

# Phylogenetic relationships among European and Asian representatives of the genus *Aspidogaster* Baer, 1827 (Trematoda: Aspidogastrea) inferred from molecular data

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## Abstract

In the present study, phylogenetic relationships of European and Far Eastern representatives of the genus *Aspidogaster* Baer, 1827 were analysed: *A. conchicola* Baer, 1827, *A. limacoides* Diesing, 1834, *A. ijimai* Kawamura, 1915 and *A. chongqingensis* Wei, Huang & Dai, 2001. Based on ITS1–5.8S–ITS2 rDNA sequence data, an obvious differentiation was seen between specimens of *A. limacoides sensu stricto* from the European part of Russia and *A. limacoides sensu Chen et al.*, 2010 from China (13.7%); the latter parasites were recognized as *A. chongqingensis*. *Aspidogaster chongqingensis* was more closely related to *A. ijimai* than to *A. limacoides s. str.* Specimens of *A. ijimai* from the Amur River, Khanka Lake (Russian Far East) and China were grouped into a single clade with low intra specific molecular differentiation ( $d = 0–0.3\%$ ). Specimens of *A. conchicola* from the European part of Russia, the Russian Far East and China also formed a single distinct clade. Genetic differentiation between European and Chinese samples of this species was two times lower ( $d = 0.45\%$ ) than between Russian Far East and European or Chinese samples ( $d = 0.96\%$ ), suggesting a long-term separate existence of *A. conchicola* in the Russian Far East.

## Introduction

Aspidogastrea is a minor group of flatworms that infect poikilothermic animals, including molluscs, fish and reptiles, and, as an exception, some crustacean species in

marine and freshwater environments (Alves *et al.*, 2015). According to the most popular opinion, this group is considered as a subclass within the class Trematoda (Skrjabin, 1952; Dollfus, 1958; Rhode, 2002). In contrast to members of the other subclasses, Aspidogastrea have a simple life cycle with no parthenogenetic stages. For this reason, and due to a number of morphological features, few authors have considered aspidogastreans as a distinct class of flatworms (Timofeeva, 1975). A number of

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freshwater aspidogastreae have a rather wide geographical distribution (Achmerov, 1956; Vosnesenskaya, 1968; Stromberg, 1970; Nagibina & Timofeeva, 1971; Strelkov, 1971; Shimazu, 2003; Schludermann *et al.*, 2005; Zhang, 2006; Popiolek *et al.*, 2007; Yuryshynets & Krasutska, 2009; Shedko *et al.*, 2010; Alves *et al.*, 2015). However, there is still no molecular evidence of conspecificity of species from European and Asian territories. Underestimation of this fact has led to inappropriate conclusions about the phylogeny and distribution of endemic aspidogastreae species (Chen *et al.*, 2010). Molecular studies of trematode phylogeny are commonly based on ribosomal DNA sequences, including 18S, 28S rDNA and internal transcribed spacer (ITS) regions (Jousson *et al.*, 2000; Lockyer *et al.*, 2003; Olson *et al.*, 2003; M.-X. Chen *et al.*, 2007; Petkevičiūtė *et al.*, 2010).

Nucleotide sequences of the ITS1–5.8S–ITS2 fragment of the ribosomal cluster were used in our study to evaluate the phylogenetic relationships of European and Far Eastern representatives of the genus *Aspidogaster* Baer, 1827: *A. conchicola* Baer, 1827; *A. limacoides* Diesing, 1834; *A. ijimai* Kawamura, 1915; and *A. chongqingensis* Wei, Huang & Dai, 2001.

## Materials and methods

### Sample collection and identification

Aspidobothrean trematodes were obtained during parasitological field work in 2009–2011 from the European part of Russia (Rybinsk reservoir (58°5'N 38°17'E) and Tvertza River (56°56'N 35°41'E)) and from the Russian Far East (Khanka Lake, Primorskyi Region (44°31'N 132°22'E), and two locations of the stream canal of the Amur River – 140 km downstream from Khabarovsk city (49°13'N 136°14'E) and near Nikolaevsk-na-Amure city (53°6'N 140°41'E)).

