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New species of *Parasaccocoelium* (Haploporidae) and new genus *Pseudohaplosplanchnus* (Haplosplanchnidae) from mullet fish in the Far East of Russia and Vietnam: morphological and molecular data

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Research Paper

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Abstract

A description and the molecular characterization of two new species in the Haploporidae and Haplosplanchnidae families are provided herein. *Parasaccocoelium armatum* n. sp. was collected from the intestine of a *Mugil cephalus* Linnaeus, 1758 from the Primorsky region, Russia, and *Pseudohaplosplanchnus catbaensis* n. g. n. sp. was collected from *Moolgarda seheli* (Forsskål, 1775) in the coastal waters of Cat Ba Island, Vietnam. The morphological features of *P. armatum* n. sp. closely resemble those of *Parasaccocoelium polyovum*, but these species differ from one another by hermaphroditic sac and vitellaria area length and by maximal egg size. The main difference between *P. armatum* n. sp. and *P. polyovum* is the presence of an armed hermaphroditic duct in the new species. Molecular data support the case for inclusion of the studied trematodes in *P. armatum* n. sp. Worms *P. catbaensis* n. g. n. sp. from the mullet from Vietnam are morphologically close to *Haplosplanchnus* (Haplosplosplanchninae). The only difference between *P. catbaensis* n. g. n. sp. and species of *Haplosplanchnus* is the presence of few (1–7) large eggs, measuring 135–142 × 92–104 µm, versus numerous small eggs with a maximal size of 75 × 50 µm. Phylogenetic analysis showed that there is a contradiction between the morphological similarity of the worms and their position in the Haplosplanchnidae system, based on the genetic data. Results of this study indicate that *P. catbaensis* n. g. n. sp. is genetically distant from other representatives of *Haplosplanchnus*, despite their morphological similarity. According to the molecular data, *P. catbaensis* n. g. n. sp. is close to *Hymenocotta mulli* Manter, 1961 (Hymenocottinae). However, these species are considerably different to each other morphologically. Molecular data argue for the possibility of establishing a new subfamily for *P. catbaensis* n. g. n. sp. However, considering earlier studies of Haplosplanchnidae, we support the view that creating new subfamilies within this family is unreasonable because of the lack of molecular data for most haplosplanchnid species, which are necessary to resolve the problematic systematics and phylogeny of this family.

Introduction

Zhukov (1971) established the new genus *Parasaccocoelium* Zhukov, 1971 as a member of Haploporidae Nicoll, 1914. The type species of this genus, *Parasaccocoelium mugili* Zhukov, 1971, was found in the intestine of *Planiliza haematocheila* (Temminck & Schlegel, 1845) in the Japan Sea Basin (Zhukov, 1971). Overstreet & Curran (2005) decided that the genus *Parasaccocoelium* Zhukov, 1971 was invalid and transferred a single species of this genus, *P. mugili*, to the genus *Pseudohapladena* Yamaguti, 1952. Later, on the basis of morphological and molecular data, the validity of the genus *Parasaccocoelium* and the type species *P. mugili* was confirmed, and two new species, *Parasaccocoelium haematocheilum* Besprozvannykh, Atopkin, Ermolenko & Nikitenko, 2015 and *Parasaccocoelium polyovum* Besprozvannykh, Atopkin, Ermolenko & Nikitenko, 2015 (Besprozvannykh *et al.*, 2015) were described from intestines of mullet from south of the Russian Far East.

Concerning Haplosplanchnoidea Poche, 1926, most of the representatives of this subfamily were detected in mullet from the Indo-Western Pacific (Madhavi, 2005), as was the case for *Parasaccocoelium* species. Species identification of most worms in both *Haplosplanchnus* Looss, 1902 and other genera of the Haplosplanchnidae Poche, 1926 were based only on morphometric data. Until now, molecular data have been available for a few species only (Cribb *et al.*, 2001; Olson *et al.*, 2003; Besprozvannykh *et al.*, 2016; Huston *et al.*, 2017, 2018). In

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Table 1. List of taxa incorporated in the molecular analysis of the family Haloporidae, with the number of DNA sequences given in parentheses.

