of basin r. Arsenievka	Host: <i>Stenothyra</i> Location lake in basin r. Karasik	Location lake in basin r. Karasik	Host: <i>Stenothyra</i> China	Host: <i>Bithynia</i> Ukraine		
Cyst	293–323	277–323	308–323	308–323	255–267	260–300	310–350	308–385	308–385	308–385	308–385	400	250–305			
Body length	539–878	570–616	493–754	493–754	1100	500–810	780–920	500–810	500–810	500–810	570–678	620	420–600			
Body width	200–231	262–277	277–308	277–308	460	210–390	290–340	210–390	290–340	290–340	339–370	380	170–230			
Oral sucker length	112	119–123	127–135	127–135	120	100–110	89–140	100–110	89–140	100–110	112–116	110	65–100			
Oral sucker width	135	104–119	135–146	135–146	120	100–130	130–150	100–130	130–150	130–150	127–135	120	65–100			
Ventral sucker length	146	116	150–158	150–158	150	89–134	150–160	89–134	150–160	150–160	181–196	160	110–140			
Ventral sucker width	154	142	162–169	162–169	150	110–160	160–180	110–160	160–180	160–180	193–204	170	110–140			
Pharynx length	62–65	58–62	50–65	50–65	90	45–56	56–78	45–56	56–78	56–78	69–77	40	35–45			
Pharynx width	62–65	54–58	58–69	58–69	60	56–67	67–78	56–67	67–78	67–78	54–69	50	35–45			
Ovary length	58	85	31–58	31–58	80	28–67	34–89	28–67	34–89	34–89	92–96	–	30–40			
Ovary width	100	116	54–96	54–96	60	72–100	73–112	72–100	73–112	73–112	100–104	–	30–45			
Testis length	100	92–96	116–135	116–135	160	72–139	110–150	72–139	110–150	110–150	135–154	–	60–70			
Testis width	116	89	65–85	65–85	100	80–110	56–67	80–110	56–67	56–67	119–181	–	60–70			

Molecular data. The nucleotide sequences of the 28S rRNA gene fragments 1001–1097 bp in length were generated for 13 specimens of *Parasymphylodora ussuriensis* sp. nov. Two sequences of *Parasymphylodora ussuriensis* sp. nov. from the Arsenyevka River basin were 1020 and 1035 bp, and one specimen from the Bolotnaya River basin was 1001 bp in length. Thirty-five singleton and one parsimony-informative variable sites were found.

Parasymphylodora sp. 1

Host: *Carassius gibelio* (Cyprinidae).

Site: Intestine.

Locality: Lake Magdykovoye in the Bolshaya Ussurka River basin 45°57'45"N, 133°56'06"E, Arsenyevka River 45°43'05"N, 133°33'34"E, Primorsky Region, south of the Russian Far East.

Molecular data: The nucleotide sequences of 28S rRNA gene fragment 1097 bp in length were generated for four specimens of *Parasymphylodora* sp. There were four singleton variable sites.

Remarks

Adult worms *Parasymphylodora* (= *Asymphylodora*) *japonica* and *P.* (= *Asymphylodora*) *markewitschi* were reported from cyprinid fishes in the south of the Russian Far East (Besprozvannykh, 2005). Their first intermediate hosts were, respectively, snails of the genus *Parafossarulus* and snails of the genus *Boreoelona*, belonging to the family Bithyniidae. Their second intermediate hosts were different species of branchial and pulmonary snails. The identification of these species in that study was based on the morphometric similarity of the worms with, respectively, *Asymphylodora japonica* from Japan and *A. markewitschi* from Europe. An additional criterion for the identification of the former species was that their first intermediate hosts were snails, *Parafossarulus* spp., as is the case with *A. japonica*.

