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Author for correspondence:
Y. V. Tatonova, E-mail: ytatonova@gmail.com

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Stephanoprora amurensis sp. nov., *Echinochasmus milvi* Yamaguti, 1939 and *E. suifunensis* Besprozvannykh, 1991 from the Russian southern Far East and their phylogenetic relationships within the Echinochasmidae Odhner 1910

Y. V. Tatonova[1], A. V. Izrailskaia[1,2] and V. V. Besprozvannykh[1]

[1]Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch, Russian Academy of Sciences, 100-letiya Street, 159, Vladivostok 690022, Russia and [2]Far Eastern Federal University, School of Biomedicine, Sukhanova Street, 8, Vladivostok 690091, Russia

Abstract

Mature worms of *Stephanoprora amurensis* sp. nov. were obtained in an experimental study of its life cycle. In the Russian southern Far East, this trematode circulates using freshwater snails *Parajuga subtegulata*, freshwater fish and birds as the first, second intermediate and final hosts, respectively. *Stephanoprora amurensis* sp. nov. differs from the well-known representatives of *Stephanoprora* in a number of morphometric indicators of the developmental stages. The validity of the species was also confirmed by nuclear and mitochondrial DNA markers. In addition, new genetic data were obtained for *Echinochasmus suifunensis* and *Echinochasmus milvi*. An analysis of phylogenetic relationships within Echinochasmidae based on the 28S rRNA gene and ITS2 region identified two clusters, one of which combines species of *Echinochasmus* with 20–22 collar spines and short-tailed cercariae, and the other which includes *Stephanoprora* spp. and a number of representatives of *Echinochasmus* with 24 collar spines and long-tailed cercariae. The results of phylogenetic analysis based on ITS2 data show interfamily level of differences between the two clusters and intergeneric differentiation between the three subclusters uniting the species of *Stephanoprora* and *Echinochasmus*.

Introduction

The family Echinochasmidae Odhner 1910 comprises numerous species that parasitize mammals, birds and, less commonly, reptiles in the mature stage (Tkach *et al.*, 2016). Many of these trematodes are cosmopolitan. For most of them, the taxonomic status is determined based only on the morphology of adult individuals and is not genetically confirmed. There are data in the GenBank with established species affiliation for six *Echinochasmus* and three *Stephanoprora* representatives. This leads to certain difficulties with solving the problems of taxonomy and phylogenetic relationships in the Echinochasmidae system for these worms (Tkach *et al.*, 2016; Besprozvannykh *et al.*, 2017). In the Russian southern Far East, 13 *Echinochasmus* and one species of *Stephanoprora* were recorded. Of these, *Stephanoprora chasanensis* Besprozvannykh, Rozhkovan, Ermolenko, 2017 and five species of *Echinochasmus* from this region revealed a natural infection of the first intermediate hosts, and their life cycles were completed in a laboratory (Besprozvannykh, 1989, 1991, 2009, 2011). In addition, genetic data were obtained for *Echinochasmus milvi* Yamaguti, 1939 and *S. chasanensis* (Besprozvannykh *et al.*, 2017).

In the present work, during parasitological studies of freshwater prosobranch molluscs of the family Semisulcospiridae in the Razdolnaya River (Primorsky Region, Russia), we found snails that emitted two types of long-tailed cercariae, which were morphologically similar to Echinochasmidae. Subsequent experimental completion of their life cycles, as well as the study of developmental stages, showed that one of the cercariae belongs to a new species of *Stephanoprora*, and others are *Echinochasmus suifunensis* Besprozvannykh, 1991. Previously, adult *E. suifunensis* worms have already been obtained from studies of the life cycle of trematodes in the Russian southern Far East (Besprozvannykh, 1991). For both species, molecular data were obtained for adult worms. In addition, new genetic data were provided for adult worms of *E. milvi*. To analyse the phylogenetic relationships of Echinochasmidae representatives, we used markers of nuclear (ITS1, ITS2 rDNA regions and 28S rRNA gene) and mitochondrial (*cox1* gene) DNA.

Materials and methods

Life cycle and morphology of worms

Long-tailed cercariae of two morphologically different species were isolated from two snails, *Parajuga subtegulata* Prozorova et Starobogatov (Semisulcospiridae), collected in the

Razdolnaya River (Primorsky Region, Russia). Cercariae of one of these species were also found in snails *Parajuga amurensis* (Gerstfeldt) from the Ussuri River. To determine the second intermediate host, the infected snails of *Parajuga* emitting cercariae were placed separately in containers (1000 mL volume) together with ten specimens of the freshwater fish *Rhodeus sericeus sericeus* (Pallas, 1776) in each. After 8 h of exposure, fish from both experiments were placed separately in two aquaria. The fish used in the experiments were caught in an artificial pond. Fifty fish from this pond were previously dissected to confirm the absence of trematode metacercariae. Two fish from each aquarium were dissected on the fourth day to establish the level of infection. Before infection of the definitive host, the remaining fish were dissected on the 25th day after the beginning of the experiment. Metacercariae were found only on the gills of all fish. Fish gills from both experiments were then fed separately to two laboratory chickens. Adult worms were found in the small intestines of the chickens 8 days later. The experiments were carried out at room temperature, from 18 to 22°C.

Measurements of rediae and metacercariae were taken on live specimens. Cercariae were measured after fixation with 4% hot formalin. Adult worms recovered from experimental chickens were fixed with 70% ethanol and then placed in 96% ethanol. Whole mounts were made by staining specimens with alum carmine, dehydrating the worms in a graded ethanol series and clearing in clove oil. Clove oil treatments were followed by mounting specimens in Canada balsam under a coverslip on a glass slide. All measurements are given in micrometres (μm).

In addition to the sexually mature worms obtained in the experiment, the study used trematode slides of *E. milvi* (No. 20 – Tr, Besprozvannykh, 1989), and *E. suifunensis* (holotype No. 26 – Tr, paratypes; Nos. 27, 28 – Tr, Besprozvannykh, 1991) from 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

Using the HotSHOT method (Truett *et al.*, 2000), DNA samples were extracted from adult worms: four samples of *Stephanoprora amurensis* sp. nov. and two samples of *E. suifunensis* from the Razdolnaya River obtained in the experiment, as well as two *E. milvi* samples from the Komissarovka River obtained by Besprozvannykh *et al.* (2017); these were deposited in the Zoological Museum of Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences.

Information about the primers for amplification and sequencing of nuclear markers (ITS1, ITS2 rDNA regions and 28S rRNA gene), the composition of the reaction mixture and the polymerase chain reaction cycling conditions can be found in Tatonova *et al.* (2020). The amplification and sequencing of the partial *cox1* gene were performed using the following primers: JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′, forward) and JB4.5 (5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′, reverse) (Bowles *et al.*, 1993) for the genus *Echinochasmus*, and CO1-Fw (5′-GGG CAT CCT GAG GTT TAT G-3′, forward) and CO1-Rv (5′-AAC AAA TCA TGA TGC AAA AGG TA-3′, reverse) for *S. amurensis* sp. nov. (Katokhin *et al.*, 2008). The annealing temperature was 50 and 55°C for the first and second primer pairs, respectively.