Species	Author	GenBank accession numbers
Waretrematinae		
Parasaccocoelium armatum n. sp. (*n* = 2)	This study	MT298950–MT298951
Parasaccocoelium polyovum (*n* = 2)	This study	MT298952–MT298953
Parasaccocoelium polyovum (*n* = 2)	Beprozvannykh *et al.*, 2015	HF548476–HF548477
Parasaccocoelium haematochelium (*n* = 2)	Beprozvannykh *et al.*, 2015	HF548467–HF548468
Parasaccocoelium mugili (*n* = 1)	Beprozvannykh *et al.*, 2015	HF548473
Elonginurus mugilus (*n* = 2)	Atopkin *et al.*, 2019	MH763761–MH763762
Capitimitta darwiensis (*n* = 1)	Pulis & Overstreet, 2013	KC206497
Capitimitta costata (*n* = 1)	Pulis & Overstreet, 2013	KC206498
Skrjabinolecithum spasskii (*n* = 3)	Atopkin *et al.*, 2015	HE806371,HG530228,LK022754
Skrjabinolecithum pyriforme (*n* = 1)	Besprozvannykh *et al.*, 2017a	HE806361
Skrjabinolecithum spinosum (*n* = 1)	Besprozvannykh *et al.*, 2017b	MF176831
Carassotrema sp. (*n* = 1)	Ding, 2018, unpublished	MH285255
Carassotrema koreanum (*n* = 1)	Atopkin *et al.*, 2019	MH763760
Spiritestis herveyensis (*n* = 1)	Pulis & Overstreet, 2013	KC206500
Outgroup		
Forticulcitinae		
Forticulcita apiensis	Andres *et al.*, 2015	KP761087
Forticulcita plantata	Andres *et al.*, 2015	KP761086
Xiha fastigata	Andres *et al.*, 2015	KP761088

2018, Huston *et al.* established the new genus *Trigonocephalotrema* Huston, Cutmore & Cribb, 2018 with three new species, which were included into the Haplosplanchnidae on the basis of morphological and molecular characteristics. Differentiation at molecular level between *Trigonocephalotrema* and other representatives of the Haplosplanchnidae required the erection of a new subfamily for this new genus. Given the low number of species within this family for which morphological and molecular data are available, the authors found this insufficient for haplosplanchnid systematics and retained the generic status of *Trigonocephalotrema* trematodes, further proposing to avoid the concept of subfamilies for the Haplosplanchnidae.

In the present study, we provide morphological and molecular data for a new species of the genus *Parasaccocoelium* collected from *Mugil cephalus* from south of the Far East of Russia, and also for worms of a new genus of Haplosplosplanchninae Poche, 1926 and we present molecular data for *Hymenocotta mulli* collected from *Moolgarda seheli* off the coast of Vietnam.

Material and methods

Collection of trematodes

Adult worms were collected from the intestines of mullet fish (Mugilidae) from coastal waters of the Primorsky region of the south of the Russian Far East and Cat Ba Island, Vietnam. Worms from the fish, previously defined under a microscope, were rinsed in saline, killed in hot distilled water and preserved in 70% ethanol. After fixation, they were replaced in 96% ethanol. Whole-mounts were made by staining specimens with alum carmine, dehydrating the worms in graded ethanol series and clearing in clove oil. The clove oil treatment was followed by mounting the specimens in Canada balsam under a coverslip on a glass slide. All measurements are given in micrometres.

DNA extraction, amplification and sequencing

Two adult specimens of *Parasaccocoelium armatum* n. sp., five specimens of *Pseudohaplosplanchnus catbaensis* n. g. n. sp. and two specimens of *H. mulli* from 96% ethanol were used for molecular analysis (table 1). Total DNA was extracted from flukes using a 'hot shot' technique (Truett, 2006).

Nuclear 18S ribosomal DNA (rDNA) and 28S rDNA fragments were successfully amplified using polymerase chain reaction (PCR). Then, 18S rDNA was amplified with the primers 18S-E (5′ CCG AAT TCG ACA ACC TGG TTG ATC CTG CCA GT 3′) and 18S-F (5′ CCA GCT TGA TCC TTC TGC AGG TTC ACC TAC 3′), as described earlier (Littlewood & Olson, 2001). Initial PCR reaction was performed in a total volume of 20 µl containing 0.25 mM of each primer pair, 25 ng of total DNA in water, 5× Taq buffer, 1.25 mM dNTPs, 1.5 mM magnesium and one unit of Taq polymerase. Amplification of a 2000-bp fragment of 18S rRNA gene was performed in a GeneAmp 9700 (Applied Biosystems, Waltham, Massachusetts, USA), with a 5-min denaturation at 96°C, 35 cycles of 1 min at 96°C, 20 s at 58°C and 5 min at 72°C, and a 10-min extension at 72°C. Negative and positive controls using both primers were used.