Adult worms *Parasymphylodora khankensis* sp. nov. examined in our study morphometrically corresponded to the worms denoted as *Parasymphylodora* (= *Asymphylodora*) *markewitschi* from the south of the Russian Far East (Besprozvannykh, 2005). Comparative morphometric analysis of the worms from our material with other related species also indicated their close similarity to *P.* (= *Asymphylodora*) *markewitschi*. The only considerable metric difference between these two species concerned the size of the eggs (Besprozvannykh et al., 2012; Lambert, 1976) (Table 2). *Parasymphylodora khankensis* sp. nov. and *P.* (= *Asymphylodora*) *markewitschi* also differ from each other by the following morphological

characteristics: pharynx transversally oval vs. pharynx round or elongated oval, intestine extending to level of middle post-testicular field vs. intestine extending to level of testis, three-lobed ovary vs. entire ovary. On the basis of existing morphometric differences between the worms and the geographic isolation of their populations, we considered that the worms from our material and those previously found in the south of the Russian Far East and assigned by Besprozvannykh (2005) to *P. (=Asymphylogora) markewitschi* belong to one and the same species, *Parasymphylogora khankensis* sp. nov. Thus, the Russian Far East region can be excluded from the geographic distribution area of *A. markewitschi*.

The worms of *Parasymphylogora ussuriensis* sp. nov. from our material were identical morphometrically with the specimens of *P. (=Asymphylogora) japonica* from cyprinid fishes in the south of the Russian Far East, reported by Besprozvannykh (2005), as well as with specimens of *P. (=Asymphylogora) japonica* from Japan (Shimazu, 2016). There was no gap in the values of most of the metric parameters of the studied worms except the metraterm length (Table 2). At the same time, the worm *Parasymphylogora ussuriensis* sp. nov. had a number of morphological differences from *P. (=Asymphylogora) japonica* reported from Japan by Shimazu (2016): ovary round or transversally oval, consisting of three subglobular lobes vs. ovary subglobular to pyriform, sometimes of irregular outline; vitelline follicles 4–8 vs. vitelline follicles 14–16; intestines extending to the level of the middle or posterior border of the testis vs. intestines sometimes extending to the midlevel of the post-testicular region; cirrus sac reaching the level of the posterior end of the ovary or anterior end of the testis vs. cirrus sac usually reaching to the ovary. On the basis of these differences, the trematodes found in our study should be recognized as distinct species. This conclusion agrees with the data on the geographic distribution: *P. (=Asymphylogora) japonica* and *Parasymphylogora ussuriensis* sp. nov. exist under conditions of long-term isolation.

The results of a comparative analysis of adult *Parasymphylogora khankensis* sp. nov. and *Parasymphylogora ussuriensis* sp. nov. from the same definitive hosts and the same region indicated the absence of a hiatus for most of the metric parameters. They differed only in the length of vitellaria fields, the size of vitellaria follicles, and the sucker ratio (Table 2). *Parasymphylogora ussuriensis* sp. nov. and *Parasymphylogora khankensis* sp. nov. also differed by the arrangement of vitellaria fields (anterior field reaching level of ovary or posterior end of ventral sucker vs. anterior field reaching level of middle of ventral sucker), the number of vitellaria follicles (4–8 vs. 10–12), cirrus sac length (reaching posterior end of ovary

or anterior edge of testis vs. not reaching anterior edge of ovary), and intestine length (extending to level of middle or posterior level of border of testis vs. extending to level of middle post-testicular field). The metacercariae of these species differ in the size of the ventral sucker (Table 3) and the presence of numerous papillae and large needle-like spines on the tegument of the posterior end of the body in *Parasymphylogora ussuriensis* sp. nov. (Fig. 4f). Moreover, the first intermediate hosts of *Parasymphylogora ussuriensis* sp. nov. and *Parasymphylogora khankensis* sp. nov. are snails from different genera: *Parafossarulus* and *Boreoelona*, respectively. After emergence from the snail, the cercaria of *Parasymphylogora ussuriensis* sp. nov. leave it and settle on the substrate, whereas the cercaria of *Parasymphylogora khankensis* sp. nov. do not leave the snail but move to its tentacle tips and wait there for the second intermediate host (Besprozvannykh, 2005). These differences indicate that the trematodes under comparison belong to different species. This conclusion is supported by the molecular data.