### Morphological data

Most trematode specimens were killed with hot tap water without crushing, and were flattened under slight pressure, fixed in 70% ethanol, stained with alum carmine and, after dehydrating and clearing, were mounted in Canada balsam. Species identification was performed according to different authors (Kawamura, 1915; Timofeeva, 1973; Tang & Tang, 1980; Bykhovskaya-Pavlovskaya, 1987; Pavljuchenko, 2007). Thus, we obtained aspidogastreae specimens that unambiguously belonged to *A. conchicola*, *A. limacoides* s. str. and *A. ijimai* (figs 1–3, table 1). Specimens of *A. ijimai* collected for the present study possessed a spined cirrus (fig. 3), which is in contrast to the original description of this species (Kawamura, 1915). We have studied specimens of *A. ijimai* from the type host (*Cyprinus carpio* (L.) s. lato) and type location (Biwa Lake, Japan), which were provided by Dr T. Shimazu. These specimens are morphologically similar to those from Primorye, including details of the cirrus structure. Voucher specimens of the studied species were deposited in the Museum of Helminthological Collections at the Centre for Parasitology of the A.N. Severtsov Institute of Ecology and Evolution, Moscow, Russia (IPEE RAS): *A. conchicola*, inventory number 14257; *A. limacoides* s. str., 14258; and *A. ijimai*, 14259 (table 1).

### DNA extraction, amplification and sequencing

Total DNA was extracted from separate mature worms fixed in 96% ethanol using a 'hot shot' technique, which has been described previously (Truett, 2006). The nuclear ITS1–5.8S–ITS2 was amplified using the polymerase chain reaction (PCR) with the universal primers BD1 (5'-GTCC TAACAAGGTTTCCGTA-3') and BD2 (5'-TATGCTTAA (G/A)TTCAGCGGGT-3') (Luton *et al.*, 1992). The initial PCR reaction was carried out in a total volume of 20 µl containing 0.25 mM of each primer pair, 1 µl DNA in water, 1 × *Taq* buffer, 1.25 mM deoxynucleoside triphosphates (dNTP), 1.5 mM MgCl<sub>2</sub> and 1 unit of *Taq* polymerase. The amplification of a 1200-bp fragment of ITS1–5.8S–ITS2 was performed in a GeneAmp 9700 (Applied Biosystems, Foster City, California, USA) with a 3-min denaturation hold at 94°C; 40 cycles of 30 s at 94°C, 30 s at 54°C and 2 min at 72°C; and a 7-min extension hold at 72°C. Negative and positive controls were amplified using both primers. The PCR products were directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems), as recommended by the manufacturer, with the internal sequencing primer 3S (5'-GGTACCGGTGGATCACGTGGCTAGTG-3') (Luton *et al.*, 1992). The PCR products were analysed using an ABI 3130 genetic analyser at the Institute of Biology and Soil Sciences, Far Eastern Branch of Russian Academy of Sciences. The sequences have been submitted to the European Nucleotide Archive (ENA)/GenBank with the following accession numbers: HE863950–HE863971, HE866756–HE866757.

### Alignment and phylogenetic analysis

The ribosomal DNA sequences were assembled with SeqScape v.2.6 software (Applied Biosystems) and aligned with sequences of aspidogastreae from China, retrieved from the GenBank database using ClustalW DNA weight matrix within MEGA 5.0 software alignment explorer (Tamura *et al.*, 2011).

Regions that could not be unambiguously aligned were excluded from the analyses. A number of variable, parsimony-informative sites, nucleotide compositions and substitution ratio analyses were performed using MEGA 5.0. Genetic divergence was estimated by calculating genetic *p*-distance (*d*) values. Phylogenetic analysis of the nucleotide sequences was undertaken, using maximum likelihood (ML) and Bayesian (BI) methods. Prior to analysis, the nucleotide substitution model was estimated using Akaike's information criterion (AIC) for ML (Akaike, 1974) and Bayesian information criterion (BIC) for BI (Huelsenbeck *et al.*, 2001) using the jModeltest v.3.07 software (Darriba *et al.*, 2012). The models TIM1 + G (Posada, 2003) and HKY + G (Hasegawa *et al.*, 1985) were estimated as those fitting the data best for ML and BI analyses, respectively. Phylogenetic trees were reconstructed with PhyML 3.1 (Guindon & Gascuel, 2003) and MrBayes v.3.1.2 software (Huelsenbeck *et al.*, 2001). A Bayesian algorithm was performed using the MCMC option with ngen = 1,000,000, nruns = 2, nchains = 4 and samplefreq = 100. Burn-in values were 250,000 for 'sump' and 'sumt' options. Optimization of the Bayesian inference algorithm was performed by setting up priors using the Tracer v.1.5.0