The 28S rDNA was amplified with the primers DIG12 (5′-AAG CAT ATC ACT AAG CGG-3′) and 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) (Tkach *et al.*, 2003) with an annealing temperature of 55°C. PCR products were directly sequenced using an ABI

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Table 2. List of taxa incorporated in the molecular analysis of the superfamily Haplosplanchnoidea, with the number of DNA sequences given in parentheses.

Species	Author	GenBank accession numbers	
		18S	28S
Haplosplanchnoidea			
Pseudohaplosplanchnus catbaensis n. g. n. sp. (*n* = 4)	This study	MT298954–MT298957	MT298959-–MT298962
Haplosplanchnus pachysomus (*n* = 4)	Besprozvannykh *et al.*, 2016	LK932143–LK932146	LK932149–LK932152
Haplosplanchnus pachysomus (*n* = 1)	Blasco-Costa *et al.*, 2008, unpublished	FJ211224	FJ211241
Provitellotrema crenimugilis (*n* = 2)	Besprozvannykh *et al.*, 2016	LK932147–LK932148	LK932153–LK932154
Haplosplanchnus purii (*n* = 1)	Blasco-Costa *et al.*, 2008, unpublished	FJ211225	FJ211242
Schikhobalotrema sparisomae (*n* = 1)	Blasco-Costa *et al.*, 2008, unpublished	FJ211223	FJ211240
Schikhobalotrema huffmani (*n* = 2)	Huston *et al.*, 2017		
Schikhobalotrema sp. (*n* = 1)	Cribb *et al.*, 2001; Olson *et al.*, 2003	AJ287574	AY222238
Trigonocephalotrema euclidi (*n* = 1)	Huston *et al.*, 2018	MG386254	MG386255
Trigonocephalotrema hipparchi (*n* = 1)	Huston *et al.*, 2018	MG386257	MG386258
Trigonocephalotrema sohcahtoa (*n* = 1)	Huston *et al.*, 2018	MG386260	MG386261
Trigonocephalotrema sp. (*n* = 1)	Huston *et al.*, 2018	MG386263	MG386264
Hymenocotta mulli (*n* = 1)	Cribb *et al.*, 2001; Olson *et al.*, 2003	AJ287524	AY222239
Hymenocotta mulli (*n* = 1)	This study		
Hymenocotta mulli (*n* = 1)	This study		
Outgroup			
Echinostomatoidea			
Psilochasmus oxyurus	Olson *et al.*, 2003; Tkach *et al.*, 2000	AY222135	AF151940
Echinostoma trivolvis	Olson *et al.*, 2003	AY222132	AY222246
Paramphistomoidea			
Diplodiscus subclavatus	Cribb *et al.*, 2001; Olson *et al.*, 2003	AJ287502	AY222212
Solenorchis travassosi	Olson *et al.*, 2003	AY222110	AY222213
Pronocephaloidea			
Catatropis indicus	Olson *et al.*, 2003	AY222114	AY222220
Lankatrema mannarense	Olson *et al.*, 2003	AY222116	AY222222

Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, Massachusetts, USA), as recommended by the manufacturer, with the internal sequencing primers described by Tkach *et al.* (2003) for 28S rDNA. PCR product sequences were analysed using an ABI 3130 genetic analyser (Applied Biosystems, Waltham, Massachusetts, USA) at the Federal Scientific Center of the East Asia Terrestrial Biodiversity FEB RAS. Sequences were submitted to the GenBank database (National Center for Biotechnology Information (NCBI)).

Alignments and phylogenetic analysis

rDNA sequences were assembled with SeqScape v.2.6 software, provided by Applied Biosystems (Waltham, Massachusetts, USA) . Alignments and estimations of the number of variable sites and sequence differences were performed using the MEGA 7.0 software (Kumar et al., 2016). The values of genetic *p*-distances were calculated for the 28S rDNA fragment. Phylogenetic relationships were obtained using a concatenated data set of the complete 18S rRNA gene and partial sequences of the 28S rRNA gene. Phylogenetic analysis was performed using the Bayesian algorithm with the MrBayes version 3.1.2 software (Huelsenbeck *et al.*, 2001). The best nucleotide substitution models – the TIM3 + G (Darriba *et al.*, 2012) for Waretrematinae and TVM + I+G (Posada, 2003) for Haplosplanchnoidea – were estimated with jModeltest version 2.1.5 software (Darriba *et al.*, 2012). Bayesian analysis was performed using 10,000,000 generations with two independent runs. Summary parameters and the phylogenetic tree were calculated with a burn-in of 25% of generations. The significance of the phylogenetic relationships was estimated using posterior probabilities (Huelsenbeck *et al.*, 2001). GenBank sequence data for representatives of Waretrematinae and Haplosplanchnoidea and outgroup taxa used in molecular analysis, including references and accession numbers, are given in the tables 1 and 2.

