Parasitology International 71 (2019) 167–176

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Redescription and supplementary molecular characteristics of *Aspidogaster ijimai* Kawamura, 1915 (Trematoda, Aspidogastrea, Aspidogastridae), a parasite of *Cyprinus carpio* Linnaeus, 1758 *s. lato* (Actinopterygii) and freshwater bivalves in East Asia

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ARTICLE INFO

Keywords:
Morphology
Aspidogastridae
ITS
28S
DNA sequences
Phylogeny

ABSTRACT

A detailed morphological description and ribosomal DNA (rDNA) sequence molecular data for *Aspidogaster ijimai* from Japan are provided. Morphological analysis, including a description of the cirrus-sac, indicate the conspecificity of Japanese and continental East Asian *A. ijimai* specimens. Analysis of ITS1-5.8S-ITS2 rDNA sequences of Japanese, Chinese and Russian specimens confirmed the morphological results. Phylogenetic analysis using ITS rDNA sequences confirmed that *A. ijimai* is a sister species for *Aspidogaster chongqingensis*. Median-joining network analysis showed an initial molecular differentiation step of Russian specimens from group of Japanese-Chinese samples. Our 28S rDNA results based on maximum likelihood (ML) and Bayesian (BI) phylogenetic analyses indicated well-supported monophyly of the *Aspidogaster conchicola* + *A. ijimai* group, a finding that indicates that these species are congeneric. At the same time, our data demonstrated that the genus *Lobatostoma* is paraphyletic and the family Aspidogastridae is polyphyletic, results that confirm previous studies.

1. Introduction

Aspidogastreans are parasitic flatworms that mainly infect mollusks, fish and freshwater turtles [1]. These worms are characterised by the presence of a longitudinal ventral row of suckers, septa or a ventral adhesive disc divided into alveoli [2,3]. The life cycle of these parasites is devoid of parthenogenetic stages [4].

Aspidogaster ijimai Kawamura, 1915 is one of six species of the genus *Aspidogaster* Baer, 1827 reported for East Asia [1,5,6]. This species spread in the Russian Primorye and Khabarovsk Regions [6], Japan [7,8], South Korea [9] and China [10–15]. The first description of *A. ijimai* was performed for worms from the carp, *Cyprinus carpio* Linnaeus, 1758 *s. lato* (Actinopterygii), collected from Lake Biwa, west-central Honshu, Japan [7]. Most of later reports about this species were provided for the same host species (reviewed by [1]). However, Zhang in 2006 found this parasite in the bivalve clam *Corbicula fluminea* (Müller, 1774) [10].

A morphological description of *A. ijimai* was presented by different authors [7,9–12]. However, these studies contained discrepant data on detailed cirrus and excretory system morphology. Molecular-based phylogenetic analyses, provided by Atopkin and collaborators [6], confirmed conspecificity of different *A. ijimai* isolates from Russia and China. At the same time, conspecificity of Japanese and continental East Asian *A. ijimai* specimens has not proven with molecular data yet. Herein we represent the new morphological and molecular data on this species from the type locality with analysis of interrelationships within the Aspidogastridae.

2. Materials and methods

2.1. Sample collection and morphological observation

Juvenile (without eggs) and subgravid (with a few eggs) *A. ijimai* specimens were collected from *C. carpio s. lato* caught in a canal near Lake Biwa or a fishing harbour at Harie in Lake Biwa, Takashima City, Shiga Prefecture, Japan (35°21′54″N, 136°03′16″E) during December 2 and 5, 2016. There are two carp strains in Lake Biwa (Lake Biwa wild strain and Eurasian strain), both of which are recognised as almost

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https://doi.org/10.1016/j.parint.2019.04.017
Received 31 January 2019; Received in revised form 22 March 2019; Accepted 18 April 2019
Available online 20 April 2019
1383-5769/

Table 1
List of the representatives of the genus *Aspidogaster* incorporated in analysis of ITS1-5.8S-ITS 2 rDNA sequences (*n* – number of replicates).

Species	*n*	Host species	Location	Author	NCBI reg. number
Aspidogaster ijimai	12	*Cyprinus carpio* Linnaeus, 1758 *s. lato* (Cyprinidae, Actinopterygii)	Lake Biwa, Takashima City, Shiga Prefecture, Japan	Original data	MK387320–MK387330
A. ijimai	1	*Id.*	Khanka Lake, Primorskyi Region, Russia	[6]	HE866757
A. ijimai	8	*Id.*	Amur River, near Nikolaevsk–na–Amure city, Khabarovsk Region, Russia	[6]	HE863950– HE863957
A. ijimai	1	*Id.*	Amur River, near Khabarovsk, Khabarovsk Region Russia	[6]	HE866756
A. ijimai	4	*Id.*	Danjiangkou Reservoir, Danjiangkou, Hubei; Jaingkou Reservoir, Xinyu, Jiangxi; Niushan Lake, Wuhan, Hubei; Jialing River, Beibei, Chongqing, China	[15]	DQ345320–DQ345323
Aspidogaster chongqingensis	1	*Coreius guichenoti* (Sauvage et Dabry de Thiersant, 1874) (Cyprinidae, Actinopterygii)	Jialing River, Beibei, Chongqing, China	[15] (as *A. limacoides*)	DQ345319
A. chongqingensis	1	*Spinibarbus sinensis* (Bleeker, 1871) (Cyprinidae, Actinopterygii)	Jialing River, Beibei, Chongqing, China	[15]	DQ345324
Aspidogaster conchicola	4	*Colletopterum anatinum* (Linnaeus, 1758) (Unionidae, Bivalvia)	Tvertza River, Tver Region, Russia	[6]	HE863959–HE863962
A. conchicola	4	*Cristaria herculea* (Middendorff, 1847) (Unionidae, Bivalvia)	Khanka Lake, Primorskyi Region, Russia	[6]	HE863958, HE863963–HE863965
A. conchicola	1	*Mylopharyagodon pieus* (Richardson, 1846) (Cyprinidae, Actinopterygii)	Danjiangkou Reservoir, Danjiangkou, Hubei; Liangzi Lake, E'zhou, Hubei, China	[15]	DQ345317, DQ345318
Aspidogaster limacoides	4	*Rutilus rutilus* (Linnaeus, 1758) (Cyprinidae, Actinopterygii)	Rybinsk Reservoir, Yaroslavl Region, Russia	[6]	HE863966–HE863969
A. limacoides	2	*Blicca bjoerkna* (Linnaeus, 1758) (Cyprinidae, Actinopterygii)	Rybinsk Reservoir, Yaroslavl Region, Russia	[6]	HE863970–HE863971
Outgroup					