Adults and metacercariae of *Parasymphylogora ussuriensis* sp. nov. from, respectively, cyprinid fishes and *Parafossarulus* snails formed a distinct, highly supported group within the clade I, and so did adults and metacercariae of *Parasymphylogora khankensis* sp. nov. from, respectively, *C. gibelio* and *Boreoelona* spp. within the clade I (Fig. 2). Genetic differentiation between these two new trematode species, *Parasymphylogora ussuriensis* sp. nov. and *Parasymphylogora khankensis* sp. nov., was $2.34 \pm 0.52\%$. The data for each of these species agree with the molecular characteristics of *Parasymphylogora ussuriensis* sp. nov. and *Parasymphylogora khankensis* sp. nov., respectively, collected from the basins inhabited only by *Parafossarulus* snails (the first intermediate host of *Parasymphylogora ussuriensis* sp. nov.) and by *Boreoelona* snails (the first intermediate host of *Parasymphylogora khankensis* sp. nov.). Four trematode specimens ex *C. gibelio* from Lake Magdykovoye, the Bolshaya Ussurka River basin, and the Arsenyevka River, denoted as *Parasymphylogora* sp. 1, were closely related to *Parasymphylogora ussuriensis* sp. nov., *P. khankensis* sp. nov. (Fig. 2). *Parasymphylogora* sp. differed from *Parasymphylogora ussuriensis* sp. nov. and *Parasymphylogora khankensis* sp. nov. by $1.59 \pm 0.41\%$ and $2.29 \pm 0.48\%$, respectively.

Parasymphylogora lacustris sp. nov.

ZooBank registration LSID: urn:lsid:zoobank.org:act:C9AE38B0-7679-41D3-97DF-D1D880A17DA3

Host: *Carassius gibelio* (Cyprinidae).

Site: Intestine.

Type-locality: Pond in the basin of the Bolshaya Ussurka River, Primorsky Region, the south of the Russian Far East; 45°57'34"N, 133°56'07"E.

Other locality: Lake in the Bolshaya Ussurka River basin, Primorsky Region, south of the Russian Far East; 45°57'45"N, 133°56'06"E.

Type-deposition: Holotype no. 209-Tr, paratype no. 210-216-Tr.

This material is deposited in the parasitological collection of the Zoological Museum (Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia); e-mail: petrova@ibss.dvo.ru. Deposited 22.11.2021.

Etymology: The species name refers to the characteristics of water body type, where the parasite was detected.

Adult worm (material examined: 8 specimens) (Fig. 3c; Table 2). Body elongated with rounded anterior and tapered posterior ends. Tegument with small fine spines. Oral sucker subterminal, round. Ventral sucker round or transversally oval, larger than oral sucker, in anterior part of middle third of body. Prepharynx short. Pharynx rounded or transversally oval. Oesophagus equal to or slightly longer than pharynx. Oesophagus bifurcating usually anteriorly or at level of anterior half of ventral sucker. Caeca extending usually to level of middle or posterior border of testis or slightly posterior of testis. Testis single elongated oval, consisting of two large lobes in middle of hindbody. Cirrus sac mostly covered by ventral sucker, slightly curved, expanded proximally, usually reaching anterior edge of ovary. Internal seminal vesicle bipartite. Prostatic cells few. Ejaculatory duct with no clearly visible spines. Genital pore sinistral, lateral, at level of middle of ventral sucker. Ovary transversally oval, consisting of two lobes, pre-testicular, located dextrally of submidline or on midline. Laurer's canal present. Seminal receptacle not clearly visible. Uterus occupying all post-testicular space, sometimes extending to level of ventral sucker. Metraterm short, elliptical, with spines. Eggs oval, yellow, operculated, embryonated. Vitelline follicles large, three or four on either side of body. Vitelline fields extending from level of posterior end of ventral sucker to middle or posterior half of testis. Excretory vesicle I-shaped, reaching to middle or anterior border of testis.