Results

Parasaccocoelium armatum n. sp.

Taxonomic summary

Type host. Mugil cephalus Linnaeus, 1758.

Number of fish examined. 97.

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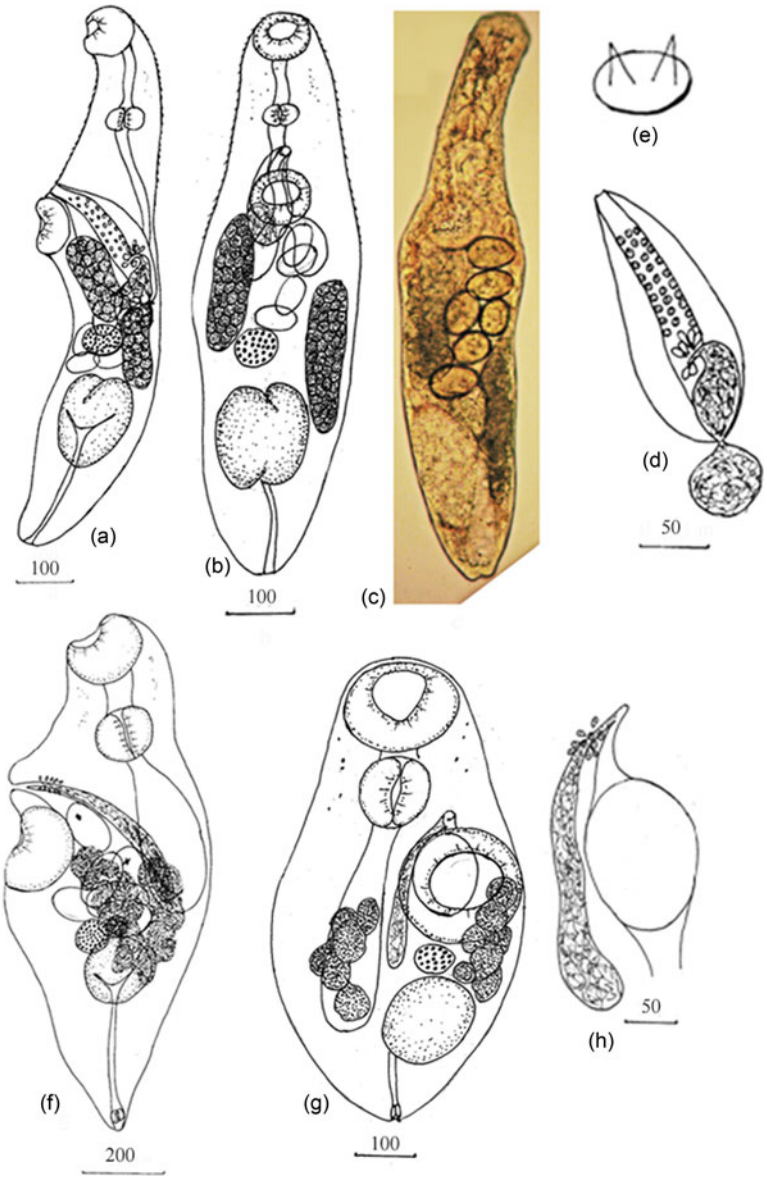

Fig. 1. *Parasaccocoelium armatum* n. sp.: (a) holotype, lateral view; (b, c) ventral view; (d) hermaphroditic sac; (e) pad with two spines. *Pseudohaplosplanchnus catbaensis* n. g. n. sp.: (f) holotype, lateral view; (g) ventral view; (h) terminal genitalia. Scale-bars in μm.

Infection of fish. 1.

Intensity of infection. 17 worms.

Site. Intestine.

Type locality. Primorsky region, Kievka River (42°85′20″N, 13° 38′390″E).

Type deposition. Type number 152-Tr, paratype number 153-156-Tr. This material is held in the parasitological collection of the Zoological Museum (Federal Scientific Center of the East Asia Terrestrial Biodiversity Far Eastern Branch of Russian Academy of Sciences, Vladivostok, Russia; e-mail: petrova@ibss.dvo.ru). Deposited: 2019.05.05.

Etymology. Species was named because of presence of arming of hermaphroditic canal.