(*continued on next page*)

Table 1 (*continued*)

Species	*n*	Host species	Location	Author	NCBI reg. number
Multicalyx elegans	1	*Callorhinchus milii* Bory de Saint-Vincent, 1823 (Callorhinchidae, Chondrichthyes)	Australia: Hobart, Tasmania	Gao, Chen and Nie (unpublished)	DQ345325

Table 2
List of the representatives of Aspidogastrea incorporated in phylogenetic analysis based on 28S rDNA sequences (*n* – number of replicates).

Species	*n*	Host species	Location	Author	NCBI reg. number
Aspidogastrea					
Aspidogaster ijimai (Kawamura, 1915)	3	*Cyprinus carpio* Linnaeus, 1758 *s. lato* (Cyprinidae, Actinopterygii)	Lake Biwa, Takashima City, Shiga Prefecture, Japan	Original data	MK387331–MK387333
Aspidogaster conchicola (Baer, 1827)	1	*Quadrula pustulosa* (Lea, 1831) (Unionidae, Bivalvia)	USA	[29]	AY222162
Cotylaspis sp.	1	*Sternotherus odoratus* (Latreille, 1801) (Kinosternidae, Reptilia)	USA	Aymond, Caoili, Tkach and McAllister (unpublished)	KM457145
Cotylaspis sp.	1	*Pelodiscus sinensis* (Wiegmann, 1835) (Trionychidae, Reptilia)	Viet Nam	[29]	AY222165
Cotylogaster basiri (Siddiqi et Cable, 1960)	1	*Pogonias cromis* (Linnaeus, 1766) (Sciaenidae, Actinopterygii)	USA	[29]	AY222164
Lobatostoma kemostoma (MacCallum et MacCallum, 1913)	1	*Trachinotus carolinus* (Linnaeus, 1766) (Carangidae, Actinopterygii)	Brazil	[30]	KF561238
Lobatostoma manteri (Rohde, 1973)	1	*Trachinotus blochii* (Lacepède, 1801) (Carangidae, Actinopterygii)	Australia	[31]	AY157177
Multicalyx elegans (Olsson, 1869)	1	*Callorhinchus milli* Bory de Saint-Vincent, 1823 (Callorhinchidae, Chondrichthyes)	Australia	[29]	AY222163
Multicotyle purvisi (Dawes, 1941)	1	*Siebenrockiella crassicollis* (Gray, 1831) (Geoemydidae, Reptilia)	Malaysia: Malaya	[29]	AY222166
Neosychnocotyle maggiae Snyder et (Tkach, 2007)	1	*Id.*	Australia: Northern Territory	[32]	EF015578
Rugogaster hydrolagi (Schell, 1973)	1	*C. milii*	Australia	[31]	AY157176
Sychnocotyle kholo (Ferguson, Cribb et Smales, 1999)	1	*Carettochelys insculpta* Ramsay, 1886 (Carettochelyidae, Reptilia)	Australia: Queensland, Ross River	[32]	EF015579
Outgroup (Digenea)					
Brachylaima thompsoni (Sinitsin, 1931)	1	Blarina brevicauda (Say, 1823) (Soricidae, Mammalia)	Wisconsin, USA	[33]	AF184262
Leucochloridium perturbatum (Pojmanska, 1969)	1	*Turdus merula* Linnaeus, 1758 (Turdidae, Aves)	Czech Republic	[29]	AY222169
Leucochloridium paradoxum (Carus, 1833)	1	*Succinea* sp. (Succineidae, Gastropoda)	Leningrad Oblast, Russia	[34]	KP938187
Zeylanurotrema spearei (Cribb et Barton, 1991)		*Bufo marinus* (Linnaeus, 1758) (Bufonidae, Amphibia)	Australia	[29]	AY222170