Molecular data. The nucleotide sequences of the 28S rRNA gene fragment (1094 bp in length) were generated for two specimens of *Parasymphylodora lacustris* sp. nov. The two sequences differ by three variable sites.

Remarks

The trematode denoted as *Parasymphylodora lacustris* sp. nov. was obtained from *Carassius gibelio* from two water bodies of the Bolshaya Ussurka River basin, one of which is inhabited by both *Parafossarulus* and *Boreoelona* snails, while the other is inhabited only by *Boreoelona* snails (Fig. 1). This trematode possesses a number of morphometric parameters similar to those of *Parasymphylodora ussuriensis* sp. nov., *Parasymphylodora khankensis* sp. nov., *P. (=Asymphylodora) japonica*, and *P. (=Asymphylodora) markewitschi*. Specimens of *Parasymphylodora lacustris* sp. nov. differ from *A. japonica* by the size of the cirrus sac, the metraterm length, the forebody/body length ratio, and the number of vitellaria follicles; from *P. (=Asymphylodora) markewitschi* by testis length and egg size; from *Parasymphylodora ussuriensis* sp. nov. by the ovary width, the cirrus sac length, the egg size, and the forebody and body length ratio; from *Parasymphylodora khankensis* sp. nov. by egg size, the length of vitellaria fields, and the number of vitellaria follicles (Table 2). Another characteristic justifying the species status of *Parasymphylodora lacustris* sp. nov. is the two-lobed ovary, as compared with the entire ovary of *P. (=Asymphylodora) japonica* and *P. (=Asymphylodora) markewitschi* and the three-lobed ovary in *Parasymphylodora ussuriensis* sp. nov. and *Parasymphylodora khankensis* sp. nov. On the basis of our comparative analyses, we conclude that the trematode under consideration is a representative of a new species. This conclusion is confirmed by the molecular data generated in our study. *Parasymphylodora lacustris* sp. nov. together with existing species *Parasymphylodora parasquamosa* and *Parasymphylodora markewitschi* was united into the same group within the *Parasymphylodora s. lato* clade on the phylogenetic tree based on molecular data; the genetic distance between them was $5.83 \pm 0.66\%$. Genetic differentiation between this clade and the sister clade containing three new far eastern species, *Parasymphylodora s. lato*, was $7.67 \pm 0.78\%$. These parameters correspond to the intergeneric differentiation level within the Lissorchiidae.

Asymphylodora khasanensis sp. nov.

ZooBank registration LSID: urn:lsid:zoobank.org:act:E075FD8F-53B0-4BE6-B5C7-077E4587DABF

Host: *Stenothyra recondita* Lindholm, 1929 (Stenothyridae).

Site: Pallium fold.

Type locality: Lake in the Karasik River basin, Khasansky district, Primorsky Region, south of the Russian Far East, 42°34'38"N, 130°41'16"E.

Type deposition: Holotype no. 217-Tr, paratype no. 218-221-Tr.

This material is deposited in the parasitological collection of the Zoological Museum (Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia); e-mail: petrova@ibss.dvo.ru. Deposited 22.11.2021.

Etymology: The species name refers to the administrative region where these worms were first detected.

Progenetic worm (material examined: 5 specimens)

(**Fig. 3d; Table 2**). Body small, elongated, with tapered anterior and posterior ends. Tegument with small fine spines. Oral sucker subterminal, round. Ventral sucker round, with minute, barely visible spines on internal wall, larger than oral sucker, at border of anterior and posterior body halves. Prepharynx absent. Pharynx rounded. Oesophagus long, bifurcating at level of middle of ventral sucker. Caeca extending to level of anterior end or anterior half of testis. Testis single elongated oval, consisting of two large lobes in middle of hind-body. Cirrus sac mostly covered by ventral sucker, curved, expanded proximally. Internal seminal vesicle bipartite. Prostatic cells few. Ejaculatory duct with spines. Genital pore sinistrally lateral, at level of middle of ventral sucker. Ovary round or transversally oval, consisting of two lobes, at midline, partially covered by testis. Uterus mainly located at level of testis and ovary. Metraterm short, with spines. Eggs oval, yellow, operculated. Vitelline follicles 8–9 on either side of body, from level of posterior end of ventral sucker to level of middle testis. Excretory vesicle sac-shaped, reaching level of posterior third of testis, sac-shaped, lined with numerous epithelial cells.

