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Restoration of the genus *Parasaccocoelium* Zhukov, 1971 (Digenea: Haploporidae) and a description of two new species from mugilid fish in the Far East of Russia

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Abstract

New data relating to the fauna of Haploporidae (Digenea) in mugilid fish from Primorsky Region of the south of the Russian Far East are presented. In the most recent revision of the family Haploporidae Nicoll, 1914, *Parasaccocoelium* Zhukov, 1971 was synonymized with *Pseudohapladena* Yamaguti, 1952 (Overstreet & Curran, 2005). Here, we restore the genus *Parasaccocoelium*. The species described by Zhukov can be distinguished from the species *Pseudohapladena sensu* Yamaguti, 1952 using a combination of the following features: (1) diffuse or absent eye-spot pigment; (2) subterminal or terminal oral sucker; (3) present or absent genital atrium; and (4) vitellarium with two large and compact vitelline masses, or vitelline follicles tubular and divided into two groups. For the species of *Pseudohapladena*, i.e. *P. scatophagi*, Yamaguti noted that the vitelline follicles occupy almost the entire post-testicular region, but in the present specimens, the vitelline follicles do not occupy the post-testicular region. Based on the diagnosis of *Pseudohapladena* by Overstreet & Curran (2005), the genus *Parasaccocoelium* differs in the following features: (1) a single testis that is longitudinal, longitudinally oval, spherical, transversally oval, irregular, bilobed or V-shaped, or a testis that is subspherical to irregular to elongate; (2) the vitellarium in the lateral fields formed from large compact follicles that have an irregular form and extend between the anterior margin of the ventral sucker or pharynx and posterior margin of the testis, and the vitelline follicles do not occupy post-testicular regions; or the vitellarium consists of elongate groups of follicles that usually extend at least one-half of a body length and extend to the posterior end of the body in some and to the posterior margin of the testis in others; (3) the eggs are operculate, unembryonated and developed when laid, or the eggs in some cases are only operculate; and (4) a developed miracidium with or without an eye spot. New data have been added to the description of *Parasaccocoelium mugili* Zhukov, 1971 based on new material, and two new species of this genus, *Pa. haematocheilum* n. sp. from *Liza haematocheila* and *Pa. polyovum* n. sp. from *L. haematocheila* and *Mugil cephalus*, are described using morphological and molecular approaches.

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We support the taxonomic status of the genus *Parasaccocoelium* and its inclusion within the sub-family Waretrematinae, and we consider *Pseudohapladena lizae* Liu et Yang, 2002 to be a junior synonym of *Parasaccocoelium mugili* Zhukov, 1971.

Introduction

Until recently, data regarding the digenean fauna of mugilid fish species from the southern Russian Far East were limited to the description of two species of flukes in the redlip mullet *Liza haematocheila* (Temminck and Schlegel), *Skrjabinolecithum spasskii* Belous, 1954 and *Parasaccocoelium mugili* Zhukov, 1971 (Belous, 1954; Skrjabin, 1956; Zhukov, 1971). Overstreet & Curran (2005) synonymized *Parasaccocoelium* Zhukov, 1971 with *Pseudohapladena* Yamaguti, 1952, which also included *Ps. scatophagi* Yamaguti, 1952 (type species), *Ps. pearsoni* (Martin, 1973), *Ps. martini* (Madhavi, 1979), *Ps. huidongensis* (Lu, 1993), *Ps. megaorchis* Liu et Yang, 2002 and *Ps. lizae* Liu et Yang, 2002, which are parasites of mugilid and scatophagid fish species in the Indo-West Pacific Region (Overstreet & Curran, 2005). *Parasaccocoelium* was synonymized with *Pseudohapladena* based on a longer prepharynx than pharynx and relatively short sac-like caeca ending near the mid-body, and other signs that are characteristic of *Pseudohapladena*.

While investigating the parasite fauna of the redlip mullet *L. haematocheila* and the striped mullet *Mugil cephalus* L., we found several species of haploporids. Based on newly obtained material from type hosts near the type locality, we added new data to the description of the type species of *Parasaccocoelium* and restored the classification of this genus. We have included *Pa. mugili* and two new species in *Parasaccocoelium*.

Materials and methods

Collection of trematodes

Trematodes were recovered from two striped mullet from Kievka River, one from Razdolnaya River basin and two from Karasyk River, and five redlip mullet from each of three river sites. Fresh specimens of fish were dissected and the intestines removed for examination and identification of trematodes.

Worms from the fish species were rinsed in distilled water, identified to species, killed in boiling distilled water and preserved in 70% ethanol. Whole-mounts for adult descriptions were made by staining the specimens with alum carmine, dehydrating the worms in a graded ethanol series and clearing in xylene, followed by mounting in Canada balsam under a coverslip on a slide. To establish the time of development of the miracidia, the eggs, which were collected from mature worms, were placed in distilled water with the temperature at approximately 20°C. All measurements are given in micrometres (μm). Mean and standard deviation of coefficient of variation (CV) were calculated according to Steel & Torrier (1980).

Descriptions were based on 100 whole-mounts of *Pa. mugili*; 12 of *Parasaccocoelium haematocheilum* n. sp. and 34 of *Parasaccocoelium polyovum* n. sp. were studied.