Genetic analysis

The nucleotide sequences were manually assembled in MEGA version 5.03 (Tamura *et al.*, 2011). The *p*-distances between species were also analysed using the same program. Phylogenetic reconstructions used aligned sequences of 427 and 1151 bp from ITS2 and 28S, respectively, based on the Bayesian inference (BI) method in MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). The list of samples used in the study is presented in Table 1. According to the Akaike criteria in Modeltest version 3.7 (Darriba *et al.*, 2012), TVM + G and GTR + I + G were the optimal models for determining genetic distances for the ITS2 region and 28S rDNA sequences, respectively. The BI analysis was performed using 400 000 and 1 200 000 generations of the Markov chain Monte Carlo test for the ITS2 region and 28S rRNA gene, respectively. This number of generations was sufficient as the S.D. value was <0.01. A total of 25% samples were excluded to construct the consensus trees. The chain was sampled every 100th generation. The sequences of the ITS1 rDNA region and the *cox1* mtDNA gene were also used to identify differences between the related species *E. suifunensis* and *E. milvi*.

Results

Stephanoprora amurensis *sp. nov.*

Host: *Gallus gallus* dom. (experimental host).
Site: small intestine.
Intensity of infection: 12 specimens.
First intermediate host: *Parajuga subtegulata* Prozorova et Starobogatov.
Other first intermediate host: *Parajuga amurensis* (Gerstfeldt).
Second intermediate host: *Rhodeus sericeus sericeus* (experimental host).
Site: gills.
Type locality: the Razdolnaya River, Primorsky Region, southern Far East, Russia (43°20′N, 131°47′E).
Other locality: the Ussuri River (right tributary of the Amur River), Primorsky Region, southern Far East, Russia; 45°15′N, 133°30′E.
Type-deposition: holotype No. 143-Tr, paratype No. 144-147-Tr. This material is held 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@biosoil.ru. Deposited: 2018.22.09.
Etymology: The species' name refers to the Amur River, in the basin of which the parasite was first discovered.

Adult worm (based on seven specimens; Fig. 1A–C; Table 2)

Body elongated, spined from anterior end to level of posterior testis. Most densely spines from anterior end of body to level of middle of ventral sucker. Oral sucker subterminal. Head-collar with 22 spines, arranged in single row interrupted dorsally. Prepharynx short, pharynx oval, oesophagus longer than prepharynx. Intestinal bifurcation just anterior to ventral sucker. Caeca narrow, reach level of posterior end of vitellarium. Ventral sucker in anterior third of body. Testes two, tandem, elongate, ovoid, in middle third of body. Distance between testes present or absent. Cirrus-sac oval, at median line of body and partly dorsal to ventral sucker. Internal seminal vesicle bipartite. Genital pore between oesophageal bifurcation and anterior margin of ventral sucker. Ovary round or transversely-oval, on median line of body anterior to anterior testis. Uterine seminal receptacle and Mehlis' gland between ovary and anterior testis. Uterus short, in space between caeca, posterior margin of ventral sucker and anterior margin of anterior testis. Vitelline fields of transversely-oval follicles, between middle of anterior testis and posterior end of body, uniting posterior to testes. Vitelline reservoir on median line of body

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Table 1. List of analysed sequences

Family/Species	Developmental stage	Locality	References	GenBank accession numbers			
				28S rRNA	ITS2 rDNA	ITS1 rDNA	*cox1* mtDNA
Echinochasmidae							
Microparyphium facetum	Adult	USA	Tkach *et al.* (2016)	KT956933	–	–	–
Echinochasmus bursicola	Adult	Ukraine	Tkach *et al.* (2016)	KT956938	–	–	–
Echinochasmus coaxatus	Adult	Ukraine	Tkach *et al.* (2016)	KT956928	–	–	–
	Adult	Ukraine	Stanevičiūtė *et al.* (2015)	–	KJ542641	–	–
Echinochasmus beleocephalus	Adult	Ukraine	Tkach *et al.* (2016)	KT956929	–	–	–
Echinochasmus japonicus	Adult	Vietnam	Besprozvannykh *et al.* (2017)	JQ890579–JQ890583	KT873310–KT873314	–	–
Echinochasmus mordax	Adult	Ukraine	Tkach *et al.* (2016)	KT956931	–	–	–
Echinochasmus donaldsoni	Adult	USA	Tkach *et al.* (2016)	KT956930	–	–	–
Echinochasmus sp. 3	Adult	USA	Tkach *et al.* (2016)	KT956932	–	–	–
Echinochasmus milvi	Adult	Russia	Besprozvannykh *et al.* (2017)	KT873315–KT873319	KT873315–KT873319	–	–
	Adult	Russia	This study	MT447054, MT447055	MT447046,MT447047	MT447046, MT447047	MT444915, MT444916
Echinochasmus suifunensis	Adult	Russia	This study	MT447056, MT447057	MT447048,MT447049	MT447048, MT447049	MT444917, MT444918
Echinochasmus sp. 2	Adult	Hungary	Molnár *et al.* (2016)	–	KT989664–KT989667	–	–
	Cercaria	Hungary	Molnár *et al.* (2016)	–	KT989660	–	–
	Metacercaria	Hungary	Molnár *et al.* (2016)	–	KT989661–KT989663	–	–
Echinochasmus sp. 1	Parthenitae	Lithuania	Stanevičiūtė *et al.* (2015)	JQ088098	FJ756940	–	–
Stephanoprora sp. 1	Adult	USA	Tkach *et al.* (2016)	KT956936	–	–	–
Stephanoprora sp. 2	Adult	USA	Tkach *et al.* (2016)	KT956937	–	–	–
Stephanoprora uruguayense	Adult	Brazil	Unpublished	–	KP068005,KP068006, KJ957828	–	–
Stephanoprora pseudoechinata	Adult	USA	Tkach *et al.* (2016)	KT956934	–	–	–
	Adult	Ukraine	Tkach *et al.* (2016)	KT956935	–	–	–
	Parthenitae	Ukraine	Stanevičiūtė *et al.* (2015)	–	KJ542638	–	–
	Adult	Ukraine	Stanevičiūtė *et al.* (2015)	–	KJ542639	–	–
Stephanoprora amurensis sp. nov.	Adult	Russia	This study	MT447050–MT447053	MT447042–MT447045	MT447042–MT447045	MT444911–MT444914
Stephanoprora chasanensis	Adult	Russia	Besprozvannykh *et al.* (2017)	KT873320, KT873321	KT873320,KT873321	–	–
Outgroup (Psilostomatidae)							
Sphaeridiotrema pseudoglobulus	Adult	USA	Tkach *et al.* (2016)	KT956957	–	–	–
Psilochasmus oxyurus	Adult	Ukraine	Tkach *et al.* (2000)	AF151940	–	–	–
Psilostomum brevicolle	Adult	Ukraine	Tkach *et al.* (2016)	KT956950	–	–	–
Outgroup (Notocotylidae)							
Notocotylus attenuatus	Adult	Ukraine	Tkach *et al.* (2001)	AF184259	–	–	–
Outgroup (Echinostomatidae)							
Echinostoma trivolvis	Adult	USA	Detwiler *et al.* (2010)	–	GQ463126	–	–