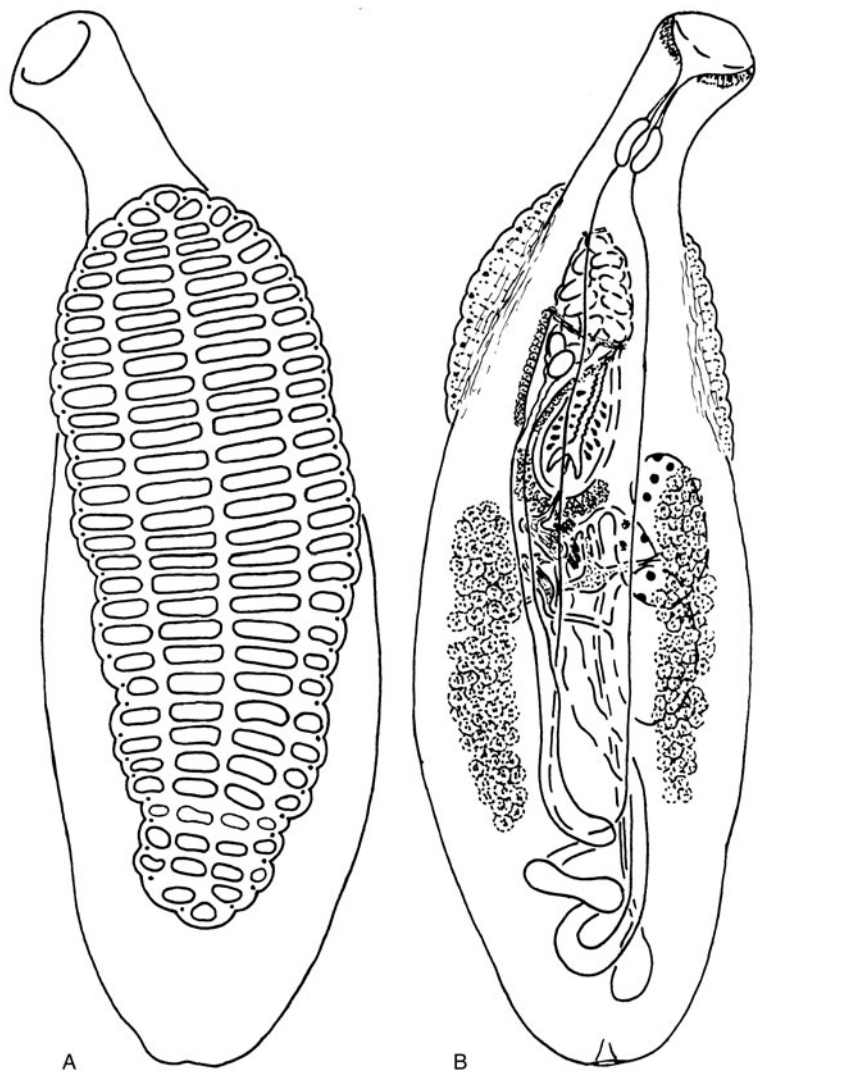


Fig. 1. *Aspidogaster conchicola* from *Colleopterum anatinum*, Tvertza River: (A) ventral view; (B) dorsal view. Scale bar: 1 mm.

software (Rambaut & Drummond, 2009). Phylogenetic relationship significance was estimated using a bootstrap analysis (Felsenstein, 1985) with 100 replications and posterior probabilities (Huelsenbeck *et al.*, 2001) for ML and BI analyses, respectively. Nucleotide sequences of the ITS1–5.8S–ITS2 fragment of the ribosomal cluster from the GenBank database were used in our study to evaluate the phylogenetic relationships of European and Far Eastern representatives of the genus *Aspidogaster* Baer, 1827: *A. conchicola* Baer, 1827; *A. limacoides* Diesing, 1834; *A. ijimai* Kawamura, 1915; and *A. chongqingensis* Wei, Huang & Dai, 2001 (table 1).

## Results

The amplification procedure produced a 1500-bp fragment of the ITS1–5.8S–ITS2 rDNA for all *Aspidogaster* specimens. After assembly and alignment procedures, the

resulting ITS rDNA sequences were 1437–1517 bp in length for different species. The fragment contained 442 variable and 414 parsimony-informative sites.