Molecular and phylogenetic analyses

Adult *Parasaccocoelium* specimens ($n = 14$), including *Pa. mugili* ($n = 4$), *Pa. haematocheilum* n. sp. ($n = 7$) and *Pa. polyovum* n. sp. ($n = 5$), from the Kievka River, Primorsky Region, were used for molecular analysis (table 1). Total DNA was extracted from flukes, which were fixed in 96% ethanol, using a 'hot shot' technique (Truett, 2006).

Nuclear 28S rDNA was successfully amplified using polymerase chain reaction (PCR) with the following 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). The initial PCR reaction was performed in a total volume of 20 μl containing 0.25 mM of each primer pair, 1 μl of DNA in water, 1 × *Taq* buffer, 1.25 mM deoxynucleoside triphosphates (dNTPs), 1.5 mM magnesium and 1 unit of *Taq* polymerase. The amplification of a 1200-bp fragment of 28S rDNA was performed in a GeneAmp 9700 (Applied Biosystems, Grand Island, New York, USA), with a 3-min denaturation at 94°C, 40 cycles of 30 s at 94°C, 30 s at 52°C and 2 min at 72°C, and a 7-min extension at 72°C. Negative and positive controls using both primers were used. The PCR products were directly sequenced using an ABI Big Dye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems), as recommended by the manufacturer, with the internal sequencing primers of 300F, ECD2, 900F and 1200R (Tkach *et al.*, 2003). The PCR products were analysed using an ABI 3130 genetic analyser at the Institute of Biology and Soil Sciences, Far Eastern Branch, Russian Academy of Sciences. The sequences have been submitted to the European Nucleotide Archive (ENA), with the following accession numbers: HF548461–HF548478 (table 1). The ribosomal DNA sequences were assembled with SeqScape v. 2.6 software and aligned using MEGA 5.22 (Tamura *et al.*, 2011) alignment explorer with the default options. The regions that could not be unambiguously aligned were excluded from the analyses. Calculation of a number of variable and parsimony-informative sites and ancestral sequence test were performed using MEGA v. 5.22. Genetic divergence was estimated using genetic p-distance values, which were calculated including all substitution types. The phylogenetic analysis of the nucleotide sequences was performed using Bayesian algorithms with MrBayes v. 3.1.2 software (Huelsenbeck, 2000). The best nucleotide substitution model, general time reversible with estimates of invariant sites and gamma-distributed among-site variation (GTR + I + G), was estimated with Modeltest v. 3.7 software (Posada & Crandall, 1998). Bayesian analysis was performed using 10,000,000 generations and with four independent runs. Burn-in (sump and sumt) values were 10,000. The significance of the phylogenetic relationship was estimated by posterior probabilities (Huelsenbeck *et al.*, 2001). The test on ancestral sequence inferring was performed on the basis of an MP phylogenetic tree using a maximum parsimony algorithm with MEGA v. 5.22

Table 1. List of taxa incorporated in the molecular analysis of subfamilies of Haploporoidea Nicoll, 1914.

Species	*N*	Author	Accession numbers in the European Nucleotide Archive (ENA)
Haploporidae Nicoll, 1914			
Waretrematinae Srivastava, 1937			
Parasaccocoelium mugili	4	This study	HF548468–HF548471
Pa. haematocheilum n. sp.	7	This study	HF548461–HF548467
Pa. polyovum n. sp.	5	This study	HF548474–HF548478
Capitimitta costata	1	Pulis & Overstreet (2013)	KC206497
Capitimitta darwinensis	1	Pulis & Overstreet (2013)	KC206498
Intromugil alachuaensis	1	Pulis *et al.* (2013)	KC430095
Intromugil mugilicolus	1	Pulis *et al.* (2013)	KC430096
Spiritestis herveyensis	1	Pulis *et al.* (2013)	KC206500
Haploporinae Nicoll, 1914			
Saccocoelium brayi	1	Blasco-Costa *et al.* (2009)	FJ211234
S. cephalic	1	Blasco-Costa *et al.* (2009)	FJ211233
S. obesum	2	Blasco-Costa *et al.* (2009)	FJ211259–FJ211260
S. tensum	2	Blasco-Costa *et al.* (2009)	FJ211257–FJ211258
Dicrogaster contracta	2	Blasco-Costa *et al.* (2009)	FJ211261–FJ211262
D. perpusilla	1	Blasco-Costa *et al.* (2009)	FJ211238
Haploporus benedeni	1	Blasco-Costa *et al.* (2009)	FJ211237
Lecithobotrys putrescen	1	Blasco-Costa *et al.* (2009)	FJ211236
Chalcinotrematinae Overstreet & Curran, 2005			
Saccocoelioides sp.	1	Curran *et al.* (2006)	EF032696
Megasoleninae Manter, 1935			
Hapladena nasonis	1	Olson *et al.* (2003)	AY222265
Forticulcitinae Blasco-Costa, Balbuena, Kostadinova & Olson, 2009			
Forticulcita gibsoni	1	Blasco-Costa *et al.* (2009)	FJ211239
Atractotrematidae Yamaguti, 1939			
Pseudomegasolena ishiga	1	Olson *et al.* (2003)	AY222266
Atractotrema signai	1	Olson *et al.* (2003)	AY222267
Monorchidae Odhner, 1911			
Monorchis monorchis		Tkach *et al.* (2001)	AF184257

software. The phylogenetic relationships among the *Parasaccocoelium* species were inferred from our data and the nucleotide sequences of 28S rDNA from other trematode specimens obtained from the National Center for Biotechnology Information (NCBI) GenBank database (Tkach *et al.*, 2001; Olson *et al.*, 2003; Curran *et al.*, 2006; Blasco-Costa *et al.*, 2009; Pulis & Overstreet, 2013; Pulis *et al.*, 2013). The other sequences were from the Haploporidae and Atractotrematidae families. *Monorchis monorchis*, family Monorchidae, was used as an outgroup (table 1).