Metacercaria (material examined: 6 specimens) (Figs 4g, h; Table 3). The cyst is thin-walled, easily destroyed by pressure. Metacercaria similar in size to progenetic form, has all organs of reproductive system except eggs in uterus.

Molecular data. The nucleotide sequences of the 28S rRNA gene fragment (1093 bp in length) were generated for two specimens of *Asymphylogora khasanensis* sp. nov. There was a single variable site.

Remarks

Morphologically, the worms denoted as *Asymphylogora khasanensis* sp. nov. agree with the diagnostic characteristics of the genus *Asymphylogora* (Asymphylogorinae) (Bray, 2008). However, the analysis of 28S rDNA sequences revealed an intergeneric differentiation level between *Asymphylogora khasanensis* sp. nov. and other

representatives of the Asymphylogorinae ($6.32 \pm 0.74\%$ – $12.56 \pm 0.94\%$), including the type species *Asymphylogora* (s. str.) *tincae* but excepting the worms known as *A. progenetica* Sercowa, Bykhovskiy, 1940 and *A. percotti* Besprozvannykh et al., 2012. The latter two species were within the same clade as *Asymphylogora khasanensis* sp. nov., differing from it at the intra-generic level ($4.16 \pm 0.68\%$). This means that these three species belong to the same genus by molecular criterion. However, we consider these trematodes as members of the *Asymphylogora s. lato* because of their morphological identity with other trematodes from the Clade III at generic level. Among the worms grouped into the clade III (*Asymphylogora s. lato*) there are species with a sac-like excretory bladder (e.g. *A. tincae*) and a tubular excretory bladder, as is characteristic of most members of the Asymphylogorinae.

Progenesis, which was noted in *Asymphylogora khasanensis* sp. nov., is also characteristic of *Asymphylogora progenetica* from Europe, *Asymphylogora amnicola* Stunkard, 1959 from America (Stunkard, 1959) and *A. stenothyrae* from China (Tang, 1980). There are no molecular data on the latter two species. Morphologically, they are similar to *Asymphylogora khasanensis* sp. nov. (as is *Asymphylogora progenetica*). The values of most metric parameters have no hiatus (Table 2). *Asymphylogora khasanensis* sp. nov. differs from *Asymphylogora progenetica* only by the forebody length and the ovary width (Kudlai, 2010), the pharynx size (Sercowa & Bykhovskii, 1940), and the egg size; from *A. amnicola*, by the size of the suckers, the pharynx size and the egg size; from *A. stenothyrae*, only by the body and egg sizes (Table 2). Morphological differences between *Asymphylogora khasanensis* sp. nov. and *Asymphylogora progenetica*, *A. amnicola*, and *A. stenothyrae*: intestinal bifurcation at the level of ventral sucker vs. intestinal bifurcation anterior to ventral sucker; cirrus sac covered by ventral sucker vs. cirrus sac sinistral to ventral sucker (*Asymphylogora progenetica*, *A. stenothyrae*); vitelline follicles not reaching the level of posterior end of ventral sucker vs. vitellaria follicles reaching the level of posterior end of ventral sucker; testis oval, two-lobed vs. testis oval. Alongside with these differences, metacercariae of *Asymphylogora khasanensis* sp. nov. differs from *N. progenetica* and *A. stenothyrae* by most metric parameters (Table 3), and by the presence of the primordium of vitellarium, which was not noted in the latter two species; from *A. amnicola* metacercariae, by cyst size: 308–385 vs. 190–220 (Stunkard, 1959). Though East Asiatic worms in the Russian Far East and China use snails from the same genus *Stenothyra* as the first intermediate host, morphological differences between progenetic worms *Asymphylogora khasanensis* sp. nov. and *A. stenothyrae*

together with their geographic isolation indicate that these digeneans belong to different species. It is difficult to estimate the generic status of *A. amnicola* and *A. stenothyrae* because of the lack of molecular data, but on the basis of morphological characteristics and progenesis, it cannot be ruled out that these two species belong to the genus *Asymphylogadora s. lato*.