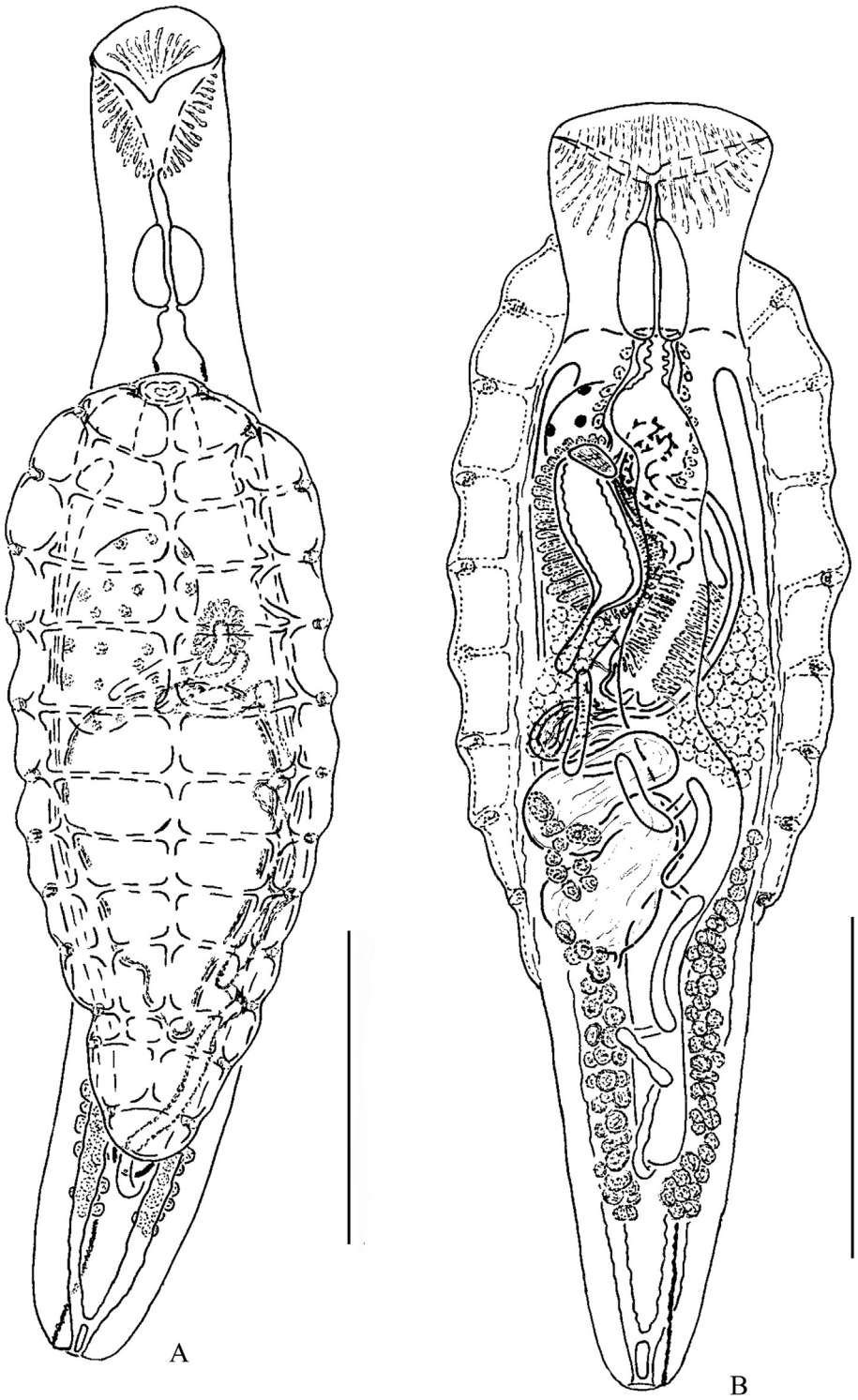

Fig. 1. *Aspidogaster ijimai*, whole specimens. A – neotype, ventral view. B – neoparatype, dorsal view. Scale bars 0.5 mm.

different biological species [16]. Strain affiliation in fish was not identified during this study. Six carp individuals (standard length 11.1–47.4 cm: average 18.1 cm) were investigated. Four-hundred thirty-six individuals of *A. ijimai* were obtained only from the large-sized fish (standard length 47.4 cm) (prevalence 16.7%).

Worms collected for morphological study were fixed in hot 4% formaldehyde and stained with acetocarmine. For a detailed morphological study, we prepared several slides of isolated reproductive organs extracted from the bodies using needles. All the lengths in morphological descriptions are in μm (unless otherwise noted). The forebody was measured from the anterior end to the proximal base of the ventral adhesive disc, the hindbody (from the distal base of ventral disc to posterior end). Specimens destined for molecular analysis were fixed in 96% ethanol and stored at −18 °C.