Genetic divergence between the ITS sequences from *Aspidogaster* species and sequences from *Multicalyx elegans* was estimated by the calculation of p-distances (table 2). The genetic p-distance between *A. limacoides* s. str. from the European part of Russia and *A. limacoides sensu* Chen *et al.*, 2010 from China was 13.7%, whereas between *A. limacoides sensu* Chen *et al.*, 2010 and *A. chongqingensis* from China it was only 0.09%. The genetic divergence between *A. ijimai* and *A. limacoides* s. str. from the European part of Russia was two times higher ( $d = 13.4\%$ ) than between *A. ijimai* and *A. limacoides sensu* Chen *et al.*, 2010 from China ( $d = 6.6\%$ ). Genetic differentiation between *A. ijimai* specimens from the Russian Far East and China was 0.28%. The highest values of p-distances were obtained between *A. conchicola* and other *Aspidogaster* species:  $d = 16.9\text{--}18.5\%$ .



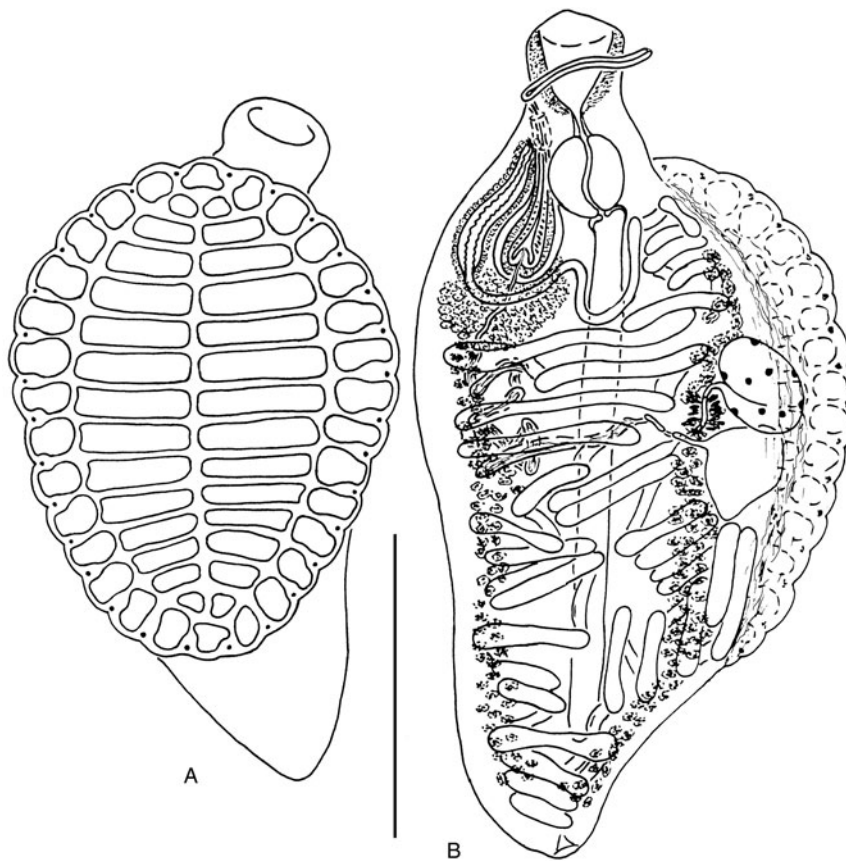


Fig. 2. *Aspidogaster limacoides* from *Rutilus rutilus*, Rybinsk Reservoir: (A) ventral view; (B) dorsal view. Scale bar: 1 mm.

Intraspecific molecular differentiation was revealed between *A. conchicola* and *A. ijimai* from different localities. Genetic *p*-distance values within *A. conchicola* ranged from 0.45% (between specimens from China and European part of Russia) to 0.94–0.96% (between specimens from the Russian Far East and the European part of Russia or China). The variation within *A. ijimai* ranged from 0 (Amur River, Khabarovsk/Khanka Lake) to 0.3% (Amur River, Nikolaevsk-na-Amure/China). Transition/transversion bias (*R*) between different *Aspidogaster* species ranged from 1.14 to 2.97 (table 3). A minimal range of *R* values was observed by pairwise comparison of *A. conchicola* with other *Aspidogaster* species (*R* = 1.14–1.25).