Asymphylostrema macrocetabulum (Belous, 1953).

Host: *Misgurnus anguillicaudatus* (Cobitidae).

Site: intestine.

Locality: Lake in the Komissarovka River basin, Primorsky Region, south of the Russian Far East; 44°56'47"N, 131°39'32"E.

This material is deposited in the parasitological collection of the Zoological Museum (Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia); e-mail: petrova@ibss.dvo.ru. Deposited 22.11.2021.

Immature worm (material examined: 3 specimens) (Fig. 3e). Body 497–578 long and 258–300 wide, flask-shaped. Tegument with small fine spines. Oral sucker 131–146 long and 131–154 wide, subterminal, round. Pharynx 46–62 length and 50–62 width, round. Oesophagus bifurcating at level of anterior half of ventral sucker. Ventral sucker 196–235 long and 172–239 wide, round, at 1/3 larger than oral sucker, in middle part of body. Intestinal caeca extending to posterior end of body. Testes two, 116–135 long and 65–81 wide, entire, oval, obliquely adjacent to each other in hind-body. Cirrus sac 96–119 long and 35–46 wide, claviform, adjoining ventral sucker sinistrally. Genital pore sinistrally lateral, at level of middle of ventral sucker. Ovary 62–77 length and 77–100 width, three-lobed, adjacent to testes. Vitelline follicles at level of ovary and anterior half of testes.

Molecular data. The nucleotide sequences of the 28S rRNA gene fragment (1095 bp in length) were generated for three specimens of *A. macrocetabulum*. There were 17 variable singleton sites.

Remarks

Trematodes obtained in our study from *Misgurnus anguillicaudatus* were morphologically identical to *Asymphylostrema macrocetabulum* from the same fish species in the Lake Khanka basin (Dvoryadkin & Besprozvannykh, 1985). Molecular-based phylogenetic analysis indicated that *A. macrocetabulum* had a close

relationship with *Lissorchis* spp., with the mean value of genetic p-distance being $5.47 \pm 0.62\%$ (Fig. 2).

Discussion

Morphological and molecular characteristics in the taxonomy of Asymphylogadora s. lato and Parasympylodora s. lato

The analysis of the morphological characteristics of the worms belonging to *Asymphylogadora s. lato* did not reveal any generic differences in most parameters. On the basis of this analysis, all these trematodes were united into one and the same genus, with the same diagnosis as in Bray (2008). The only morphological indicator previously used for distinguishing asymphylogadorine genera was the shape of the excretory bladder: sac-like in *Asymphylogadora* and tubular in *Parasympylodora* (Kulakova, 1982). In the light of new morphological and molecular data, the shape of the excretory bladder should no longer be considered an important character. Some species of *Asymphylogadora s. lato*, considered here, include species with a sac-like excretory bladder (*Asymphylogadora khasanensis* sp. nov.) and species with a tubular one (e.g. *Asymphylogadora percottii*; (Besprozvannykh et al., 2012)). The reports of the excretory bladder shape in *Asymphylogadora progenetica* are contradictory: it is tubular according to Kulakova (1982) but sac-like according to Petkevičiūtė et al. (2022). This means that the morphological generic diagnosis according to Bray (2008) corresponds to all the representatives of the Asymphylogadorinae. Differentiation between the genera was revealed only at the molecular level. The molecular characteristics of each genus based on the 28S rRNA gene partial sequences provided in our study are effective criteria for the differentiation of the genera within *Asymphylogadora sensu lato*.