Family Haploporidae Nicoll, 1914; Subfamily Waretrematinae Srivastava, 1937; Genus *Parasaccocoelium* Zhukov, 1971

Diagnosis of the genus

Body oval, fusiform or retort-shaped; anterior end retractable or not, posterior retractable. Eye-spot pigment dispersed. Oral sucker subterminal. Ventral sucker larger than oral sucker placed on border of anterior and middle third of body, or in middle third of body. Prepharynx and the oesophagus variable in length. Pharynx transversally oval or spherical. Caeca short, sac-shaped. Testis single, variable in form: longitudinal, longitudinally oval, spherical, transversally oval, irregular, bilobed or V-shaped. Ovary and testis in end of middle or in posterior third of body. Hermaphroditic sac sacciform or oval. Posterior margin of sac does not cross level of posterior margin of ventral sucker. External seminal vesicle sac-shaped, club-shaped or tubular. Genital pore anterior to ventral sucker. Genital atrium absent. Ovary from large cells, pretesticular, medial. Uterus short, extending between posterior end of hermaphroditic sac and middle of testis. Eggs operculate, light yellow, embryonated when laid. Developed miracidium with eye spot. Vitellarium in lateral fields, from large compact follicles of irregular form, extends between anterior margin of ventral sucker or pharynx and posterior margin of testis. Vitelline follicles absent in post-testicular region. Excretory bladder Y-shaped. In mugilid fish species. Type species: *Parasaccocoelium mugili* Zhukov, 1971.

Parasaccocoelium mugili *Zhukov, 1971*

Species description

Synonyms. *Pseudohapladena mugili* (Zhukov, 1971) Overstreet et Curran, 2005; *Pseudohapladena lizae* Liu et Yang, 2002.

Type host. Redlip mullet *Liza haematocheila*.

Other host. Striped mullet *Mugil cephalus*.

Site. Intestine.

Intensity of infection. In redlip mullets, from 1 to 254; in striped mullets, up to 10 worms per fish.

Localities. Razdolnaya River basin (43°20′N, 131°47′E), Karasyk River (42°32′N, 130°38′E), Kievka River (42°52′N, 133°39′E). Flukes were found in both fish species in each river.

Material examined. 100 specimens.

Adult worm (fig. 1). Body pyriform when posterior end retracted, as typical in life, or fusiform. Small spines on tegument, except in last sixth of body length, can be lost during fixation and staining. Eye-spot pigment dispersed in anterior third of body. Ventral sucker larger than oral sucker, about one-third body length from anterior end. Prepharynx usually conspicuous, sometimes almost overlapping anterior edge of ventral sucker, or else very short. Pharynx spherical or transversally oval. Oesophagus short. Caeca sac-shaped, terminating near or slightly posterior to posterior margin of ventral sucker. Testis single, oval, spherical or irregular in shape. Hermaphroditic sac (fig. 1d) dorsal to ventral sucker and not extending beyond posterior margin of sucker, oval in dorsal or ventral view, but sac-shaped in lateral view, with an internal seminal vesicle, the duct of which extends into the proximal part of the hermaphroditic canal and prostate cells occupy the length of this canal. Hermaphroditic canal with muscular walls. External seminal vesicle sac-shaped, somewhat variable in volume and position. Genital pore with muscular sphincter, median, immediately anterior to ventral sucker. Ovary round or oval, somewhat variable in position but usually overlapping testis, median or submedian. Uterus short, between hermaphroditic sac and anterior margin of testis, contains single large, light-yellow, operculate egg with curved knob at aboperculate pole (fig. 1c) and with 1–2