The phylogenetic relationships of *Aspidogaster* species were reconstructed using ML and BI methods (fig. 4). Both phylogenetic tree topologies showed the differentiation of *Aspidogaster* species into four clades, corresponding to different species, and were highly statistically supported. The first contained specimens of *A. conchicola*, which were subdivided according to geographical origin with high statistical support. Specimens of *A. ijimai* formed the second distinct clade with high support. *Aspidogaster ijimai* was subdivided into three groups, corresponding to 'Russian' and 'Chinese' samples. It is notable that Chinese samples of *A. ijimai* formed a distinct

compact group within the Russian *A. ijimai* cluster. The third clade included *A. limacoides sensu Chen et al., 2010* and *A. chongqingensis* from China, and the fourth clade contained *A. limacoides s. str.* from the European part of Russia.

## Discussion

Our results showed considerable molecular differentiation between *A. limacoides s. str.* from the European part of Russia and *A. limacoides sensu Chen et al., 2010* from China. *Aspidogaster limacoides s. str.* is reliably known only from European, and Central and Western Asian territories (reviewed by Alves *et al., 2015*). Records of *A. limacoides* in China were presented by several authors (Jin *et al., 1993*; Wang *et al., 1997*; Zhang *et al., 1999*; Chen *et al., 2010*), but these data are not supported by the morphological description of the parasite (Kawamura, 1915; Timofeeva, 1973; Tang & Tang, 1980; Bykhovskaya-Pavlovskaya, 1987; Pavljuchenko, 2007). These circumstances raised some doubts about the presence of *A. limacoides s. str.* in China. Our molecular data also indicate that the reports of *A. limacoides sensu Chen et al., 2010* from China are not reliable. These worms are conspecific with *A. chongqingensis* from *Spinibarbus sinensis* (Bleeker, 1871) caught from the Jialing