Description

Based on five specimens (fig. 1a–e; table 3). Body elongated, fusiform with spines from anterior end to ventral sucker. Forebody and posterior end of body capable of retracting into inside of body. Eyespot pigment dispersed in forebody. Oral sucker subterminal. Prepharynx long or short. Pharynx transversally oval. Oesophagus shorter, equal or longer than prepharynx, bifurcating at level of or posterior to posterior margin of ventral sucker. Caeca short, sac-shaped terminate near posterior margin middle third of body. Ventral sucker larger than oral sucker, on border of anterior and middle third of body, or in beginning middle third of body. Testis single, V-shaped or from two equal lobes in posterior third of body. Hermaphroditic sac sac-shaped, extends posteriorly beyond ventral sucker, contains internal seminal vesicle, some prostatic cells and long hermaphroditic canal. Hermaphroditic canal thick-walled, muscular and armed with six rows of pads. Pads with two spines on reticular sclerotized base. External seminal vesicle round, extended to ovary. Genital pore, immediately anterior to ventral sucker. Ovary round or oval anterior to testis. Uterus short, located from hermaphroditic sac up to anterior margin of the testis, containing 2–7 eggs. Uterine seminal receptacle present. Metraterm short, with thin walls. Eggs light yellow, oval, operculate, at various stages of embryogeny. Vitellarium in

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Table 3. Measurements (in micrometres) of adult worms of new species.

	Parasaccocoelium armatum n. sp. *n* = 5			*Parasaccocoelium polyovum* (Besprozvannykh *et al.*, 2015)	*Pseudohaplosplanchnus catbaensis* n. g. n. sp. *n* = 5		
	Holotype	Range	Mean		Holotype	Range	Mean
Body length	955	847–955	909	327–832	1278	785–1278	1133
Body width	200	200–246	223	146–262	462	431–554	487
Forebody length	281	184–322	270	123–269	474	316–539	447
Body/forebody length ratio %	29.4	28.0–34.3	29.7	–	37.0	37.0–44.9	39.5
Oral sucker length	81	58–89	70	50–77	166	166–212	190
Oral sucker width	81	58–89	75	60–85	166	166–212	190
Ventral sucker length	96	73–100	87	58–92	193	193–277	223
Ventral sucker width	96	77–100	89	58–92	193	193–277	223
Ventral/oral sucker length ratio	1:1.19	1:1.12–1.33	1:1.24	1:0.73–1.20	1:1.20	1:1.08–1.31	1:1.17
Ventral/oral sucker width ratio	–	1:1.05–1.53	1:1.19	1:0.74–1.06	–	1:1.08–1.31	1:1.17
Prepharynx length	100	35–131	88	19–92	69	19–69	50
Pharynx length	39	27–39	31	27–42	116	116–135	126
Pharynx width	65	54–65	58	39–58	146	119–158	141
Oesophagus length	227	81–227	149	27–46	–	–	–
Ovary length	58	58–77	64	23–50	77	54–116	81
Ovary width	62	46–73	57	27–58	77	73–92	82
Testis length	169	162–200	171	85–223	154	154–200	175
Testis width	123	112–123	132	58–123	154	154–173	162
Hermaphroditic sac length	181	181–212	200	96–173	–	–	–
Hermaphroditic sac width	81	77–85	80	50–96	–	–	–
Pars prostatica length	–	–	–	–	196	173–196	184
Vitelline field sinistral length	192	162–262	207	104–146	–	–	–
Vitelline field dextral length	169	169–204	198	50–96	–	–	–
Post-testicular length	158	112–158	140	42–339	–	–	–
Armed hermaphroditic duct	+	+	+	–	–	–	–
Egg length	65–69	65–69	–	61–85	135–142	135–142	–
Egg width	35–39	35–39	–	39–50	92–104	92–104	–

two lateral fields formed from compact follicles of round forms, extending between posterior half of ventral sucker and testis, and can partly cover ovary and testis. Vitelline fields located diagonally relative to each other. Anterior-edge dextral vitelline field at level of posterior half of ventral sucker. Anterior-edge sinistral vitelline field at level of posterior-end dextral vitelline field. Excretory bladder Y-shaped.

Molecular data

Two sequences of 28S rDNA fragments 1086 bp in length of *P. armatum* n. sp. contained no variable sites. The sequences were submitted to the NCBI database with accession numbers MT298950–MT298951.