2.2. DNA extraction, amplification and sequencing

Total DNA was extracted from eleven 96% ethanol-fixed separate

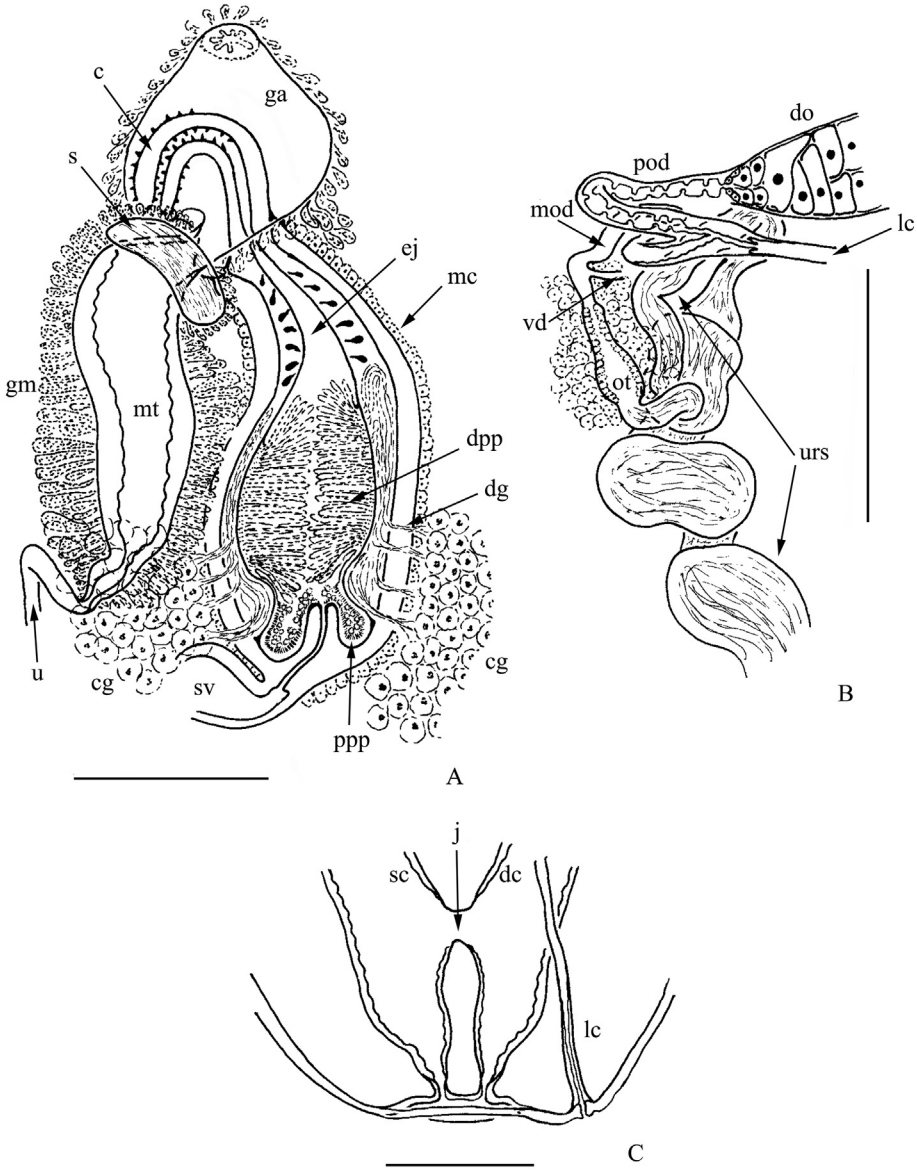

Fig. 2. *Aspidogaster ijimai*, details of the structure of the reproductive and excretory systems. A – terminal genitalia, dorsal view; B – ovarian complex, dorsal view; C – terminal part of excretory system, dorsal view; c – cirrus, cg – clusters of external prostatic cells, dc – dextral collecting duct, dg – bunches of external prostatic cells ducts, do – distal part of ovary, dpp – distal part of pars prostatica, ej – ejaculatory duct, ga – common genital atrium, gm – gland cells covering metraterm, j – jumper, lc – Laurer's, canal, mc – cell bodies (myocytons?) covering cirrus-sac, mod – middle portion of oviduct, mt – metraterm, ot – ootype with Mehlis' gland, pod – proximal portion of oviduct, ppp – proximal part of pars prostatica, s – sphincter, sc– sinistral collecting duct, sv – fragment of external seminal vesicle, u – uterus, urs –uterine seminal receptacle, vd – common vitelline duct. Scale bars: A, B – 0.15 mm; C – 0.05 mm.

Table 3
Genetic distances between representatives of the genus *Aspidogaster* calculated on the basis of ITS1-5.8S-ITS2 rDNA nucleotide sequences (*p*-distance values (%) – below diagonal; std.err. values – above diagonal).

		1	2	3	4	5	6
1	*A. ijimai* from Russia		0.12	0.12	0.68	1.00	1.01
2	*A. ijimai* from China	0.31		0.03	0.68	0.99	1.01
3	*A. ijimai* from Japan	0.26	0.05		0.69	0.99	1.01
4	*A. chongqingensis*	7.31	7.46	7.46		0.97	1.12
5	*A. limacoides*	17.84	17.90	17.88	18.74		1.04
6	*A. conchicola*	20.62	20.88	20.85	22.03	18.82	

subgravid worms using a “hot shot” technique, which was described previously [17]. The nuclear 28S ribosomal DNA (rDNA) was amplified using polymerase chain reaction (PCR) with the primers DIG12 (5′-AAGCATATCACTAAGCGG-3′) and 1500R (5′-GCTATCCTGAGGGAA ACTTCG-3′), which were described earlier [18]. Ribosomal ITS1-5.8S-ITS2 was amplified with the universal primers BD1 (5′-GTCGTAACAA GGTTTCCGTA-3′) and BD2 (5′-TATGCTTAA(G/A)TTCAGCGGGT-3′) [19]. The initial PCR was performed in a total volume of 20 µl that contained 0.25 mM of each primer pair, 1 µl DNA in water, 1× Taq buffer, 1.25 mM dinucleotide triphosphates (dNTPs), 1.5 mM $MgCl_2$ and 1 unit of *Taq* polymerase. The amplification of a 1200-base pair (bp) fragment of ITS1-5.8S-ITS2 was performed in a GeneAmp 9700 (Applied Biosystems) with a 3 min denaturation hold at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 55 °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, as recommended by the manufacturer, with the internal sequencing primers [18,19]. The PCR products were analysed using an ABI 3130 genetic analyser at the Institute of Biology and Soil Sciences FEB RAS. The sequences have been submitted to GenBank with the following accession numbers: MK387320–MK387330, MK387331–MK387333 (Tables 1 and 2).

2.3. Alignment and phylogenetic analysis

Ribosomal DNA sequences were assembled with SeqScape v.2.6 software and aligned with sequences of Chinese Aspidogastrids, retrieved from the Genbank database using the ClustalW DNA weight matrix within MEGA 7.0.26 software alignment explorer [20]. Genetic divergence was estimated by calculating genetic p-distance (d) values using MEGA 7.0.26 software. Phylogenetic analysis of nucleotide