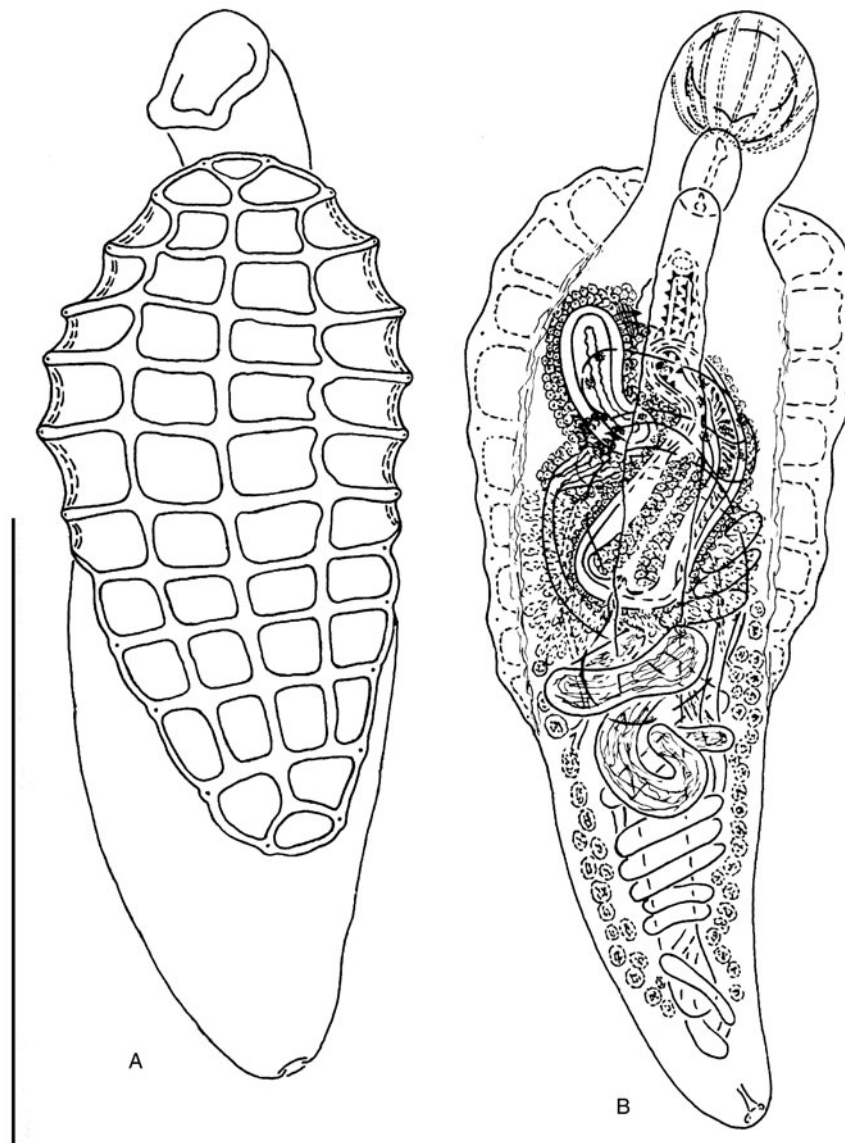


Fig. 3. *Aspidogaster ijimai* from *Cyprinus carpio s. lato*: (A) ventral view; (B) dorsal view. Scale bars: (A) 0.4 mm; (B) 1 mm.

River of Chongqing, China, as evidenced by the extremely low genetic p-distance values, corresponding to intra-specific genetic variation for trematodes (Jousson *et al.*, 2000; D. Chen *et al.*, 2007). Based on ITS1–5.8S–ITS2 rDNA sequence data, *A. chongqingensis* is phylogenetically closer to *A. ijimai* than to *A. limacoides s. str.* (fig. 4). These results are unexpected because *A. chongqingensis* is morphologically closer to *A. limacoides s. str.* in a number of significant features: size ratio of the buccal funnel and pharynx, degree of development of the external field of prostatic cells and presence of an unarmed cirrus (fig. 2) (Wei *et al.*, 2001).

The species *A. conchicola* possesses maximal values of intraspecific molecular differentiation among the investigated species of aspidogastreaans (table 2, fig. 4).

Specimens from the Russian Far East were collected from the Amur River basin (Khanka Lake), whereas samples from China (Hubei province) and the European part of Russia were from different river systems, which have no connections with the Amur River. Genetic p-distance values between different geographical samples indicate that *A. conchicola* from the Russian Far East has existed separately from European and Chinese specimens for twice as long as the two latter groups have been separated from each other. Despite the wide distribution of *A. conchicola* in molluscs, this species has also been found in freshwater fishes and even aquatic reptiles (reviewed by Alves *et al.*, 2015; see also Vosnesenskaya, 1968; Dvodryadkin, 1976). For this reason, there may have been a possible exchange of *A. conchicola* specimens

Table 1. List of the representatives of the genus *Aspidogaster* incorporated in sequence analysis (*n*, number of replicates; voucher accession numbers are in bold).

Species	<i>n</i>	Sample number	Host species	Location	Author	ENA/NCBI reg. number
<i>A. limacoides</i> s. str.	4	1523	<i>Rutilus rutilus</i> (Cyprinidae, Teleostei)	Rybinsk Reservoir, Yaroslavl Region, ER*, <b>voucher no. 14258</b>	Original data	HE863966–HE863969
<i>A. limacoides</i> s. str.	2	1524	<i>Blicca bjoerkna</i> (Cyprinidae, Teleostei)	Rybinsk Reservoir, Yaroslavl Region, ER	Original data	HE863970–HE863971
<i>A. conchicola</i>	4	1447–49	<i>Colletopterum anatinum</i> (Unionidae, Bivalvia)	Tvertza River, Tver Region, ER, <b>voucher no. 14257</b>	Original data	HE863962–HE863965
<i>A. conchicola</i>	4	1727, 1239	<i>Cristaria herculea</i> (Unionidae, Bivalvia)	Khanka Lake, Primorskyi Region, RFE	Original data	HE863958, HE863961–HE863965
<i>A. ijimai</i>	1	1782	<i>Cyprinus carpio</i> (Cyprinidae, Teleostei)	Khanka Lake, Primorskyi Region, RFE, <b>voucher no. 14259</b>	Original data	HE866757
<i>A. ijimai</i>	8	1406/2	<i>Cyprinus carpio</i> (Cyprinidae, Teleostei)	Amur River, near Nikolaevsk-na-Amure city, Khabarovsk Region, RFE	Original data	HE863950–HE863957
<i>A. ijimai</i>	1	1444/1	<i>Cyprinus carpio</i> (Cyprinidae, Teleostei)	Amur River, near Khabarovsk, Khabarovsk Region RFE	Original data	HE866756
<i>A. limacoides</i> sensu Chen et al., 2010	1	–	<i>Coreius guichenoti</i> (Cyprinidae, Teleostei)	Jialing River, Beibei, Chongqing, China	Chen et al., 2010	DQ345319
<i>A. chongqingensis</i>	1	–	<i>Spinibarbus sinensis</i> (Cyprinidae, Teleostei)	Jialing River, Beibei, Chongqing, China	Chen et al., 2010	DQ345324
<i>A. conchicola</i>	1	–	<i>Mylopharyngodon pieus</i> (Cyprinidae, Teleostei)	Danjiangkou Reservoir, Danjiangkou, Hubei; Liangzi Lake, E'zhou, Hubei, China	Chen et al., 2010	DQ345317–DQ345318
<i>A. ijimai</i>	4	–	<i>Cyprinus carpio</i> (Cyprinidae, Teleostei)	Danjiangkou Reservoir, Danjiangkou, Hubei; Jaingkou Reservoir, Xinyu, Jiangxi; Niushan Lake, Wuhan, Hubei; Jialing River, Beibei, Chongqing, China	Chen et al., 2010	DQ345320–DQ345323
Outgroup						
<i>Multicalyx elegans</i>	1	–	<i>Callorhynchus milii</i> (Callorhynchidae, Chondrichthyes)	Australia: Hobart, Tasmania	Gao, Chen & Nie, 2005 (unpublished)	DQ345325