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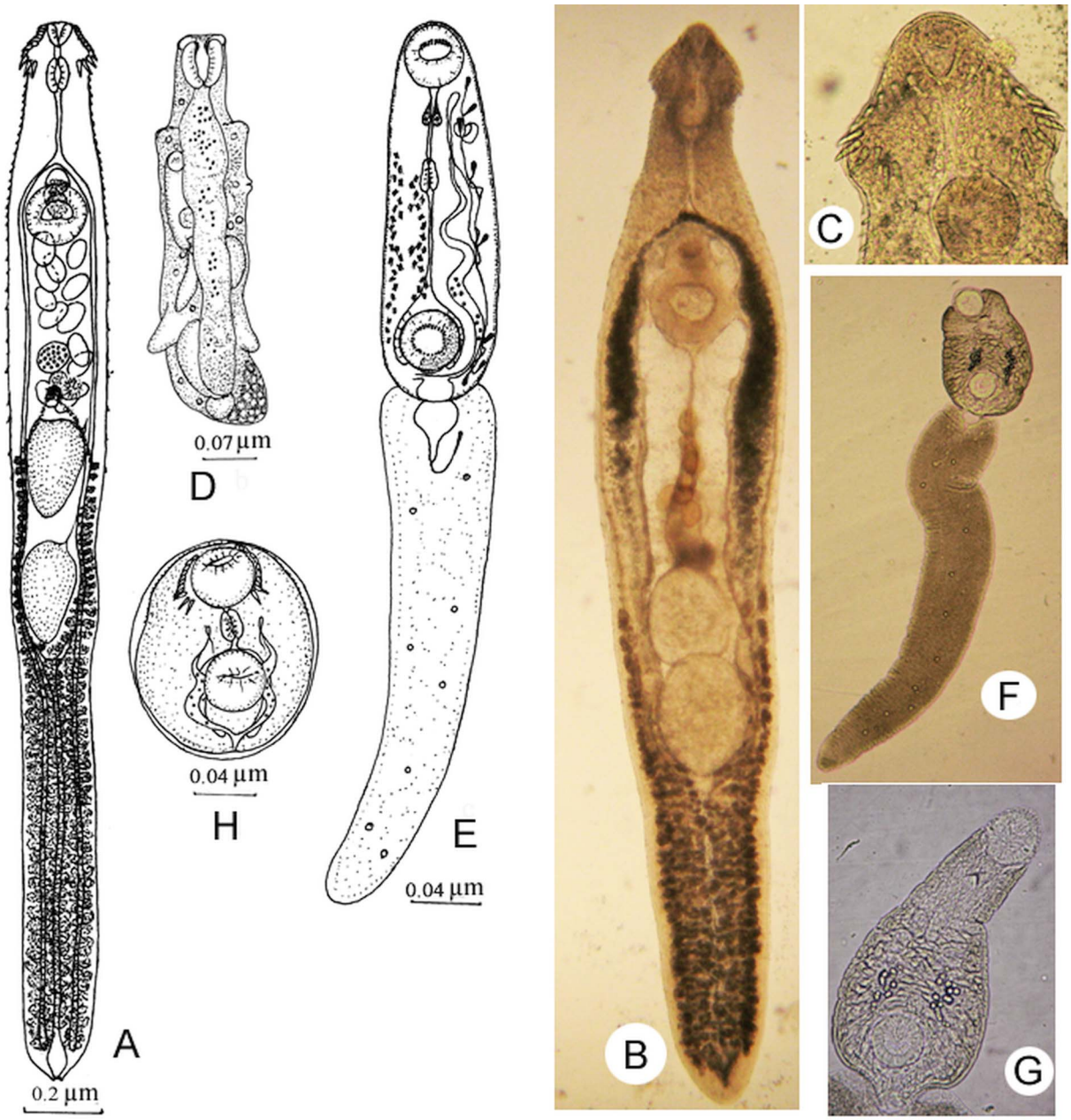

Fig. 1. *Stephanoprora amurensis* sp. nov.: (A, B) adult worm; (C) head-collar; (D) redia; (E, F) cercaria; (G) body of cercaria and (H) metacercaria.

between ovary and anterior testis. Eggs operculated, light yellow. Excretory bladder Y-shaped.

Redia (based on ten specimens; Fig. 1D)

Body sac-shaped, 0.410–0.730 × 0.145–0.156, grey, with collar and lateral appendages. Pharynx 0.056–0.072 × 0.045–0.061. Caeca long, terminate at some distance from posterior extremity of body. Intestinal contents dark grey. Birth pore posterior to collar, 0.13–0.24 from anterior end of body. Redia contains few mature cercariae and several immature cercariae at various stages of development.

Cercaria (based on ten specimens; Fig. 1E–G; Table 3)

Body without spines. Oral sucker, with ten cuticular plates in single row. Prepharynx and pharynx present, oesophagus long, caeca short, terminate at posterior margin of ventral sucker. Intestinal bifurcation anterior to ventral sucker. Ventral sucker at 0.132–0.180 from anterior end of body. Internal edge of ventral sucker with 32 cuticular plates. Undifferentiated genital primordium dorsal to ventral sucker, at level of its posterior margin. Cells of cystogenous glands in two lateral and two median rows. Lateral rows between anterior margin of pharynx and level of middle of ventral sucker, median rows between middle of pharynx and anterior margin of ventral sucker. Two drop-like cells of another type of glands on both sides from prepharynx and by five cells on both sides from oesophagus. Gland ducts located at level of oesophagus open at anterior end of body. Excretory bladder bipartite, with single caudal appendage. Caudal appendage twisted in initial part of tail or elongated, reaches middle of its length. Collecting channels of excretory system with 10–11 granules. Tail large, light grey, contains few small vacuole-like inclusions.