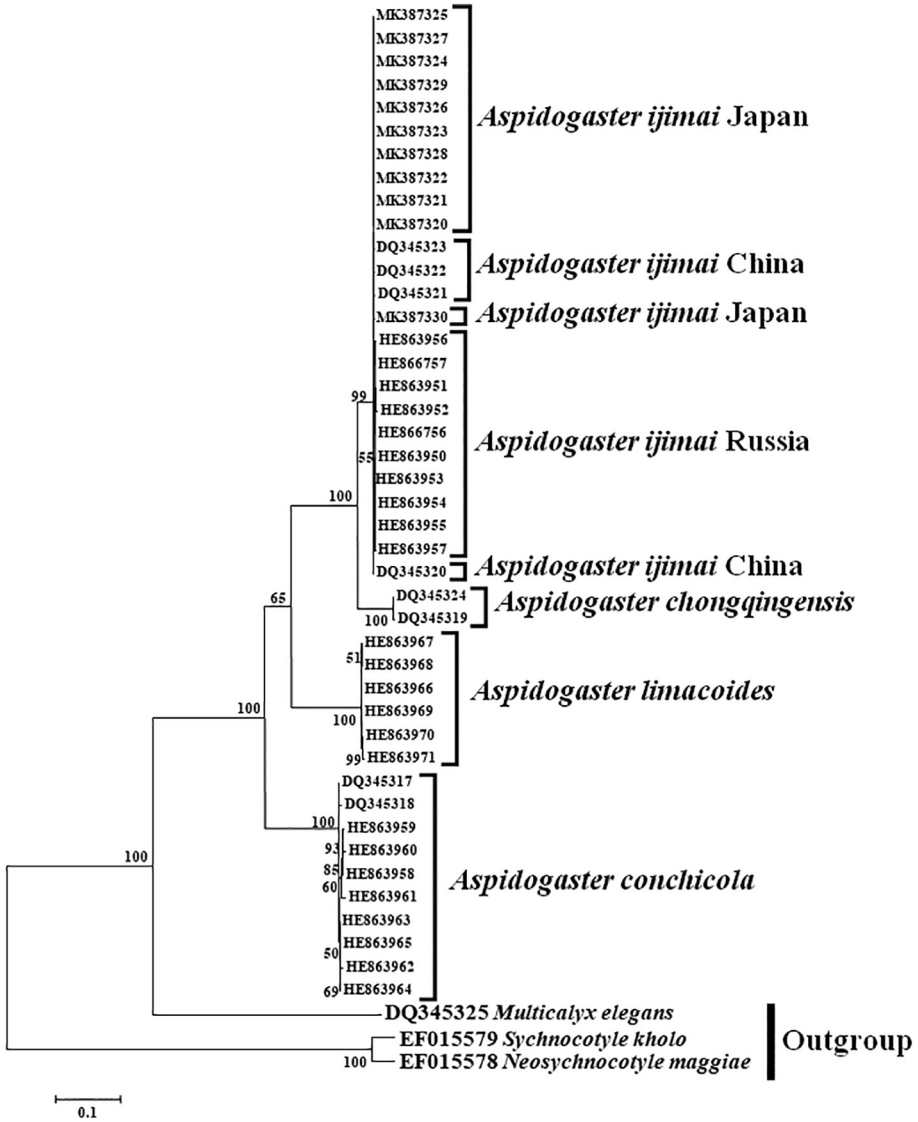

Fig. 3. Maximum Likelihood phylogenetic tree of the genus *Aspidogaster* based on ITS1-5.8S-ITS2 rDNA sequence data. Nodal numbers – bootstrap values. Only values higher of 50% are showed.

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 [21] and Bayesian information criterion (BIC) for BI [22] using jModeltest v.3.07 software [23]. The models TIM2 + G + I [24] and HKY + G [25] were estimated as those best fitting the ITS rDNA sequence data for ML and BI analyses, respectively. For 28S rDNA sequence data, the models GTR + G + I [26] and TVM + G + I [24] were estimated as optimal for ML and BI analyses, respectively. Phylogenetic trees were reconstructed with PhyML 3.1 [27] and MrBayes v.3.1.2 software [22]. A Bayesian algorithm was performed using the MCMC option with ngen = 10,000,000, nruns = 2, nchains = 4 and samplefreq = 100. Burnin values were 250,000 for “sump” and “sumt” options. Phylogenetic relationship significance was estimated using a bootstrap analysis [28] with 100 replications and posterior probabilities [22] for ML and BI analyses, respectively. ITS1-5.8S-ITS2 fragment nucleotide sequences, and partial 28S ribosomal RNA (rRNA) gene sequences of the ribosomal cluster from the GenBank database [6,15,29–34], were used in our study to evaluate the phylogenetic relationships of Aspidogastridae (Tables 1 and 2).

3. Results

3.1. Morphology

The *A. ijimai* type specimens, and other materials on this species collected by Tamiji Kawamura, were lost. Single notification about Kawamura's samples can be found in the Kyoto University Museum – a bottle (sample No. Iv 133) labeled as “*Aspidogaster ijimai*” collected on March 27, 1915 from the intestine of a carp captured at Otsu City on Lake Biwa. We studied the content of this bottle and found only a piece of the host intestine. In this case, we believe that the designation of a neotype and neoparatypes of *A. ijimai* based on our material is needed.

Family Aspidogastridae Poche, 1907.

Subfamily Aspidogastrinae Poche, 1907.

Genus *Aspidogaster* Baer, 1827.

Aspidogaster ijimai Kawamura, 1915 (Figs. 1 and 2).

Description (based on nine subgravid whole-mounted specimens – one neotype and eight neoparatypes; five slides with isolated ovarian complex and five slides with isolated terminal genitalia; measurements of the neotype in square brackets). Body elongated; length 1.86–2.6 [2.23] mm, maximum width 0.42–0.51 [0.43] mm in middle third of body. Posterior end of body with shallow pocket-shaped terminal