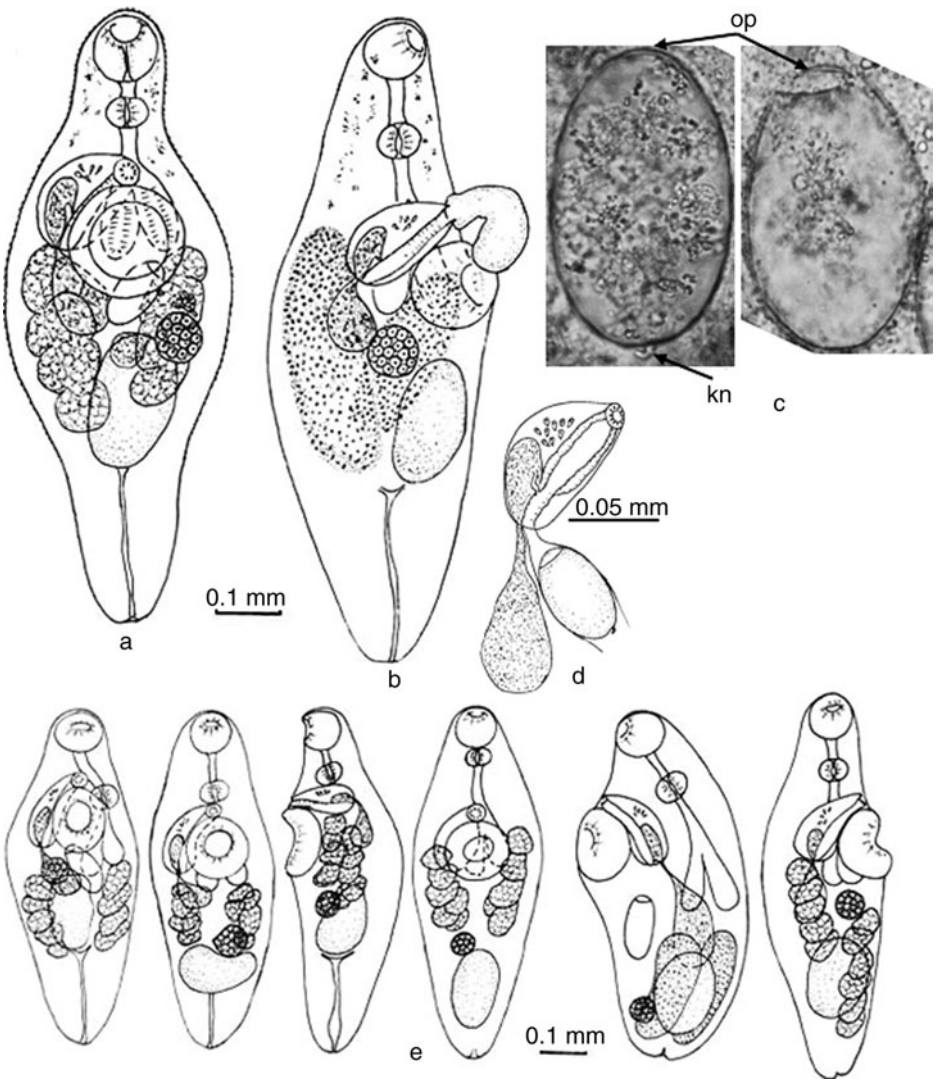

Fig. 1. *Parasaccocoelium mugili* Zhukov, 1971: (a) ventral view; (b) lateral view; (c) eggs with operculum (op) and knob (kn); (d) hermaphroditic sac; (e) variations in the position of organs.

embryonic and vitelline cells. Uterine seminal receptacle present. Metraterm short, with fine walls. Mehlis' gland consists of 6–8 cells. Vitellarium between anterior margin of ventral sucker and posterior margin of testis, or anterior margin of testis in some cases, from compact groups of six large, variably shaped follicles on each side of body, sometimes coalescing in larger worms and often appearing as homogeneous masses in whole mounts (fig. 1b). Lateral groups of vitelline cells never confluent. Excretory bladder Y-shaped.

Miracidium. Hatches after 14 days in distilled water and possesses an eye spot.

28S rRNA gene sequence data

Ribosomal 28S rRNA gene fragment of approximately 1200 bp was obtained for species of *Parasaccocoelium.* Six specimens of *Pa. mugili* were analysed. Partial 28S rDNA fragments of 1143 bp were identical from each of six *P. mugili* specimens.

Remarks

Zhukov (1971) described a new digenean species from the family Haploporidae (Waretrematinae) isolated from the intestines of red mullets that were caught in the Sea of Japan and the Yellow Sea, and erected the new genus *Parasaccocoelium* with a single species, *Pa. mugili.*

Overstreet & Curran (2005) revised the family Haploporidae and transferred *Parasaccocoelium* to a junior synonym of the genus *Pseudohapladena*. In this genus, in addition to *Ps. mugili*, they included *Saccocoelioides martini* Madchavi, 1980, *S. pearsoni* Martin, 1973 and *S. huidongensis* Lu, 1993. These species were transferred to *Pseudohapladena* because they have a prepharynx that is longer than the pharynx, short caeca, an ovary and testis that are close to each other and lie near the ends of the caeca, and elongated vitelline fields.

As proposed by Overstreet & Curran (2005), the genus *Pseudohapladena* includes seven species: the type species *Ps. scatophagi* Yamaguti, 1952 and *Ps. mugili* (Zhukov, 1971), *Ps. pearsoni* (Martin, 1973), *Ps. martini* (Madhavi, 1979), *Ps. huidongensis* (Lu, 1993), *Ps. megaorchis* Liu et Yang, 2002 and *Ps. lizae* Liu et Yang, 2002.

Overstreet & Curran (2005) changed the diagnostic features of this genus to include the following characteristics: (1) body fusiform to elongate (previously body small, elongate, covered with spines); (2) eye-spot pigment dispersed (previously eye spot absent); (3) oral sucker terminal or subterminal (previously oral sucker terminal, with subterminal aperture); (4) testis subspherical to irregular to elongate, near the middle or posterior portion of the hindbody (previously testis single, median, near posterior extremity); (5) seminal receptacle canalicular (previously seminal receptacle absent); and (6) vitellarium with elongate groups of follicles that usually extend at least one-half of a body length, extending to the posterior end of the body in some and to the posterior margin of the testis in others (previously vitellarium tubular, divided into two groups). Yamaguti (1952) described a genital atrium in the genus *Pseudohapladena*, but Overstreet & Curran (2005) did not mention this characteristic. However, Overstreet & Curran (2005) did indicate that eggs are operculate, at least in some species, and that miracidia lack eye spots.