Metacercaria (based on five specimens; Fig. 1H; Table 3)

Cyst oval, with thin wall. Body surface with few small spines. Head collar with 22 spines. Prepharynx short, pharynx spherical, caeca terminate blindly at level of excretory bladder. Oral and

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Table 2. Measurements (μm) of adult worms of the family Echinochasmidae

	S. amurensis sp. nov. (this study)			*S. aylacostoma* (Ostrowski de Núñez and Quintana, 2008)	*S. chasanensis* (Besprozvannykh *et al.*, 2017)	*E. suifunensis* (Besprozvannykh, 1991; this study)	*E. milvi* (Besprozvannykh, 1989; this study)
	Holotype	Range ($n = 7$)	Mean	Range	Range	Range	Range
Body length	2700	2320–2770	2510	1728–2256	2341–2464	790–970	493–740
Body width	250	250–350	280	208–256	493–585	185–277	169–280
Bw/Bl (%)[a]	9.3	9.3–14.1	11.2	–	20–25	21.5–32.1	33.2–43.8
Forebody length	430	410–510	450	–	554–631	330–554	173–204
Fo/Bl (%)[a]	15.9	15.9–19.8	17.9	–	23.7–25.6	38.3–58.0	34.0–39.1
Oral sucker length	61	56–84	70	60–88	116–123	33–45	31–54
Oral sucker width	61	61–89	72	63–82	116–123	42–56	31–54
Ventral sucker length	156	156–220	184	142–173	212–266	72–84	70–90
Ventral sucker width	156	156–280	195	158–180	262–293	78–85	70–90
Suckers length ratio	1:2.56	1:2.0–3.28	1:2.63	–	1:1.83–2.29	1:1.9–2.1	1:2.2–2.5
Suckers width ratio	1:2.56	1:1.86–3.73	1:2.71	–	1:2.26–2.46	1:1.7–1.9	1:2.2–2.6
Head-collar width	184	162–220	193	–	273–289	145–156	160
Collar spines length	42–50	42–50	–	23.7–37.9	42–50	22–39	17–23
Prepharynx length	17	17–56	39	0–47	39–54	–	31
Pharynx length	89	67–109	86	69–95	116–119	50–67	28–54
Pharynx width	61	61–101	79	63–85	96–100	39–56	28–54
Oesophagus length	168	127–210	164	161–221	166–212	190	70–78
Ovary length	89	61–89	75	54–95	104–112	56–60	48
Ovary width	89	84–145	100	79–117	112–139	70	50
Anterior testis length	250	168–250	207	117–189	250–296	67	81–92
Anterior testis width	150	127–250	170	139–189	223–277	123–127	140
Posterior testis length	250	178–300	231	123–261	312–331	60–70	81
Posterior testis width	140	112–250	154	139–176	231–254	110–140	110
Cirrus-sac length	127	112–190	137	120–176	254–262	100	87–89
Cirrus-sac width	67	61–84	77	95–113	119–135	45–67	56
Post-testicular field length	1060	870–1080	960	–	832–893	173–190	58–116
Pt/Bl (%)[a]	39.3	36.7–39.3	38.2	–	35.5–37.6	19.6–22.0	11.4–22.1
Eggs length	84–89	84–95	–	88–104	81–92	78	78–100
Eggs width	61	56–67	–	35–60	50–54	45	45

[a]Bw/Bl, body width as a percentage of body length; Fo/Bl, length of the forebody as a percentage of body length; Pt/Bl, post-testicular field length as a percentage of body length.

ventral suckers equal in size. Collecting channels of excretory system contain small granules.

Genetic data

The analysed lengths of nucleotide sequences were 437, 422, 1145 and 720 bp for nuclear ribosomal (complete ITS1 and partial ITS2, 28S) and mitochondrial markers (partial *cox1*), respectively. The sequences were identical for all specimens of *S. amurensis* sp. nov. At position 397 of the complete ITS1 region, intragenomic variability (T ↔ C) was detected for all samples.

Remark

The above-described mature worms of a new species, according to morphological parameters, including the number of collar spines (22), corresponded to the diagnostic features of the genus *Stephanoprora* (with the exception of the species *Stephanoprora ornata* Odhner, 1902, which has 26 spines). In addition, the parasite belonging to the genus was confirmed by the similarity of the morphology of its cercaria with the cercariae of *Stephanoprora denticulata* (Rudolphi, 1802), *Stephanoprora uruguayense* Holcman-Spector et Olague, 1989, *Stephanoprora aylacostoma* Ostrowski de Nunez et Quintana 2008 and *S. chasanensis* (Køie, 1986; Ostrowski de Núñez, 2007; Ostrowski de Núñez and Quintana, 2008; Besprozvannykh *et al.*, 2017). Among *Stephanoprora*, for which the life cycles were studied, *S. denticulata*, *S. uruguayense* and *S. chasanensis* circulate with the participation of Truncatelloidea Gray, 1840 molluscs. In contrast, *Stephanoprora* in our material uses the Cerithioidea Fleming, 1822 molluscs as the first intermediate hosts, like *S. aylacostoma* from Argentina. Between mature individuals of *S. amurensis* sp. nov. and *S. aylacostoma*, there were differences in the size of the head spines, the width of the cirrus-sac, the maximum size

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Table 3. Measurements (μm) of cercariae and metacercariae of the family Echinochasmidae

	S. amurensis sp. nov. (this study)	*S. aylacostoma* (Ostrowski de Núñez and Quintana, 2008)	*S. chasanensis* (Besprozvannykh *et al.*, 2017)	*E. suifunensis* (Besprozvannykh, 1991; this study)	*E. milvi* (Besprozvannykh, 1989)
Cercariae					
Body length	200–240	239–264	212–260	200–240	156–190
Body width	60–90	82–94	92–100	120–150	110–120
Oral sucker length	34–40	35–47	32–42	42–45	33–45
Oral sucker width	34–40	38–44	32–42	33–45	45–56
Pharynx length	19–22	16–25	23–25	22–33	17
Pharynx width	11–19	13–16	12–15	15–17	15
Ventral sucker length	31–39	41–47	39–42	39–45	45
Ventral sucker width	34–39	38–44	39–42	39–45	45–56
Tail length	290–310	1792–2224	710–880	290–330	330–440
Tail width	50–80	138–192	92–150	80–92	89–120
Numbers of granules	10–11	23–51	12–17	14–15	13–15
Cuticular formations on suckers	Present	Present	Present	Present	Present
Spines around oral sucker	0	0	0	10	0
Metacercariae					
Cyst length	120–130	120–136	119–138	145–156	100–110
Cyst width	112–123	95–113	92–108	123–130	67–72
Oral sucker length	33–40	30–36	35–44	42–45	22–25
Oral sucker width	40–45	36–44	40–45	36–47	34
Pharynx length	20–28	19–27	20–28	44–47	22
Pharynx width	20–28	13–16	20–28	30–39	16
Collar spines length	11.2	9.5–13.8	–	19–22	9–10
Ventral sucker length	33–39	32–41	33–40	39–47	22–26
Ventral sucker width	39–45	47–52	39–45	39–47	28–38
Number of granules	6–7	3–6	7–9	–	–

of the ventral sucker and testes (Table 2), as well as the presence of a space between the vitelline fields in the posterior part of the body for *S. aylacostoma*. At the cercaria stage, they differed in the size of the ventral sucker and number of granules in the excretory system, while those at the metacercaria stage had only a different pharynx width (Table 3).