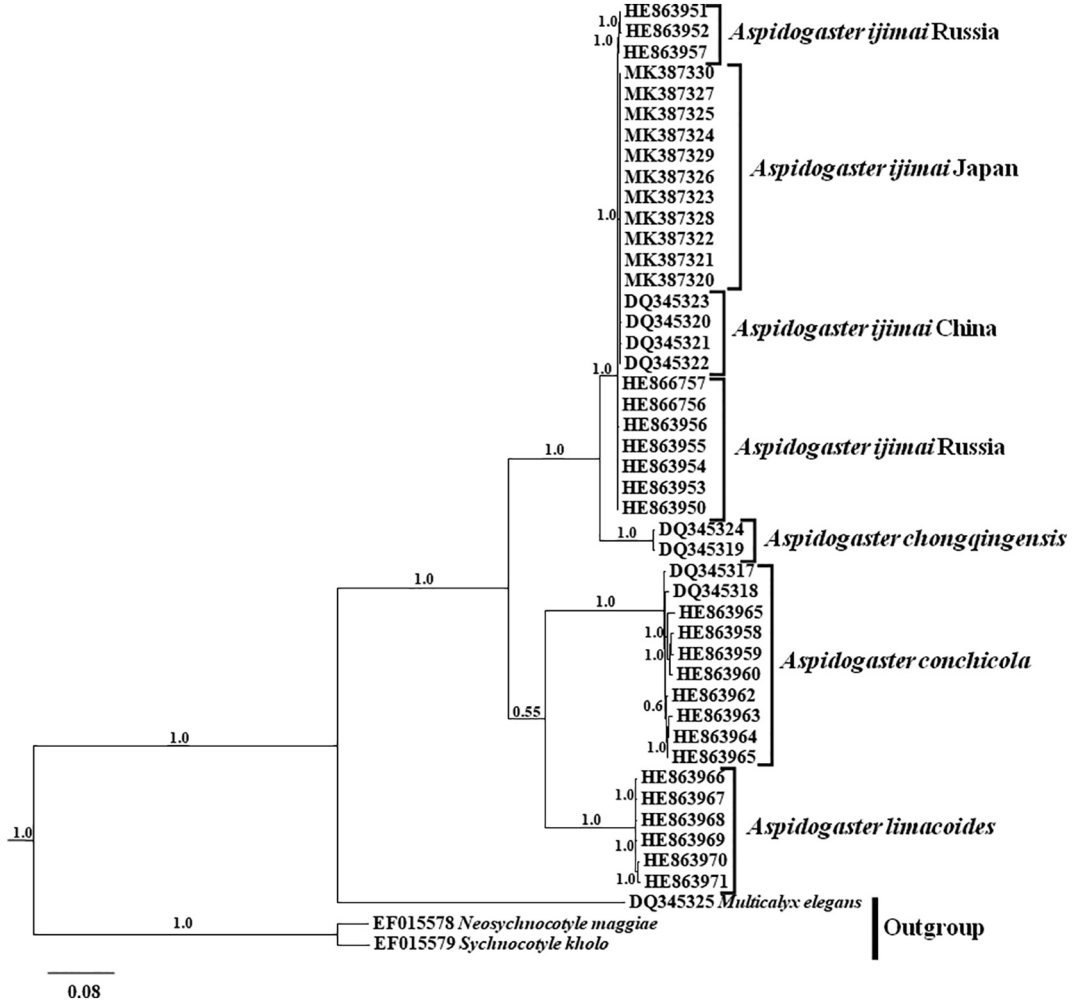

Fig. 4. Bayesian phylogenetic tree of the genus *Aspidogaster* based on ITS1-5.8S-ITS2 rDNA sequence data. Nodal numbers – posterior probabilities values.

depression. Tegument unarmed. Forebody and hindbody 16.9–28.3 [28.3]% and 12.7–22.3 [15.3]% of body length, respectively. Head lobes absent. Mouth funnel weakly muscular, terminal. Ventral adhesive disc oval, large, 1.29–1.6 [1.29] × 0.52–0.75 [0.52] mm, with 42–46 alveoli (24–26 external and 18–20 internal) and 24–26 marginal organs. Internal alveoli of ventral adhesive disc lie in two longitudinal submedan rows of 9–10 each. Prepharynx 45–97 [71]. Pharynx oval, strongly muscular, 161–180 [161] × 103–129 [116]. Oesophagus 64–103 [84]. Caecum blind and simple, dorsal to terminal genitalia; terminating near posterior end of body. Postcaecal space 303–417 [303], representing 13.6–17.7 [13.6]% of body length. Gonads ventral and postero-ventral to terminal genitalia. Testis single, oval, 509–728 [606] × 290–432 [322], in middle part of body. Vas deferens arising from antero-dorsal part of testis. External seminal vesicle long and convoluted. Cirrus-sac muscular, claviform, 490–586 [586] × 174–219 [187], whole in second quarter of body or in second quarter and partly third quarter of body; communicating with common genital atrium, and containing massive bipartite pars prostatica, ejaculatory duct and long invaginated cirrus armed with numerous spines. Proximal part of pars prostatica with fringed surface covered with spherical granules; distal part lined with numerous large finger-like processes that are supposedly associated with distal portions of ducts of external prostatic cells. External prostatic cells in two clusters lying sinistral and dextral to cirrus-sac. Ducts of external prostatic cells grouped into several bunches, each of which penetrates into the cirrus-sac through aseparate canal in the muscular wall. Cirrus-sac covered with numerous cells (probably myocytons). Common genital atrium 165–225 [165] in length, surrounded by glandular cells. Genital pore, median, immediately anterior to proximal base of ventral disc. Ovary comma-shaped, proximal portion 335–419 [354] × 155–238 [213], distal portion 174–258 [225] × 64–90 [71]; dextro-submedian, pre-testicular and contiguous with testis. Proximal portion of oviduct septate, middle portion unspecialised, distal portion expands slightly to form ootype. Mehlis' gland distinct. Laurer's canal long, opening at posterior extremity of body, sinistro-dorsally to terminal depression. Uterine seminal receptacle present. Uterus long, convoluted, terminating with muscular metraterm. Metraterm large, 325–387 [325] × 150–180 [150], with massive sphincter at distal extremity; covered with glandular cells, and opening into common genital atrium sinistro-dorsally or strictly sinistraly. Vitellarium follicular, in two lateral fields; anterior edge varies between nearly mid-level and last third of ventral disc, posterior edge varies between anterior border and almost last quarter of hindbody. One field often slightly shorter than other. Eggs oval, 64–77 × 32–39. Excretory bladder absent. Two large tubular collecting ducts running ventro-laterally from anterior end ventral disc and connected by short jumper at 61–81 [70] from posterior edge of body, and opening into terminal depression through separate almost median apertures.