We believe that a revision of the *Pseudohapladena* complex, which includes genera *Pseudohapladena*, *Skrjabinolecithum* and *Platydidymus,* is necessary. In many cases, to define the systematic status of a genus or species, Overstreet & Curran (2005) used variable features, such as the length of the prepharynx, the ratio of the prepharynx to pharynx, forms of the caeca and the hermaphroditic sac, insignificant differences in the length of the caeca, and the position of the ovary and testis relative to the end of the caeca. We noted great variation in the features of *Pa. mugili* (fig. 1e) and in the other species described herein.

The most stable features of *Pa. mugili* and other species include the lateral position of the vitellaria in the mid and posterior third of the body, with no vitelline connection behind the testis and the presence of a short uterus containing a small number of eggs. The type species of *Pseudohapladena*, *Ps. scatophagi*, and other members of this genus, such as *Ps. martini* and *Ps. megaorchis*, possess vitelline fields that join behind the testis and extend to the posterior end of the body (Zhukov, 1971; Martin, 1973; Madhavi, 1979; Liu & Yang, 2002). Thus, this species and *Pa. mugili* cannot be combined within a single genus. Additionally, *Ps. scatophagi* possesses a long uterus with many eggs. *Pseudohapladena pearsoni*, described by Martin (1973), is atypical of the genus in that vitellaria, apart from containing only a few follicles, extend from the hermaphroditic sac to the posterior end of the testis, whereas the uterus is short with only a small number of eggs. However, because of differences in the position of the vitellaria, *Ps. pearsoni* has not been transferred to the genus *Parasaccocoelium*.

It is difficult to distinguish *Ps. lizae*, described by Liu & Yang (2002) from *Liza carinatus* (Cuvier et Valenciennes) from *Pa. mugili*. Specimens of both species are similar in size and morphology, including body shape, structure and position of hermaphroditic sac, uterus, vitellarium, testis and ovary (table 2). Therefore, we consider *Ps. lizae* to be a junior synonym of *Pa. mugili*.

We consider that *Parasaccocoelium* and *Pseudohapladena sensu* Yamaguti (1952) differ in having (1) dispersed or absent eye-spot pigment; (2) a subterminal or terminal oral sucker; (3) absent or present genital atrium; and (4) a vitellarium in the form of two clusters of large, compact vitelline masses or a tubular vitellarium that is divided into two groups. In addition to these criteria, Yamaguti (1952) noted in a description of the type species *Ps. scatophagi* that vitelline follicles occupy almost the entire post-testicular region; but vitelline follicles do not occupy this zone in specimens of *Parasaccocoelium*. From this diagnosis of *Pseudohapladena*, Overstreet & Curran (2005) accordingly proposed that the genus *Parasaccocoelium* differs in these features: (1) a single testis that is longitudinal, longitudinally oval, spherical, transversally oval, irregular, bilobed or V-shaped, or a subspherical testis that is irregular to elongate; (2) vitellarium in lateral fields formed from large compact follicles with an irregular form that extend between the anterior margin of the ventral sucker or the pharynx and posterior margin of testis, and possesses vitelline follicles that do not occupy the post-testicular region; or a vitellarium that

Table 2. Comparative morphometrics (μm) of *Parasaccocoelium mugili* Zhukov, 1971 with *Pseudohapladena lizae* Liu & Yang, 2002, to show ranges, means and coefficient of variation (CV), with first values for length and second values for width; n = number of specimens examined.

	Parasaccocoelium mugili				*Pseudohapladena lizae* Liu &Yang (2002)	
	Present study ($n = 15$)			Zhukov (1971)		
Features	Mean	CV	Range	Range	Range	Mean
Body	756 × 309	16.7; 15.9	590–1020 × 220–430	470–940 × 140–460	616–1116 × 224–360	809 × 292
Body length/width ratio	1:2.45	–	1:2.0–2.95	–	–	–
Oral sucker	89 × 86	10.7; 21.8	70–154 × 61–110	62–120 × 71–83	60–112 × 94–124	88 × 103
From anterior end of body to ventral sucker	230	27.8	120–358	–	–	–
Ventral sucker	145 × 132	14.3; 21.6	110–189 × 110–185	62–200 × 92–120	74–150 × 78–162	106 × 115
Sucker length ratio	1:0.60	–	1:0.45–0.82	–	1:0.7–1:0.96	1:0.78
Sucker width ratio	1:0.72	–	1:0.54–0.98	–		
Length of prepharynx	45	71.0	12–89	–	10–50	29
Pharynx	47 × 62	17.0; 10.5	31–58 × 53–81	37–71 × 41–66	36–58 × 38–70	50 × 53
Length of oesophagus	50	25.4	39–69	46–102	100–290	173
Length of caeca	145	23.4	85–177	66–96	64–126	96
Ovary	62 × 61	21.9; 14.3	39–85 × 50–78	46–140 × 33–130	60–114 × 44–112	86 × 65
Testis	153 × 122	25.8; 26.2	100–231 × 90–200	150–230 × 48–100	166–268 × 90–188	216 × 131
Hermaphroditic sac	177 × 92	19.7; 32.6	120–239 × 56–170	87–160 × 54–96	106–172 × 74–152	132 × 93
Vitelline fields	300 × 121	37.1; 20.9	123–439 × 77–173	188–274 × 58–104	–	–
Distance from anterior end of body to ovary	409	17.2	300–523	–	–	–
Distance from anterior end of body to vitellarium	308	38	210–400	–	–	–
Distance from posterior end of body to vitellarium	196	31	110–273	–	–	–
Distance from posterior end of body to testis	168	42.7	72–296	–	–	–
Eggs	92 × 59	9.7; 9.5	89–103 × 54–69	87–100 × 41–62	64–82 × 42–64	74 × 51

consists of elongate groups of follicles that usually extend at least one-half of a body length to the posterior end of the body in some and to the posterior margin of the testis in others; (3) eggs operculate, unembryonated, developed when laid, or eggs which, in some cases, are only operculate; and (4) a developed miracidium with or without an eye spot.