Remarks

Currently, there are three species within *Parasaccocoelium* from mullet from south of the Russian Far East (Besprozvannykh *et al.*, 2015). *Parasaccocoelium armatum* n. sp. is a fourth species that has been found in mugilids from this region. *Parasaccocoelium armatum* n. sp. is most similar to *P. polyovum* based on morphology, including the form of the body, testis,

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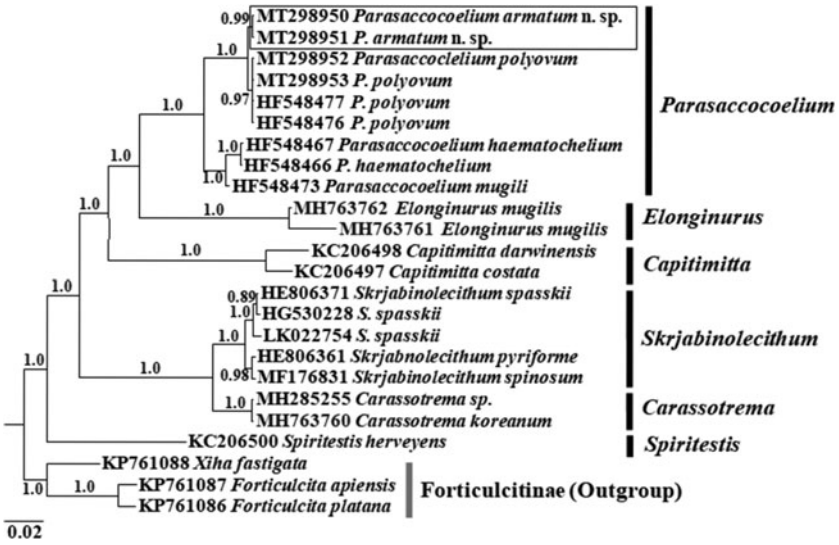

Fig. 2. Phylogenetic tree of the subfamily Waretrematinae based on the analysis of partial 28S rRNA gene sequences; nodal numbers indicate posterior probabilities for Bayesian inference algorithms.

ovary, hermaphroditic sac and its reciprocal arrangement and number of eggs within the uterus – more than four (*P. mugili*, *P. haematocheilum* have from one to four eggs). However, these species differ from each other by hermaphroditic sac and vitellaria field length and by maximal egg size (table 3). Moreover, vitellaria fields are arranged parallel one to another in *P. polyovum* and diagonally in *P. armatum* n. sp. The main difference between *P. armatum* n. sp. specimens and other *Parasaccocoelium* species is the presence of an armed hermaphroditic duct. Molecular data support the generic membership of *P. armatum* n. sp., and, associated with morphological data, indicate a close relationship between the new species and *P. polyovum* within the monophyletic *Parasaccocoelium* (fig. 2). Additionally, the genetic *p*-distance value between *P. armatum* n. sp. and *P. polyovum* (0.37% ± 0.19%) is comparable with the interspecific genetic differentiation level for the genus *Parasaccocoelium* (0.78% ± 0.26%). Nucleotide sequences of 28S rDNA of *P. armatum* n. sp. and *P. polyovum* are different by four fixed substitutions.

Family Haplosplanchnidae Poche, 1926

Subfamily Haplosplosplanchninae Poche, 1926

Pseudohaplosplanchnus n. g.

Diagnosis

Body elongated, narrowed posterior end capable of retracting into inside of body. Eyespot pigment dispersed in forebody. Oral sucker subterminal. Prepharynx short. Pharynx transversally oval. Oesophagus absent. Caecum single, reaching level of anterior border ovary. Ventral sucker at level of mid-body, larger than oral sucker. Testis single, in posterior third of body, round or oval. Seminal vesicle tubular, reaching to level posterior border ventral sucker. Pars prostatica thin-walled, surrounded by prostatic cells. Hermaphroditic duct short. Genital pore median, close to anterior border of ventral sucker. Ovary spherical, pre-testicular or contiguous with testis. Seminal receptacle round, contiguous to ovary. Uterus in middle third of body. Eggs large, few, operculated, in distal part of uterus only, containing miracidia with eyespot. Vitellaria in two lateral fields formed from follicles of irregular forms, extending between level of middle ventral sucker and posterior-end testis. Excretory bladder Y-shaped with muscular sphincter. Found in intestine of Mugilidae fishes in Halong Bay, northern Vietnam.

Taxonomic summary

Type species. Pseudohaplosplanchnus catbaensis n. sp.

Etymology. The genus was named *Pseudohaplosplanchnus* n. g. n. sp. on the basis of morphological similarity of these flukes with representatives of the genus *Haplosplanchnus*.