3.2. Taxonomic summary

Host. *Cyprinus carpio* Linnaeus, 1758 *s. lato* (Actinopterygii, Cyprinidae).

Locality. Lake Biwa near Takashima City, Japan.

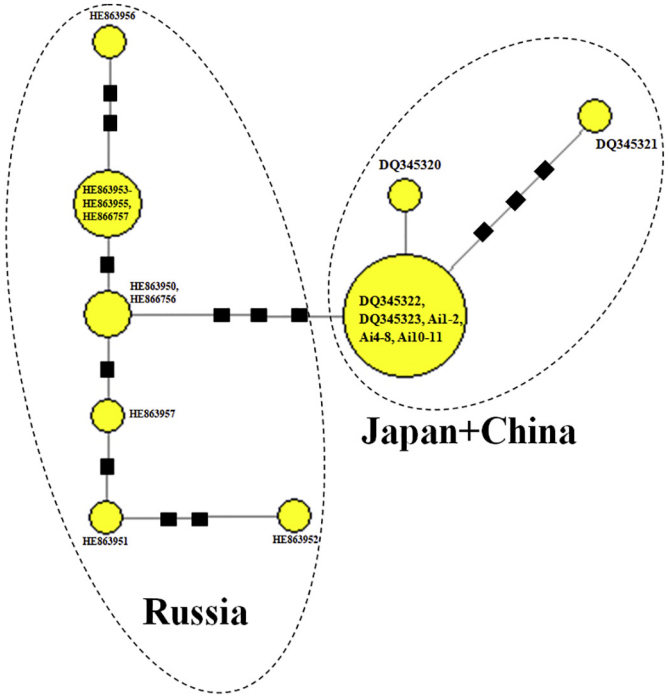

Fig. 5. Median-joining network for *Aspidogaster ijimai* based on ITS1-5.8S-ITS2 rDNA sequence data. Black box is one mutational step.

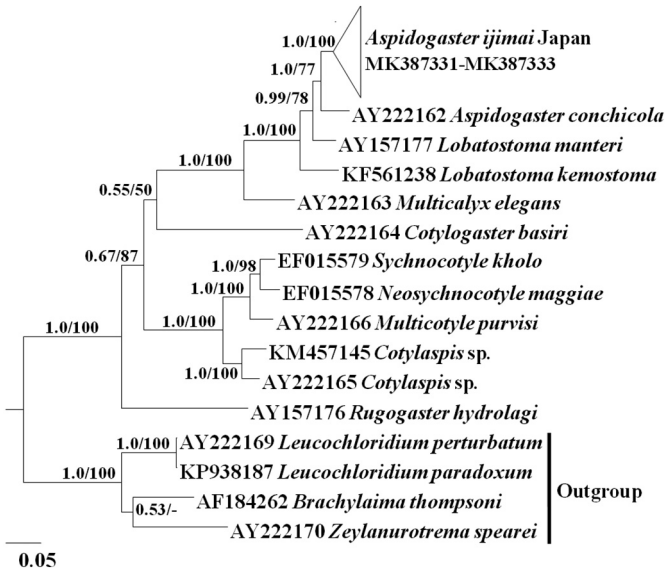

Fig. 6. Phylogenetic tree of Aspidogastrea based on partial 28S rDNA sequence data. Nodal numbers – values of bootstrap and posterior probabilities for ML/BI analyses, respectively.

Date of collection. December 2016.

Site of infection. Intestine.

Prevalence and intensity. 16.7% (1/6) and 436 ind. (n = 1).

Specimens deposited. Neotype № NSMT-Pl 6499 and one neoparatype №NSMT-Pl 6500 in the National Museum of Nature and Science, Japan, Tokyo; seven neoparatypes № 1307–1311 in the Museum of Helminthological Collections at the Centre of Parasitology of the A.N. Severtsov Institute of Ecology and Evolution (IPEE RAS), Moscow, Russia.

3.3. Molecular data

A ribosomal ITS1-5.8S-ITS2 fragment 1485 bp in length was sequenced for 11 Japanese *A. ijimai* specimens with alignment and analysis with the sequences of *A. ijimai* from the southern Russian Far East and China. Within Japanese specimens, ITS rDNA sequences were identical to each other and differed from Russian and Chinese worms by 0.26 ± 0.12% and 0.05 ± 0.03%, respectively (Table 3). *A. ijimai* from Russia and China differed from each other by 0.31 ± 0.12%. Interspecific molecular differentiation values ranged from 7.31 ± 0.68% for *A. ijimai* (Russia)/*Aspidogaster chongqingensis* Wei, Huang et Dai, 2001 to 20.88 ± 1.01% for *A. ijimai* (China)/*Aspidogaster conchicola* Baer, 1827. ML and BI phylogenetic analyses (Figs. 3 and 4, respectively), based on ribosomal ITS1-5.8S-ITS2 sequences, revealed no intraspecific structured clustering of *A. ijimai* on the background of interspecific differentiation and showed that *A. ijimai* was closely related to Chinese *A. chongqingensis*. The ML analysis showed *Aspidogaster limacoides* as a sister clade to *A. ijimai* and *A. chongqingensis*, with poor bootstrap support, and *A. conchicola* as a basal clade for other *Aspidogaster* species with high bootstrap support. BI analysis showed *A. limacoides* closely related to *A. conchicola* with extremely poor support. Both analyses confirmed monophyly of the genus *Aspidogaster*.