\* ER, European part of Russia; RFE, Russian Far East.

ENA, European Nucleotide Archive; NCBI, National Center for Biotechnology Information.

Table 2. Genetic divergence of *Aspidogaster* species, estimated with p-distance calculations by means of ITS1–5.8S–ITS2 rDNA nucleotide sequences.

Species	1	2	3	4	5	6	7	8	9	10
1. <i>A. ijimai</i> AN*	–	–	–	–	–	–	–	–	–	–
2. <i>A. ijimai</i> AK	0.06	–	–	–	–	–	–	–	–	–
3. <i>A. ijimai</i> Kh	0.06	0.00	–	–	–	–	–	–	–	–
4. <i>A. ijimai</i> Chi	0.30	0.23	0.23	–	–	–	–	–	–	–
5. <i>A. hongqingensis</i> Chi	6.37	6.30	6.30	6.49	–	–	–	–	–	–
6. <i>A. limacoides sensu</i> Chen <i>et al.</i> , 2010 Chi	6.45	6.39	6.39	6.58	0.09	–	–	–	–	–
7. <i>A. limacoides</i> ER	13.55	13.49	13.49	13.51	13.64	13.73	–	–	–	–
8. <i>A. conchicola</i> Chi	17.46	17.46	17.46	17.65	17.93	18.02	16.89	–	–	–
9. <i>A. conchicola</i> Kh	17.99	17.99	17.99	18.19	18.42	18.51	17.38	0.94	–	–
10. <i>A. conchicola</i> ER	17.61	17.61	17.61	17.80	18.02	18.10	17.00	0.45	0.96	–
11. <i>Multicalyx elegans</i> Aus	34.72	34.67	34.67	34.65	34.84	34.75	35.45	35.99	36.48	35.99

\*AN, Amur River, Nikolaevsk-na-Amure; AK, Amur River, Khabarovsk; Kh, Khanka Lake; Chi, China; ER, European part of Russia; Aus, Australia.

Table 3. Transition/transversion ratio bias ( $R^*$ ), obtained by pairwise comparison of ITS1–5.8S–ITS2 rDNA sequences of different *Aspidogaster* species.

Species	1	2	3
1. <i>A. ijimai</i>	–	–	–
2. <i>A. limacoides</i>	2.15	–	–
3. <i>A. hongqingensis</i>	2.97	1.56	–
4. <i>A. conchicola</i>	1.25	1.14	1.34

\* $R = [A^*G^*k1 + T^*C^*k2] / [(A + G)^*(T + C)]$ , where  $k1$  and  $k2$  are frequencies of transitions between purines and pyrimidines, respectively.  $R$  becomes 0.5 when there is no bias towards either transitional or transversional substitution, because when the two kinds of substitution are equally probable, there are twice as many possible transversions as transitions.