Pseudohaplosplanchnus catbaensis n. sp.

Taxonomic summary

Type host. Moolgarda seheli (Forsskål, 1775).

Number of fish examined. 80.

Infection of fish. 5.

Intensity of infection. 1–4 worms per fish.

Site. Intestine.

Type locality. Coastal water of Cat Ba Island, Ha Long Bay, northern Vietnam (20°88′40″N, 10°68′590″E).

Type deposition. Type number 157-Tr, paratype number 158-161-Tr. This material is held in the parasitological collection of the Zoological Museum (Federal Scientific Center of the East Asia Terrestrial Biodiversity Far Eastern Branch of Russian Academy of Sciences, Vladivostok, Russia; e-mail: petrova@ibss.dvo.ru). Deposited: 2019.05.05.

Etymology. Species was named with respect to first description place – Cat Ba Island, Vietnam.

Description

Based on five specimens (fig. 1f–h; table 3). Body elongated, narrowed posterior end capable of retracting into inside of body. Eyespot pigment dispersed in forebody. Oral sucker subterminal. Prepharynx short. Pharynx transversally oval. Oesophagus absent. Caecum single, reaching level of anterior border ovary. Ventral sucker in mid-body larger than oral sucker. Testis single, in posterior third of body, round or oval. Seminal vesicle tubular, reaching to level of posterior border ventral sucker. Prostatic part thin-walled, surrounded by prostatic cells. Hermaphroditic duct short. Genital pore median, close to anterior border of ventral sucker. Ovary spherical, pre-testicular or contiguous with testis. Seminal receptacle round, contiguous to ovary. Uterus in middle

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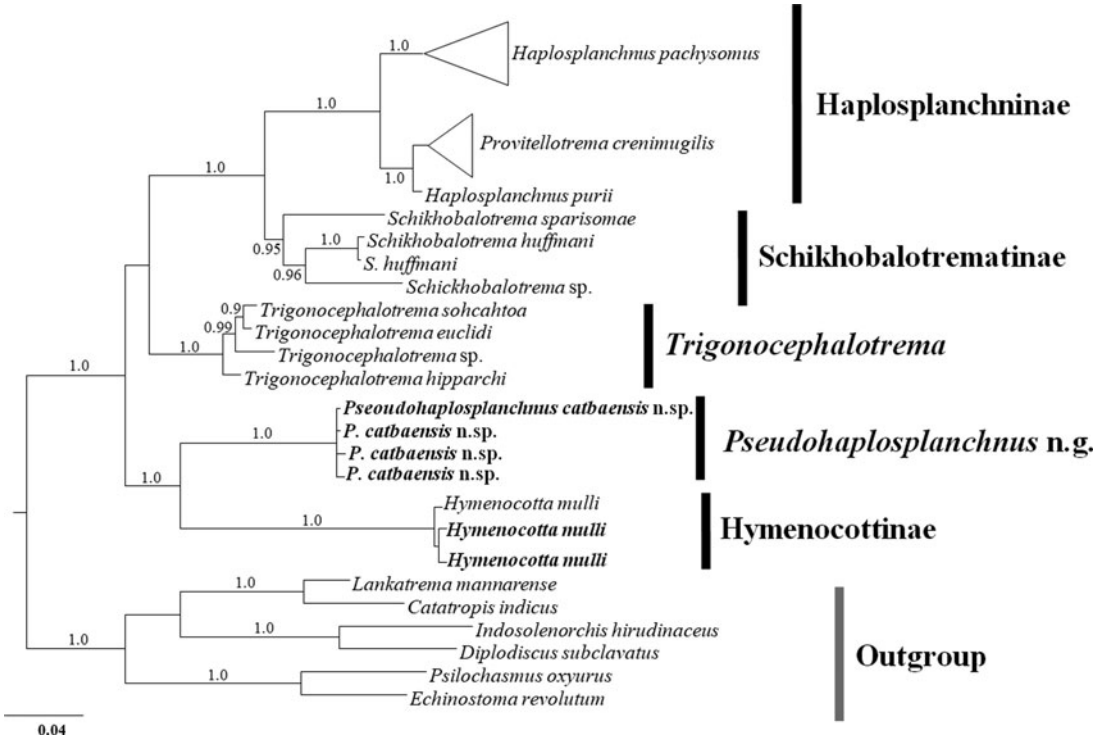

Fig. 3. Phylogenetic tree of the family Haplosplanchnidae based on the analysis of combined 18S rRNA (complete) and of 28S rRNA (partial) gene sequences; nodal numbers indicate posterior probabilities for Bayesian inference algorithms. Sequences from the present study are marked in bold.

third of body, with several loops, reaching level of ovary. Eggs 1–7 in number, large, operculated, in distal part of uterus containing miracidia, with eyespot. Vitellaria in two lateral fields formed from follicles of irregular forms extending, between level of middle ventral sucker and level of posterior-end testis. Excretory bladder Y-shaped with muscular sphincter.