To date, in the Russian southern Far East, only *Stephanoprora pseudoechinata* Olsson, 1876 and *Stephanoprora skrjabini* Dozenko, 1954 have been found in naturally infected definitive hosts (Skrjabin and Bashkirova, 1956; Oshmarin, 1963), while *S. chasanensis* was experimentally obtained in the study of the life cycle (Besprozvannykh *et al.*, 2017). The mature worms described in this study had significantly smaller body sizes than the trematodes of first two species (*S. pseudoechinata* was 3100–5920 × 319–480 μm, *S. skrjabini* was 7000 × 800 μm), and differed in metric data for body width, sizes of the oral and ventral suckers, pharynx, etc. from *S. chasanensis* (Table 2). However, at the stages of cercaria and metacercaria, the differences between the new representative of the genus and *S. chasanensis* were only in the number of granules of the excretory system (Table 3). Based on the above-mentioned facts, the trematodes of the genus *Stephanoprora* found in Russia in this study belong to a new species, *S. amurensis* sp. nov. In addition, the classification of *S. amurensis* sp. nov. and *S. chasanensis* to different species was confirmed by the participation of mollusks from different orders, Sorbeoconcha Ponder & Lindberg, 1997 and Hypsogastropoda Ponder & Lindberg, 1997, respectively, in their life cycles as the first intermediate hosts.

The identification of these worms as a new species was also reaffirmed by genetic data. In the reconstruction obtained using the 28S rRNA gene, *S. amurensis* sp. nov. entered into a cluster containing other representatives of the genus (Fig. 2). Genetic distances between species of the genus *Stephanoprora* did not exceed 0.6% according to this marker. The closest to the new species was the representative from the Russian southern Far East, *S. chasanensis*; between them, two nucleotide substitutions were revealed (T ↔ C transitions), which account for 0.2% of the differences.

On the tree based on the partial ITS2 rDNA data (Fig. 3), species of *Stephanoprora* from the Russian southern Far East (*S. amurensis* sp. nov. and *S. chasanensis*) also formed a separate branch inside the subcluster *C*, including all representatives of this genus, as well as the species *Echinochasmus* sp. 3 (FJ756940), which was apparently mistakenly assigned to *Echinochasmus* when working with parthenitae from the snail. The genetic distance between *S. amurensis* sp. nov. and *S. chasanensis* was 0.5%, while the level of differences from other species of *Stephanoprora* reached 1.3–2.6%.

Differences in the mitochondrial sequences of the *cox1* gene separated these species more significantly. Between *S. amurensis* sp. nov. and *S. chasanensis*, 2.6 and 1.3% differences were revealed at the nucleotide and amino acid levels, respectively. Data for other species were absent from GenBank.

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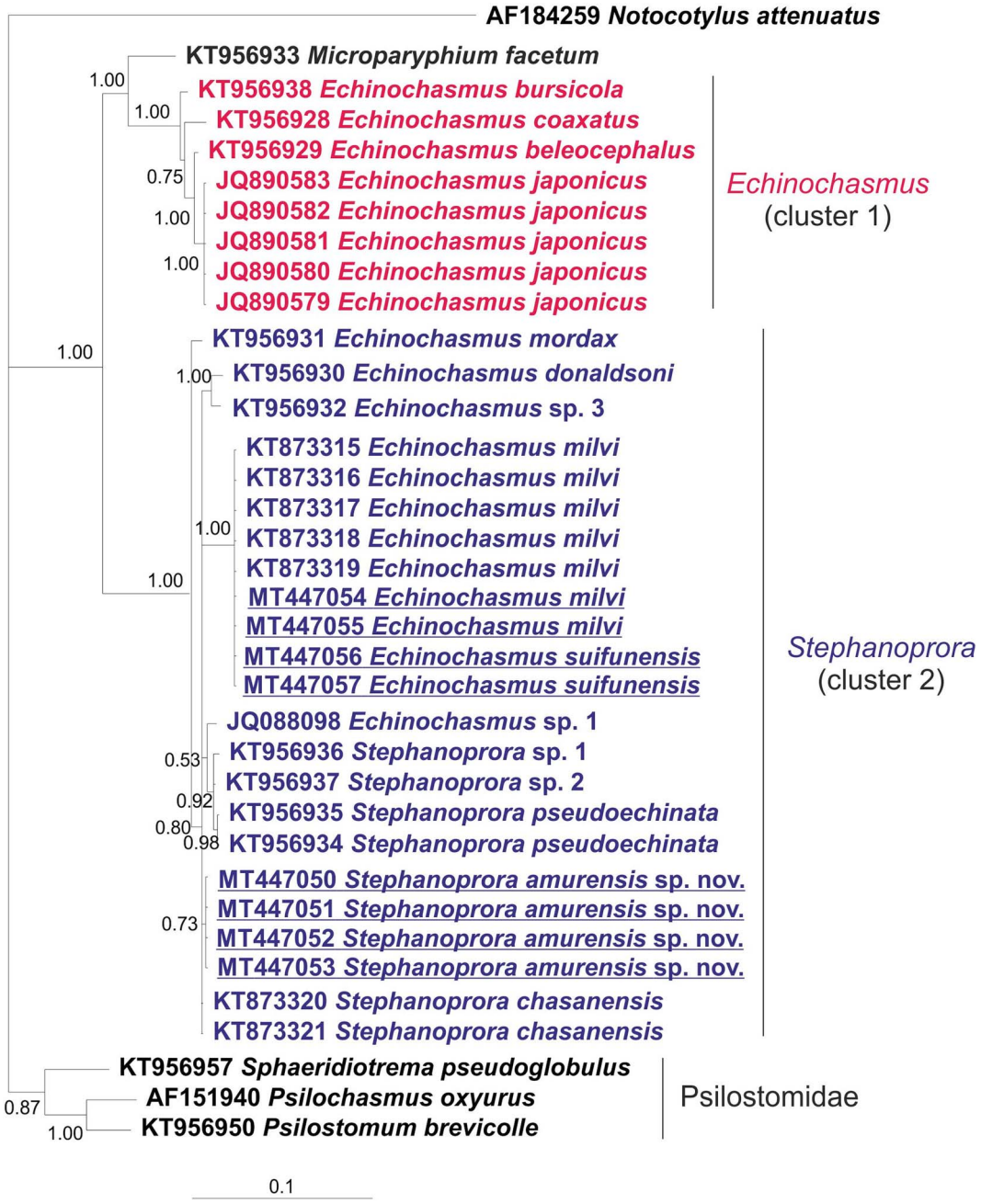

Fig. 2. The phylogeny based on 28S rRNA gene sequences using the BI method. Bayesian posterior probabilities of ≥0.50 are shown. The developmental stages and localities of echinochasmids, as well as outgroup species are listed in Table 1.