In the light of these differences, we restore the genus *Parasaccocoelium* with the type species *Pa. mugili* Zhukov, 1971.

Parasaccocoelium haematocheilum *n. sp.*

Species description

Type host. Redlip mullet *Liza haematocheila*.

Site. Intestine.

Intensity of infection. 1–10 worms per fish.

Type locality. Razdolnaya River basin, 43°20′N, 131°47′E.

Type deposition. Holotype No. 51-Tr, paratypes No. 52-Tr. 63-Tr. This material is held in the collection of the Zoological Museum, Institute of Biology and Soil Sciences, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia (e-mail: petrova@ibss.dvo.ru). Deposited 29 July 2010.

Etymology. The specific name refers to the specific name of its definitive host.

Material examined. 12 specimens.

Adult worm (fig. 2a–c). Body oval. Tegumental spines only before opening of oral sucker and around opening of ventral sucker. Pigmented eye spots mainly in anterior end of body and small pigmented spots occur in the middle third of body. Oral sucker subterminal. Ventral sucker larger than oral sucker, about one-third body length of anterior end or in middle third of body. Prepharynx short. Pharynx longitudinally oval. Oesophagus short. Caeca sac-shaped, terminating near posterior margin of ventral sucker. Ovary, testis and vitellarium usually between middle and beginning posterior third of body. Testis longitudinally or transversally elongate, from two equal or unequal lobes (holotype with lobes 69×77 and 31×39). Hermaphroditic sac (fig. 2c) oval in dorsal or ventral view but sac-shaped

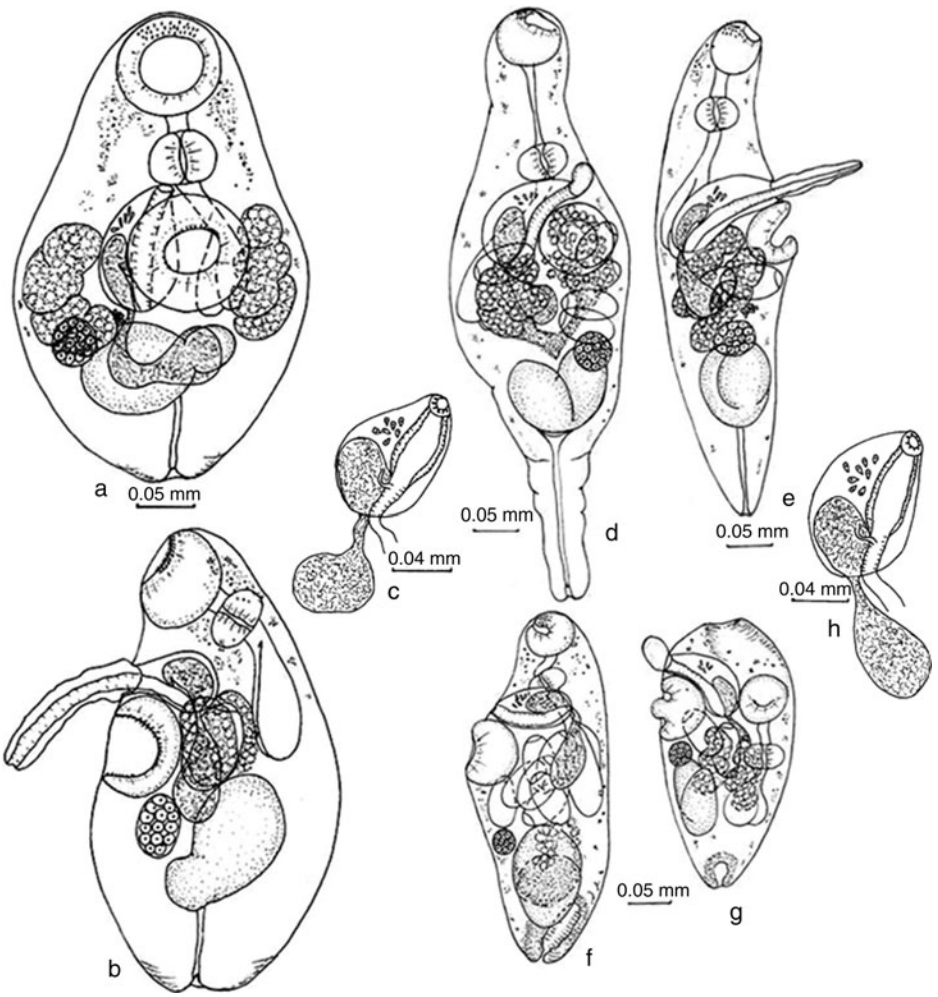

Fig. 2. *Parasaccocoelium haematocheilum* n. sp.: (a) ventral view; (b) lateral view; (c) hermaphroditic sac. *Parasaccocoelium polyovum* n. sp.: (d) ventral view; (e) lateral view; (f) posterior extremity retracted; (g) anterior extremity retracted; (h) hermaphroditic sac.