Median-joining network analysis (Fig. 5) showed the *A. ijimai* specimens grouped into two clades: Russian as well as Japanese with Chinese specimens. These two clades differ from each other by the mutational steps. The “Russian” clade was more heterogeneous compared to “Japanese-Chinese” clade and contained six sequence variants with frequencies that ranged from one to four sequences. The “Japanese-Chinese” clade contained three sequence variants, including two unique sequences from China and one variant for all Japanese and two Chinese specimens, and overall comprised 13 sequences.

The 28S-based ML and BI analyses (Fig. 6) demonstrated a close relationship between *A. ijimai* and *A. conchicola* within a terminal clade with high statistical support. This clade was closely related to *Lobatostoma manteri* Rohde, 1973, *Lobatostoma kemostoma* MacCallum et MacCallum, 1913 (both from Aspidogastridae, Aspidogastrinae), *Multicalyx elegans* Olsson, 1869 (Multicalycidae) with high support, and with *Cotylogaster basiri* Siddiqi et Cable, 1960 (Aspidogastridae, Cotylaspidinae) with low support. This large clade that comprised six species is sister to the well-supported clade of the aspidogastrids *Sychnocotyle kholo* Ferguson, Cribb et Smales, 1999, *Neosychnocotyle maggiae* Snyder et Tkach, 2007, *Multicotyle purvisi* Dawes, 1941 (Aspidogastrinae) and *Cotylaspis* spp. (Cotylaspidinae). *Rugogaster hydrolagi* Schell, 1973 (Rugogastridae) is basal relative to all other aspidogastreans. The 28S rRNA gene nucleotide sequences were identical for different Japanese *A. ijimai* specimens.

4. Discussion

The present specimens were consistent with those described by Kawamura [7] for most morphological features, namely body size, morphology of ventral adhesive disc, cirrus-sac and clusters of external prostatic cells, and vitellarium placement. Surprisingly, most authors, except [6,7], did not note a spined cirrus for *A. ijimai* (compare with [9–12]). However, taking into account conspecificity of *A. ijimai* specimens from Japan and continental Asia, we believe that data from Korean and Chinese authors for cirrus morphology for this species are not correct.

Our results provided a morphological description of Laurer's canal, the distal part of excretory system and metraterm of *A. ijimai* (Figs. 1 and 2). The position of the distal portion of Laurer's canal in the studied parasite species was only provided by Tang and Tang [12]. The authors reported that the canal extends to the posterior end of body, but did not indicate whether it has an external opening or is blindly closed as in *A. conchicola*. The morphology for the distal part of the *A. ijimai* excretory system was described in [7,10,11]. However, these authors

reported Y- or V-shaped excretory bladders, and not the presence of two ventro-lateral collecting ducts joined with a short jumper. A massive terminal sphincter for the metraterm was not previously noted.

The *Aspidogaster* genus comprises 12 valid species [1,5,6]. Alves with coauthors [1] added *Aspidogaster antipai* Lepsi, 1932 and *Aspidogaster parabramae* Tang et Tang, 1963 to this genus. However, *A. antipai* is a synonym of *A. conchicola* [35,36], and *A. parabramae* should be considered as *nomen nudum* because a description of this species is unpublished [37]. A description of the position of the distal part of Laurer's canal was provided in this study and in [38,39] for only three *Aspidogaster* species: *A. limacoides*, *A. conchicola* and *A. ijimai*. The variant of the position of the distal part of this organ, which is described in this study, is characteristic only for *A. ijimai* (Figs. 1 and 2). Additionally, a spined cirrus and excretory collecting ducts connected by a jumper are typical only for this species.

Our molecular results confirm previous data, presented by Atopkin with collaborators [6] and Chen with coauthors [15], which indicate *A. ijimai* and *A. chongqingensis* are sister species. According to Atopkin with coauthors [6], an *A. ijimai* and *A. chongqingensis* clade clustered with *A. limacoides*. However, in our study *A. limacoides* had a labile position depending on the phylogenetic algorithm used (Figs. 3 and 4).

Zamparo and Brooks [40] reported about paraphyly of the genus *Aspidogaster* on the basis of morphological studies of *A. conchicola*, *Aspidogaster indicum* Dayal, 1943, *Aspidogaster piscicola* Rawat, 1948 and other Aspidogastrinae representatives. These authors believe that *A. indicum* and *A. piscicola* are closely related to *Lobatostoma* spp. rather than to *A. conchicola*. 18S-rRNA-gene-based phylogenetic analysis, provided by Chen with coauthors [41], showed that *A. conchicola* forms a monophyletic group with *A. ijimai* with relatively low bootstrap support. Our results of 28S-based ML and BI phylogenetic analyses indicate well supported monophyly of the *A. conchicola* + *A. ijimai* group, a finding that supports these species are congeneric (Fig. 6). At the same time, our data demonstrated that the genus *Lobatostoma* Eckmann, 1932 is paraphyletic and Aspidogastridae is polyphyletic, results in line with previous studies [30,41].

In the present study, we provided new molecular data for *A. ijimai* and a detailed morphological description of this parasite. Our studies confirmed conspecificity of Japanese, Chinese and Russian *A. ijimai* specimens. Nevertheless, ITS-rDNA-based median-joining network analysis showed a tendency for molecular differentiation of *A. ijimai* specimens in Russian and Japanese + Chinese groups.

Acknowledgments

The authors wish to thank to Mr. T. Nishihira, Division of Environmental Dynamics, Graduate School of Environmental Science, University of Shiga Prefecture, for his fieldwork to obtain fish samples from Lake Biwa.

Funding

This study was funded by Russian Science Foundation (grant № 17-74-20074).

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

Sampling and field studies

All necessary permits for sampling and observational field studies have been obtained by the authors from the competent authorities.

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