Echinochasmus suifunensis *Besprozvannykh, 1991*

Host: *Gallus gallus* dom. (experimental host).
Site: small intestine.
Intensity of infection: 17 specimens.
First intermediate host: *Parajuga subextensa* Prozorova et Starobogatov.
Second intermediate host: *Rhodeus sericeus sericeus* (experimental host).
Locality: the Razdolnaya River, Primorsky Region, southern Far East, Russia (43°20′N, 131°47′E).

Echinochasmus milvi *Yamaguti, 1939*

Host: *Butorides striatus* (native host), *Mus musculus*, *Felis catus dom.*, *Anas platyrhynchos dom.*, *Gallus gallus dom.* (experimental hosts) (Yamaguti, 1939; Besprozvannykh, 1989; Besprozvannykh *et al.*, 2017)
Site: small intestine.
First intermediate host: *Semisulcospira libertina* (Koga, 1952), *Parajuga* spp. (Besprozvannykh, 1989).
Second intermediate host: predominantly, Cyprinidae (native and experimental hosts) (Koga, 1952; Besprozvannykh, 1989).
Locality: Japan, southern Far East, Russia (Yamaguti, 1939; Besprozvannykh, 1989).

Genetic data for E. suifunensis and *E. milvi*

In both *E. suifunensis* and *E. milvi*, variability between nucleotide sequences within a species was absent for the following nuclear ribosomal markers: complete ITS1 and partial 28S. The analysed length of the above-mentioned sequences was 426 and 1145 bp, respectively.

The length of partial sequences of the ITS2 rDNA region was 420 bp for *E. suifunensis*. Within the species, the ITS2 sequences were 100% identical. The size of ITS2 of *E. milvi* varied from 414 to 420 bp due to a 6 bp insertion in two samples (KT873318 and KT873319). The insertion was associated with a G/A transition at

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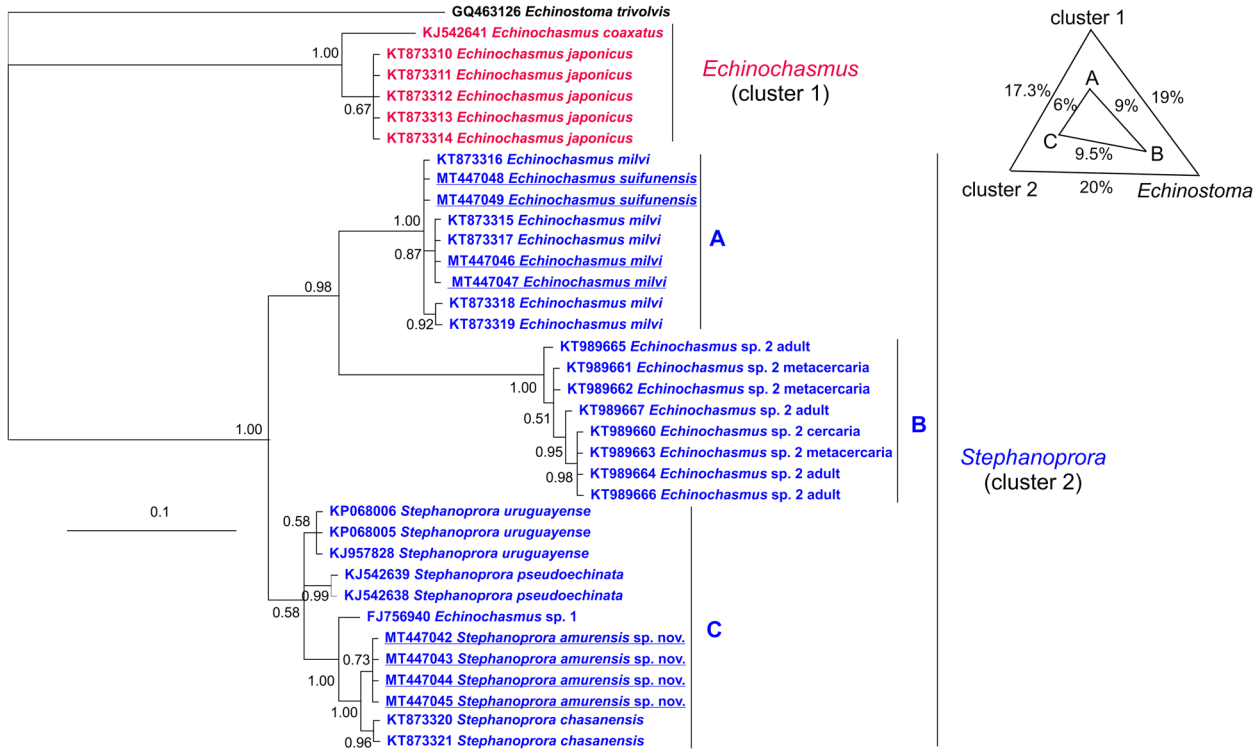

Fig. 3. The phylogeny based on ITS2 rDNA sequences using the BI method. Bayesian posterior probabilities of ≥0.50 are shown. The developmental stages and localities of echinochasmids, as well as outgroup species are listed in Table 1. The *p*-distances between clusters and subclusters are shown in the in the upper right corner.

position 87. In addition, there was one T/C transition at position 366 of the sequences with no insertion. These substitutions in the ITS2 region accounted for 0.3% of the differences within *E. milvi*.

The sequence length of the mitochondrial *cox1* gene was 432 bp for both species. The distances within *E. suifunensis* and *E. milvi* were 1.2 and 0.7%, respectively. Amino acid substitutions were not obtained within species.

Remark

Snails of *Parajuga* emitting *E. suifunensis* cercariae were collected from a type locality of species (Besprozvannykh, 1991). These cercariae, as well as the metacercariae and mature worms obtained from them, were morphometrically identical to worms described in Besprozvannykh (1991). Given this and the fact that the author of the species and life cycle studies of *E. suifunensis* is the author of this study, we used the data presented in Besprozvannykh (1991) in the discussion and also provided further detail of the metric data and photographs of the different developmental stages of worms (Fig. 4A–D) that were absent in the earlier publication. Another representative of the genus, *E. milvi* (Fig. 4E–I), was also described previously (Besprozvannykh, 1989); therefore, all of the above applies to this species.

The molecular studies showed 100% identity in the nucleotide sequences of the ITS1 rDNA region and 28S rRNA gene obtained from mature worms of *E. suifunensis* and *E. milvi*. In addition, there was an absence of species separation based on the data of the ITS2 rDNA region. The nucleotide distances, based on partial sequences of the ITS2 rDNA region (including data from GenBank), were low within *E. suifunensis* and *E. milvi* (from 0 to 0.5%); the two detected nucleotide substitutions were not fixed and did not distinguish these species. Moreover, both species have a similar life cycle in which, on the territory of the Russian southern Far East, the first and second intermediate hosts are snails of *Parajuga* and freshwater fish (with metacercariae localized on the gills).