in lateral view, dorsally from ventral sucker. Posterior margin of hermaphroditic sac does not reach level of posterior margin of ventral sucker. Hermaphroditic sac contains internal seminal vesicle (duct of vesicle occupies the beginning of hermaphroditic canal) and some prostatic cells along hermaphroditic canal. Hermaphroditic canal with muscular walls. External seminal vesicle sac-shaped, reaches testis or most often lies on level of ovary and testis. Genital pore with muscular sphincter, on midline of body immediately anterior to ventral sucker. Ovary round or oval, joined to testis or some distance from it on midline of body or to right on level of testis. Uterus short, bounded by hermaphroditic sac and anterior border of testis, contains 1–4 oval, light yellow, operculate eggs with curved knob at aboperculate pole, and with 1–2 embryonic and vitelline cells. Uterine seminal receptacle present. Metraterm short, with fine walls. Mehlis' gland present. Vitellarium in two lateral fields between anterior margin of ventral sucker and posterior margin of testis, and partly covers ovary and testis, forming 4–5 compact follicles each. Lateral groups of vitelline cells never confluent. Excretory bladder Y-shaped.

Miracidium. Hatches after 14 days in distilled water and possesses an eye spot.

28S rRNA gene sequence data

Partial 28S rDNA fragments of 1143 bp were identical from each of seven specimens of *Pa. haematocheilum* n. sp.

Remarks

Morphological features of this species are found in accordance with the generic diagnosis. Compared with the type species *Pa. mugili*, they are smaller in almost all dimensions (tables 2, 3), such as the bilobed testis and eggs in the uterus (1–4 versus only 1 in *Pa. mugili*). Molecular data also confirm an independence of described species. The 1143-bp 28S rDNA fragment of *Pa. haematocheilum* n. sp. and *Pa. mugili* contained eight variable sites. Genetic differentiation between 28S rDNA partial sequences of *Pa. haematocheilum* n. sp. and *Pa. mugili* was 0.73%.

Parasaccocoelium polyovum *n. sp.*

Species description

Type host. Redlip mullet *Liza haematocheila*.

Other host. Striped mullet *Mugil cephalus*.

Site. Intestine.

Intensity of infection. 21–104 worms per redlip mullet, 18 in striped mullet.

Type locality. Razdolnaya River Basin, 43°20′N, 131°47′E (only redlip mullet was infected).

Other locality. Kievka River, 42°52′N, 133°39′E (both fish species were infected).

Type deposition. Holotype No. 71-Tr, paratypes No. 72-Tr.–79-Tr. This material is held in the collection of the Zoological Museum, Institute of Biology and Soil Sciences, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia (e-mail: petrova@ibss.dvo.ru). Deposited: 29 July 2010.

Etymology. The specific name refers to the fact that these worms have the greatest number of eggs in the uterus among all described species of this genus.

Material examined. 34 specimens.

Adult worm (fig. 2d–h). Body fusiform or oval with retractable anterior and posterior ends, spined only on anterior end of body. Eye-spot pigment dispersed over entire body surface. Oral sucker subterminal. Prepharynx usually well developed. Pharynx transversally oval, oesophagus short, sac-shaped caeca terminate near posterior margin of ventral sucker. Ventral sucker equal to or larger than oral sucker and lies on border of anterior and middle third of body, or in beginning of middle third. Hermaphroditic sac (fig. 2h) sac-shaped, dorsally from ventral sucker. Ovary, testis and vitellarium between middle and beginning posterior third of body and the testis is V-shaped. Posterior margin of hermaphroditic sac does not cross level of posterior margin of ventral sucker. Hermaphroditic sac contains internal seminal vesicle (duct of vesicle occupies the beginning of hermaphroditic canal) and some prostatic cells along hermaphroditic canal. Hermaphroditic canal with muscular walls. External seminal vesicle, depending on number of genital products, can extended to the ovary and testis and can be tubular or sac-shaped. Genital pore with muscular sphincter, immediately anterior to ventral sucker. Ovary round or oval and level with, or situated to the left of, the anterior margin of the testis. Uterus short and, when containing up to 7 eggs, is located from hermaphroditic sac up to anterior margin of the testis or, with up to 12 eggs, to the mid-region of the testis. Uterine seminal receptacle present. Metraterm short, with fine walls. Eggs light yellow, oval, operculate, with curved knob at abopercular pole, at various stages of embryogeny. Vitellarium in two lateral fields formed from compact follicles of various forms, extending between anterior margin of ventral sucker and middle of testis, and can partly cover ovary and testis. Lateral groups of vitelline cells never confluent. Excretory bladder Y-shaped.

Miracidium. Hatches after 5 days in distilled water and possesses an eye spot.

28S rRNA gene sequence data

Partial 28S rDNA fragments of 1139 bp were identical from each of five specimens of *P. polyovum* n. sp. The shorter length of the investigated DNA fragment was due to a four-nucleotide deletion in the 28S rDNA sequences of *Pa. polyovum* n. sp. between base-pair positions 407 and 410 in comparison with the sequences of *Pa. mugili* and *Pa. haematocheilum* n. sp.

Remarks

The described species belongs to the genus *Parasaccocoelium* but differs from others by a suite of features. Compared with *Pa. mugili*, specimens of *Pa. polyovum* n. sp. differ by the smaller maximum and mean dimensions of the body and most organs (suckers, pharynx, ovary and testis), and by the smaller eggs (tables 2 and 3). Their dimensions more closely resemble those of *Pa. haematocheilum* n. sp. than *Pa. mugili*. Adult *Pa. polyovum* n. sp. have greater maximum and mean sizes of the length of the body and hermaphroditic sac, smaller

Table 3. Comparative morphometrics (μm) of *Parasaccocoelium haematocheilum* and *Pa. polyovum*, to show ranges, means and coefficient of variation (CV), with first values for length and second values for width; n = number of specimens examined.