The results of the previous studies on the life cycles of *Asymphylogadora s. lato* show that most of the species from this trematode group use prosobranch snails Bithyniidae Gray, 1857 and Stenothyridae Tryon, 1866 as the first intermediate hosts (Sercowa & Bykhovskii, 1940; Kulakova, 1972; Besprozvannykh, 2005; Tang, 1980; Besprozvannykh et al.; 2012; Shimazu, 2016). The type species, *Asymphylogadora tincae*, has been reported to use pulmonate snails. The reports refer to snails from the families Planorbidae Rafinesque, 1815, Lymnaeidae Rafinesque, 1815, and Acroloxidae Thiele, 1931 (Našincová & Scholz, 1994) and Planorbidae and Lymnaeidae (Petkevičiūtė et al., 2022). However, these reports raise some doubts. The study of Našincová and Scholz (1994) lacks an objective validation of the use of snails from these three families as the first intermediate

host by the trematode denoted as *A. tincae*. Their experimental study was performed in two unrelated stages. At the first stage, different snail species fed on the eggs of the worms collected from naturally infected fish. In this case, mixed infections of fish species with asymphyloporines cannot be excluded. At the second stage, the fish were experimentally fed on naturally infected snails (which could be infected with metacercariae of different *Asymphylogora* spp.) and then mature worms from the fish were collected. Considering the high morphological similarity of close *Asymphylogora* spp., one cannot be sure that Našincová and Scholz (1994) dealt only with *A. tincae*.

Petkevičiūtė et al. (2022) reported molecular data on the species *Asymphylogora s. lato* but did not provide any morphological validation; the molecular data on mature worms from naturally infected fish and cercariae from planorbid snails, *Anisus vortex* (Linnaeus, 1758) were identical. However, Petkevičiūtė et al. (2022) provided no molecular data on the cercariae from the lymnaeid snail *Stagnicola palustris* (Müller, 1774). This means that we cannot be sure whether these snails play the role of the first intermediate hosts of *A. tincae*. The worms from *S. palustris* might equally well belong to some other species morphologically close to *A. tincae*. To sum up, it remains unclear whether trematodes *A. tincae* implement their life cycles through pulmonate snails from different families.

Analysis of 28S rDNA sequences of trematode specimens from *Leuciscus idus* from our material showed that these worms were closely related to the cercaria of *Parasymphylogora markewitschi* ex *Bythinia tentaculata* (acc. number OP106447) from the study of Petkevičiūtė et al. (2022): genetic differentiation was 0.12%. At the same time, these specimens differed from *Parasymphylogora markewitschi* ex *Scardinius erythrophthalmus* (accession number OP106444) from that study at the interspecific level (*p*-distance value $3.89 \pm 0.7\%$). Petkevičiūtė et al. (2022) did not provide any morphological data on either adult worms or the cercaria. This means that the identification of the mature worm and the larva as *Parasymphylogora markewitschi* in the study of Petkevičiūtė et al. (2022) is highly doubtful. Therefore, we postpone the taxonomic conclusion on adult trematodes ex *L. idus* from our material until a detailed morphological analysis has been performed. In this study, we place these trematodes in the genus *Parasymphylogora s. lato*.

Thus, several taxa of the Asymphyloporinae from our study could be erected to the level of distinct genera on the basis of the available molecular data. However, the absence of morphological data on the type species of the genus *Asymphylogora*, *A. tincae*, as well as on the

representatives of *Parasymphylogora* reported by Petkevičiūtė et al. (2022), renders difficult the taxonomic interpretation of phylogenetic relationships within the Asymphyloporinae. For this reason, we consider all asymphyloporine trematode species within three genera: *Asymphylogora s. str.*, *Asymphylogora s. lato*, and *Parasymphylogora s. lato* until additional studies on other species of the Asymphyloporinae from their type locations are performed.