However, the affiliation of these trematodes to different species is not in doubt and was confirmed by the morphometric data of adult worms, cercariae and metacercariae. Mature worms of *E. suifunensis* and *E. milvi* differed in body length and the length of angular spines (Table 2); the parasites differed in body size at the cercaria stage and there were differences in all metric indicators at the metacercaria stage (Table 3). In addition, unlike *E. milvi*, cercariae of *E. suifunensis* had spines on both sides of the oral sucker. Moreover, despite the fact that the cercariae of both species united to form the 'Rattenkonig' (for *E. suifunensis*, this fact was established in the current study), they differed in the morphology of the tail and type of cercariae aggregation. *Echinochasmus suifunensis* had a transparent tail, twisted in a spiral at the end, while the tail of *E. milvi* was dull white or pigmented (dark or light brown) and there was a thin outgrowth with a bulbous tip at its distal end. In the first case, 'Rattenkonig' was formed due to the interlacing of the twisted parts of the tails of the cercariae and in the second, due to the interlacing of the bulbous outgrowths (Fig. 2D, H, I). These data indicated the validity of *E. suifunensis* and *E. milvi* at the morphological level.

At the molecular level, the validity of species was confirmed only by the sequences of the mitochondrial *cox1* gene. The distance between *E. suifunensis* and *E. milvi* was 6.7% (including one fixed nonsynonymous and 24 fixed synonymous substitutions), while the distances within these species were 1.2 and 0.7%, respectively; that is, the difference was almost 6–10 times higher than within each separate species. Moreover, one amino acid substitution (I ↔ M) was also identified between *E. suifunensis* and *E. milvi*. The presence of a large number of fixed substitutions indicated the presence of speciation processes, which have so far only been detected by analysis of the mitochondrial gene, which is more sensitive, thus revealing cryptic species with recent ancestry. Previously, using mitochondrial DNA, the presence of cryptic species was detected for different groups of worms (Blouin, 2002; Vilas *et al.*, 2005; Lavikainen *et al.*, 2010). Thus, we considered that the validity of *E. suifunensis* and *E. milvi* were confirmed by genetic data.

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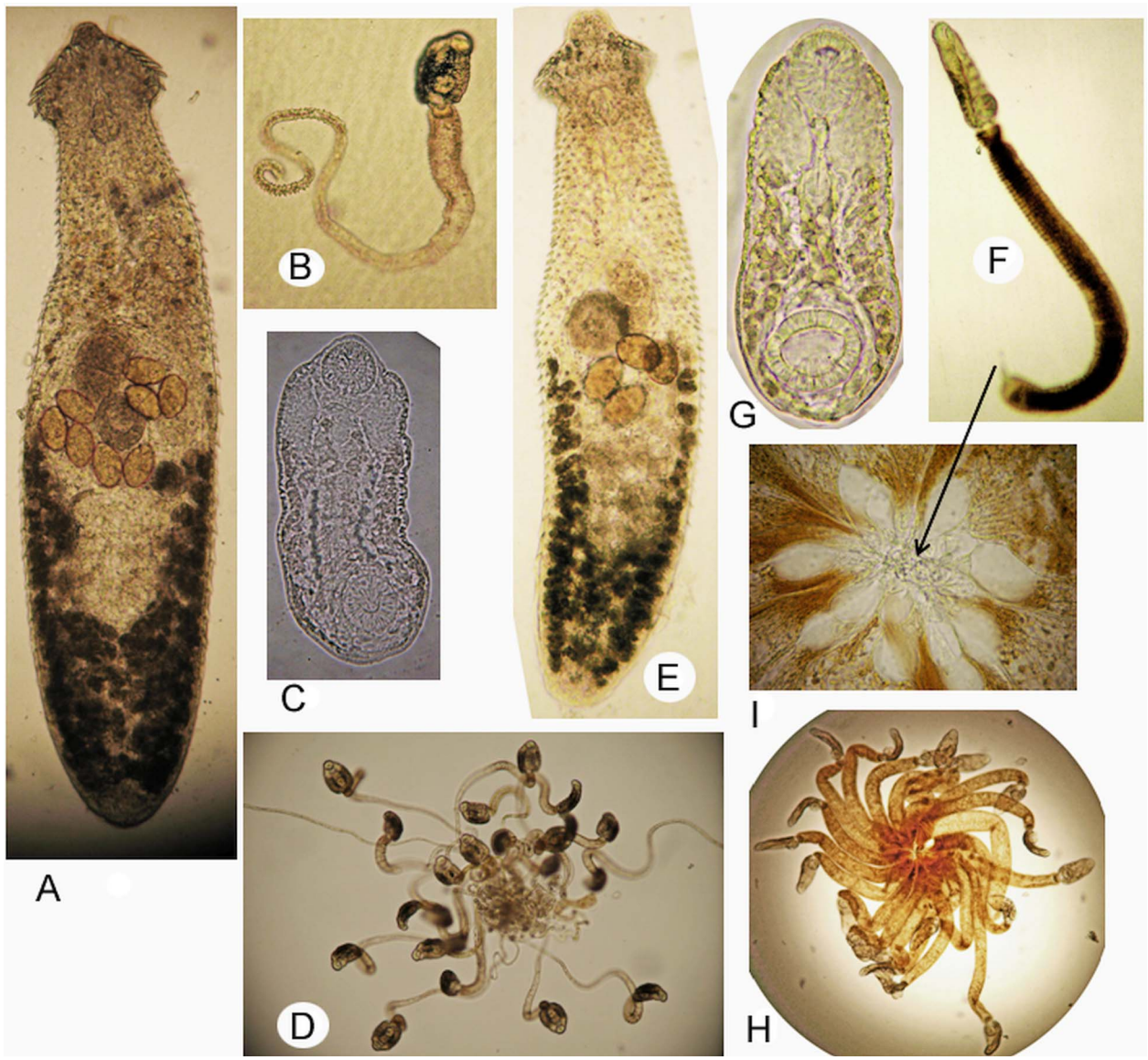

Fig. 4. *Echinochasmus suifunensis*: (A) adult worm; (B) cercaria; (C) body of cercaria; (D) 'Rattenkonig'; *Echinochasmus milvi*: (E) adult worm; (F) cercaria; (D) body of cercaria; (H) 'Rattenkonig'; and (I) interlacing of the bulbous outgrowths of tails.