Molecular data

Four successfully amplified and sequenced 18S rDNA of *P. catbaensis* n. sp. were 1785 bp in length and contained one variable singleton site. Five 28S rDNA fragments 1069 bp in length of *P. catbaensis* n. g. n. sp. comprised single variable singleton site. The sequences of 18S rDNA and 28S rDNA were submitted to the NCBI database with accession numbers MT298954-MT298957 and MT298959-MT298962, respectively.

Remarks

Pseudohaplosplanchnus catbaensis n. g. n. sp. is similar to representatives of Haplosplosplanchninae by a combination of morphological characteristics, including the presence of a single intestine, single testis and the absence of a cirrus sac. Among haplosplanchnins, these worms are morphologically closer to *Haplosplanchnus* species. The single difference between *Pseudohaplosplanchnus* n. g. and *Haplosplanchnus* is the presence of few (1–7) large eggs (135–142 × 92–104 μm) in the uterus of *Pseudohaplosplanchnus* n. g. versus numerous small eggs with a maximal size of 75 × 50 μm (Al-Bassel, 1997; Nahhas, Rhodes & Seeto, 1997) in the uterus of *Haplosplanchnus*. Despite the morphological similarity of representatives of these two genera, the validity of *Pseudohaplosplanchnus* n. g. is supported by molecular data. The 28S rDNA-based genetic distances between *Pseudohaplosplanchnus* n. g. and *Haplosplanchnus* are in the intergeneric range, indicating *Pseudohaplosplanchnus* n. g. does not belong to *Haplosplanchnus*. On the other hand, molecular data show that the new genus is closely related to *Hymenocotta* Manter, 1961 (Hymenocottinae Yamaguti, 1971) (fig. 3).

Phylogenetic analysis, based on the available molecular data for the Haplosplanchnidae, including type species for *Haplosplanchnus* and *Trigonocephalotrema*, revealed that representatives of the Haplosplanchnoidea, Schikhobalotrematinae Skrjabin & Guschanskaja, 1955 and the genus *Trigonocephalotrema* formed three distinct, highly supported clades. Of these, the Haplosplanchnoidea and Schikhobalotrematinae were closely related to each other with high statistical support, and the genus *Trigonocephalotrema* appears as a sister clade with poor support (fig. 3). Another highly supported clade contained representatives of the Hymenocottinae, including our new samples of *H. mulli* and specimens of *P. catbaensis* n. sp.

The situation with representatives of the genera *Trigonocephalotrema* and *Pseudohaplosplanchnus* n. g. is paradoxical. On the one hand, representatives of *Trigonocephalotrema* possess a combination of morphological characteristics that are representative of worms of the Hymenocottinae and Schikhobalotrematinae (*Schikhobalotrema*). In particular, specimens of *Trigonocephalotrema*, like *Hymenocotta*, possess a peculiar-shaped oral sucker. *Pseudohaplosplanchnus* n. g. worms are morphologically similar to *Haplosplanchnus* (Madhavi, 2005). On the other hand, analysis of genetic *p*-distances and phylogenetic relationships indicate a considerable level of differentiation of *Trigonocephalotrema* from both *Hymenocotta* and *Schikhobalotrema* (*p*-distance values are 14.2% ± 1.0% and 11.9% ± 0.8%, respectively), and the same for *Pseudohaplosplanchnus* n. g. and *Haplosplanchnus* (15.64% ± 1.0%). Overall, *p*-distance values and the results of phylogenetic analysis of

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Pseudohaplosplanchnus n. g. (fig. 3) indicate that a new subfamily for this genus, and for *Trigonocephalotrema*, can be proposed. However, phylogenetic analysis showed that there is a contradiction between the morphological similarity of these worms and their position in the Haplosplanchnidae system, based on the genetic data. Considering these results, we support the view of Huston *et al.* (2018), who state that erecting new subfamilies within the Haplosplanchnidae is questionable because of the lack of molecular data for most haplosplanchnid species; such data are needed to resolve the problematic systematics and phylogeny of this family.

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Conflicts of interest. 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.

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