Relationships and species diversity within the Lissorchiidae

Our phylogenetic analysis indicates that progenetic lissorchiids are an intermediate lineage within the family. *Asymphylogora percotti*, the terminal taxon within the 'progenetic' clade, does not seem to be progenetic (Besprozvannykh et al., 2012). This fact suggests that progenesis in *Asymphylogora progenetica* and *Asymphylogora khasanensis* sp. nov. is a plesiomorphic feature within this clade. On the other hand, the absence of progenesis in other lissorchiid species from the terminal and basal clades of the phylogenetic tree indicates that this character is an apomorphy within the whole family. Moreover, in light of our opinion that the group from the phylogenetic tree, containing the species *Asymphylogora percotti*, *Asymphylogora progenetica*, and *Asymphylogora khasanensis* sp. nov. should have the status of a separate genus, progenesis can be a taxon-specific feature. Additional data are necessary to solve the issue of progenesis within lissorchiids.

In general, our molecular-based phylogenetic analysis shows that the family Lissorchiidae is monophyletic, and the subfamily Asymphyloporinae is monophyletic, too, but the subfamily Lissorchiinae is not, as was also demonstrated in earlier phylogenetic studies of Lissorchiidae (Petkevičiūtė et al., 2020, 2022; Sokolov & Gordeev, 2019). Members of the Asymphyloporinae form a large, highly supported clade, while members of the Lissorchiinae are distributed across three independent clades (Fig. 2). This disagreement can be resolved by assigning the status of distinct subfamilies to the [*Lissorchis* + *Asymphyloporinae*] clade, to *Palaeorchis incognitus* Szidat, 1943, and to a marine lissorchiid, *Anarhichotrema ochotense* Shimazu, 1973. However, we abstain from this action because the available data are insufficient. The values of genetic differentiation between these three clades range from 5.06 ± 0.63 to $9.98 \pm 0.91\%$. The maximal value of this range, that between *Anarhichotrema ochotense* and the two other clades, can be accepted as intersubfamilial. However, we cannot apply this interpretation to the differentiation between [*Lissorchis* + *Asymphyloporinae*] and

Palaeorchis incognitus clades, which represents an inter-genetic level. The same situation is characteristic of other trematode families, such as the Haplosporididae and the Bucephalidae, for which the monophyly of the family and the paraphyly of the subfamilies were demonstrated based on the disagreement of morphological and molecular data (Atopkin *et al.*, 2020, 2022).

At present, there is an obvious lack of molecular data on different species of Asymphyliodoridae and Lissorchiinae, as well as other subfamilies for the trematode groups mentioned above, that is opposing the correct taxonomic and phylogenetic interpretation of molecular results in complex with morphology. Nevertheless, in our study, some trematode groups within monophyletic Asymphyliodoridae deserve generic status based on molecular data, but they are highly similar morphologically at the generic level. The current zoological nomenclature code, based only on morphological characteristics, does not allow establishing new valid genera in our case. At present, we must wait for new data on other species to resolve the question of taxonomically problematic groups of trematodes. However, we do not exclude that the availability of morphological and molecular data for all known species from their type locations will not help make a final conclusion in this respect, and then a zoological nomenclature code will need to consider molecular criteria.

In conclusion, we have to emphasize the high species diversity of Lissorchiid trematodes, uncovered with a molecular approach, in a relatively small area. Some of these species have a high specificity for the first intermediate host, for example, *Parasymphyliodora khankensis* sp. nov., which inhabits the water reservoirs with only *Boreoelona* snails and was not registered in the ponds with only *Parafossarulus* snails. Inversely, *Parasymphyliodora ussuriensis* sp. nov. was found in the ponds with only gastropods of *Parafossarulus* spp. and absent in the ponds with only *Boreoelona* spp. *Asymphyliodora khasanensis* sp. nov. was found in snails of *Stenothyra recognita* that belong to the tropical genus of estuarine snails (Likharev, 1953). Most of the trematode species reported here realize their life cycle through the same species of definitive host, *Carassius gibelio*, which is widely distributed in rivers and ponds across the Primorye Region (Antonov *et al.*, 2019). Accepting all these features, we can propose intensive processes of speciation within lissorchiid trematodes on the investigated territory through the snail's life activity rather than through *C. gibelio*, supporting the hypothesis about the key role of the first intermediate host in digenean species diversity formation. Nevertheless, detailed studies of the life cycles of the trematode species presented in this work are needed to draw final conclusions in this respect.

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