Discussion

Phylogenetic analysis of Echinostomatoidea based on molecular studies of species from different families was carried out relatively recently by Tkach *et al.* (2016). Unfortunately, notwithstanding the scope of this review, data on individual families, including Echinochasmidae, were still insignificant and more information was required for this taxonomic group. In the present study, in addition to the differences that we identified for the analysed echinochasmids, it is necessary to note some aspects in the distribution of species of this family on phylogenetic trees built using nuclear markers, 28S and ITS2. As in previously published studies (Tkach *et al.*, 2016; Besprozvannykh *et al.*, 2017), representatives of the genus *Echinochasmus* were divided into two separate clusters (Figs 2 and 3), the first of which included species whose cercariae had a short tail, including a type species, *Echinochasmus coaxatus* Dietz, 1909. In addition, all species of *Echinochasmus* of this cluster had 24 collar spines. *Echinochasmus bursicola* (Creplin, 1837) also clustered with these species and as Tkach *et al.* (2016) previously noted, was apparently mistakenly assigned to a different genus, *Uroproctepisthmium*. In the second cluster, species of *Echinochasmus* joined with representatives of another genus *Stephanoprora* (Figs 2 and 3). Most species in this cluster had 20–22 collar spines and their cercariae had a long tail. Based on the 28S rRNA gene sequences, the distances between these two clusters and the only representative of the genus *Microparyphium* were in a range from 4.5 to 5.4%, while the distances within the first and second clusters were 1.4 and 2.4%, respectively. Both clusters were also clearly separated using data from the ITS2 rDNA region (Fig. 3).

Based on the data obtained in the first cluster, the genus *Echinochasmus* combines *E. coaxatus* and *E. japonicus* using both 28S and ITS2 markers, along with *E. beleocephalus* and *E. bursicola* according to the 28S rRNA gene sequences (Figs 2 and 3). As for the species *Stephanoprora* and *Echinochasmus* being united in the second cluster by the 28S rRNA gene, the absence of any statistically confirmed differences between them makes it necessary to designate, at this stage of the research, the whole group as *Stephanoprora*-like (Fig. 2). In contrast, based on the ITS2 region, the distance between the first and second clusters of 17.3% was comparable with the distance between all representatives of the family Echinochasmidae and *Echinostoma trivolvis* (Echinostomatidae), 19.6%. At the same time, within

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cluster 2, there was also a subdivision into three more equivalent subclusters, A, B and C (Fig. 3), the distances between which are in the range from 6 to 9.5%. Such high values of differences may indicate that the species belonging to each of the subclusters belong to separate genera. Thus, there is a morphological similarity in the number of adoral spines and tail length in cercaria of species in the second cluster obtained using both nuclear markers; however, there were significant distances between subclusters on the ITS2 tree. The data obtained indicated the possibility of distinguishing at least two subfamilies that separately combined the species of the first and second clusters, as well as new genera based on the structure of the second cluster. However, we considered it premature to make a final conclusion about whether the worms belong to one or different genera, as well as to solve taxonomic problems within Echinostomatoidea at a higher level. The main reason for this is the limited amount of genetic data for both Echinochasmidae and other representatives of the subfamily.

As mentioned above, most representatives of the second cluster of the phylogenetic reconstruction based on the 28S and ITS2 markers had cercariae with long tails. However, questions remain regarding *E. mordax*, for which the morphology of cercariae was unknown, as well as *E. donaldsoni* and representatives of *Echinochasmus* with unknown species affiliation. *E. donaldsoni* had 20 adoral spines, but cercariae with short tails (Beaver, 1941). For worms designated as *E. donaldsoni*, genetic data were available for only one specimen obtained from naturally infected animals (Tkach *et al.*, 2016). Morphometric and biological descriptions were not given for confirmation of the belonging to this species. It was important to remember that the presence of morphological twins within the genera is quite widespread among digeneans. In this case, in the absence of data on the morphology of individuals of the species, assigning it the first obtained genetic data may be incorrect. This also applies to *Echinochasmus* with an unknown species affiliation: the nucleotide sequences for *Echinochasmus* sp. 1. were obtained from parthenitae (Stanevičiūtė *et al.*, 2015) and there was no morphological description for *Echinochasmus* sp. 3.

Designated difficulties may complicate the interpretation of the results, including this study. Based on the molecular data of the ITS2 rDNA region, *Echinochasmus* sp. 2, obtained experimentally by Molnár *et al.* (2016), entered the second cluster together with *Stephanoprora*. Mature worms of this species, like other echinochasmids in the cluster, had 20 adoral spines. Unfortunately, the authors (Molnár *et al.*, 2016) did not provide a detailed description of morphological characteristics for cercaria but indicated that 'the tail was almost as long as the body'. However, based on the figure in the publication, this larval stage had a tail that is characteristic of long-tailed Echinochasmidae cercariae. The tail had numerous folds formed during its contraction, as well as numerous vacuole-like inclusions, which are not typical for short-tailed *Echinochasmus* cercariae. At the same time, the cercaria shown in micrograph differs in a number of morphological features from cercaria in the figure: ventral sucker is smaller than the oral one *vs* ventral sucker is equal to or larger than the oral one; and a tail typical for short-tailed cercariae *vs* tail like long-tailed cercariae. Thus, the use of data obtained by Molnár *et al.* (2016) violates the objectivity of taxonomy assessment and analysis of phylogenetic relationships of Echinochasmidae to a certain extent. The greatest problems of taxonomy, systematics and phylogeny arise when, during the study of the life cycle of both echinostomatids and other representatives of digeneans, some developmental stages were obtained experimentally, while others were collected by dissecting naturally infected animals. These results are combined and *a priori* assigned to the same species. Considering that closely related worms belonging to different species can have similar morphometries at different stages, cases of using incorrect methodology for studying the life cycle can lead to erroneous conclusions on the taxonomy of the studied trematodes. If such studies are accompanied by obtaining genetic data, the resolution of questions of taxonomy, systematics and phylogeny becomes even more difficult.

This situation is compounded by the fact that there are no clear criteria for differentiating trematode species in different systematic groups due to a limited amount of molecular data. Our studies of echinochasmids showed that the 28S rRNA gene, as well as the ITS1 and ITS2 regions, did not resolve the problem of species differentiation for *E. milvi and E. suifunensis*. In the presence of a high level of nuclear DNA conservatism, and subject to obtaining adequate data on the life cycle, the taxonomic status can be confirmed by nucleotide sequences of the mitochondrial genome, since this is more variable. However, there are no significant criteria of these differences for establishing the taxonomic affiliation of most trematodes, since, in the structure of macro- and micro-populations of worms from various taxonomic groups, there is no data on the rate of mutation accumulation in the mitochondrial genome. Based on this, we emphasize that data for the adequate taxonomic and phylogenetic classification of digenean species should include a description of both the morphology of developmental stages and the genetic data for the studied worms. Additionally, it should particularly be done at the first acquisition of genetic data for the designated species.

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Conflict of interest. The authors declare no conflict of interest.

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

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