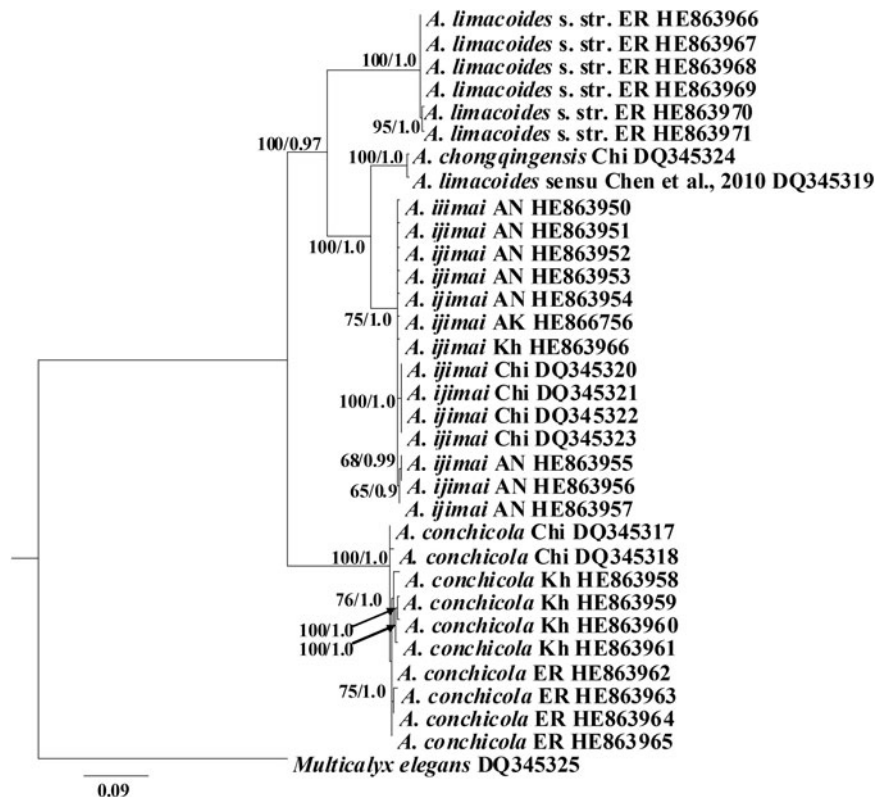


Fig. 4. Phylogenetic tree based on analysis of ITS1–5.8S–ITS2 rDNA sequences of species of the genus *Aspidogaster* using the Bayesian method of phylogenetic reconstructions. Nodal numbers give bootstrap statistical support for ML/BI analyses. AN, Amur River, Nikolaevsk-na-Amure; AK, Amur River, Khabarovsk; Kh, Khanka Lake; Chi, China; ER, European part of Russia.



between different territories in the past by host-switching processes, which have been described for some digenetic trematodes (Attwood *et al.*, 2002, 2004, 2007). Moreover, aspidogastriids are well-known for their low host specificity, which can favour their dispersion (Alves *et al.*, 2015). However, this assumption needs to be confirmed by detailed studies of the phylogeography of *Aspidogaster* species and their host fish species; facultative definitive hosts are potential distributors of these flatworms.

Molecular differentiation between *A. conchicola* and other *Aspidogaster* species studied here were also characterized by maximal values (table 2). Transition/transversion bias values (R) varied with the same pattern, suggesting a higher amount of transversion substitution type between ITS1–5.8S–ITS2 sequences of *A. conchicola* and other *Aspidogaster* species. Molluscs are obligate hosts for *Aspidogaster* species. However, the final stages of the life cycles of these worms electively occur in fish species (Timofeeva, 1975, 2005). Freshwater fish species are important for circulation of the three species examined in the present study – *A. chongqingensis*, *A. ijimai* and *A. limacoides* s. str. – and this is evidenced by the occurrence of these species in these vertebrates (Achmerov, 1956; Gao *et al.*, 2003; Alves *et al.*, 2015). *Aspidogaster conchicola* is generally known to infect freshwater bivalves and gastropods (Michelson, 1970; Dvodryadkin, 1976; Dugarov, 2010), but there are few reports about this worm within fish species (Alves *et al.*, 2015). The phylogenetic relationships of species obtained in the present study showed earlier divergence of the ancestral form of the parasite species group, namely *A. chongqingensis*, *A. ijimai* and *A. limacoides* s. str. in comparison with *A. conchicola* (fig. 4). This may be evidence that a wide inclusion of fish species into the aspidogastrian life cycle is plesiomorphic. This assumption corresponds with the hypothesis of aspidogastrian evolution, based on morphological and ecological data. These consider *A. conchicola* maturing within bivalve molluscs as a progenetic phenomenon, which appeared after fish-specific adaptation (Timofeeva, 2005).

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### Conflict of interest

None.

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