	Parasaccocoelium haematocheilum n. sp. ($n = 12$)				*Parasaccocoelium polyovum* n. sp. ($n = 10$)			
Features	Holotype	Mean	CV	Range	Holotype	Mean	CV	Range
Body	393 × 250	384 × 199	13.0; 16.0	308–466 × 158–227	677 × 215	521 × 168	27.4; 24.0	327–832 × 146–262
Body length/body width	1.58:1	1.95:1	–	1.57–2.31:1	3.15:1			
Oral sucker	85 × 85	76 × 63	17.9; 22.0	54–96 × 50–85	62 × 73	59 × 59	12.4; 10.5	50–77 × 60–85
Distance from anterior end of body to ventral sucker	154	141	14.9	119–154	219	168	26.8	123–269
Ventral sucker	96 × 96	94 × 96	3.9; 3.8	89–100 × 92–100	85 × 85	70 × 73	18.0; 10.5	58–92 × 58–92
Sucker length ratio	1:0.88	1:0.81	–	1:0.59–1.0	1:0.73	1:0.84	–	1:0.73–1.20
Sucker width ratio	1: 0.88	1:0.69	–	1:0.50–0.88	1:0.86	1:0.81	–	1:0.74–1.06
Length of prepharynx	12	15	64.6	6–31	92	46	57.0	19–92
Pharynx	50 × 58	40 × 57	11.8; 10.5	35–50 × 46–62	39 × 58	37 × 48	13.5; 13.5	27–42 × 39–58
Length of oesophagus	51	35	51.0	11–52	39	36	21.9	27–46
Length of caeca	65	71	23.5	52–100	135	101	19,4	77-135
Ovary	39 × 35	48 × 42	33.3; 18.0	23–73 × 35–58	42 × 39	36 × 39	22.2; 25.4	23–50 × 27–58
Testis	69 × 116	99 × 84	27.0; 37.0	69–142 × 54–135	96 × 108	125 × 82	32.5; 27.0	85–223 × 58–123
Hermaphroditic sac	100 × 65	101 × 68	20.4; 15.9	77–131 × 54–85	116 × 77	119 × 70	21.5; 21.4	96–173 × 50–96
Vitelline fields	116–123 × 69	158 × 52	11.2; 15.0	135–177 × 58–77	104–108 × 77–92	123 × 72	13.0; 21.5	104–146 × 50–96
Distance from anterior end of body to ovary	250	232	12.9	193–281	385	304	16.0	273–338
Distance from anterior end of body to vitellarium	166	160	22.0	116–227	216	220	11.8	193–266
Distance from posterior end of body to vitellarium	104	122	19.3	89–162	304	220	39.5	112–404
Distance from posterior end of body to testis	65	71	7.7	65–77	193	126	74.0	42–339
Eggs	–	85 × 49	8.0; 14.4	73–92 × 42–58	–	77 × 45	9.4; 9.8	61–85 × 39–50

Table 4. Ancestral sequences for species of the genus *Parasaccocoelium* with the probability of inference > 90%.

	Species	
Nucleotide position	*P. mugili*, *P. haematocheilum* n. sp.	*P. polyovum* n. sp.
407	T	T → X
408	C	C → X
409	G	G → X
410	G	G → X

maximum and mean sizes of the oral sucker, and a vitelline field that is shorter and wider than that of *Pa. haematocheilum*. In addition, *Pa. polyovum* n. sp. differs from these species by the V-shaped testis, the presence in the uterus of up to 12 eggs and the presence of pigment spots over the entire surface of the body. Molecular data support the validity of *P. polyovum* n. sp. The 1139 bp 28S rDNA fragment of *Pa. polyovum* n. sp. and 1143 bp fragment of *Pa. mugili* contained 34 variable sites, which were also parsimony-informative. 28S rDNA fragments of *Pa. polyovum* n. sp. and *Pa. haematocheilum* n. sp. contained 37 variable and parsimony-informative sites. Genetic differentiation between 28S rDNA partial sequences of *Pa. polyovum* n. sp. and *Pa. mugili* was 2.8%, and between sequences of *Pa. polyovum* n. sp. and *Pa. haematocheilum* n. sp. it was 3.1%.

Discussion

We attribute three species – *Pa. mugili* Zhukov, 1971 and two newly described species, *Pa. haematocheilum* n. sp. and *Pa. polyovum* n. sp. – to the genus *Parasaccocoelium.* Studies of the morphology of mature worms of this genus showed that certain parameters, such as the length of the prepharynx and oesophagus, the ratios of prepharynx to pharynx lengths and oesophagus to pharynx lengths, the form and size of the caeca, the seminal vesicles, the ovary, the testis, the relative position of the ovary and testis, and the relative position of the testis and ventral sucker, are very variable. The most stable characteristics of this genus are the structure of the vitellarium, which includes large compact and irregular follicles, the position of the vitellarium in the posterior part of the body up to the level of the posterior margin of the testes and no connection between the vitelline fields behind the testes, the position of the posterior margin of the hermaphroditic sac not far from the posterior margin of the ventral sucker, and a short uterus between the posterior margin of the

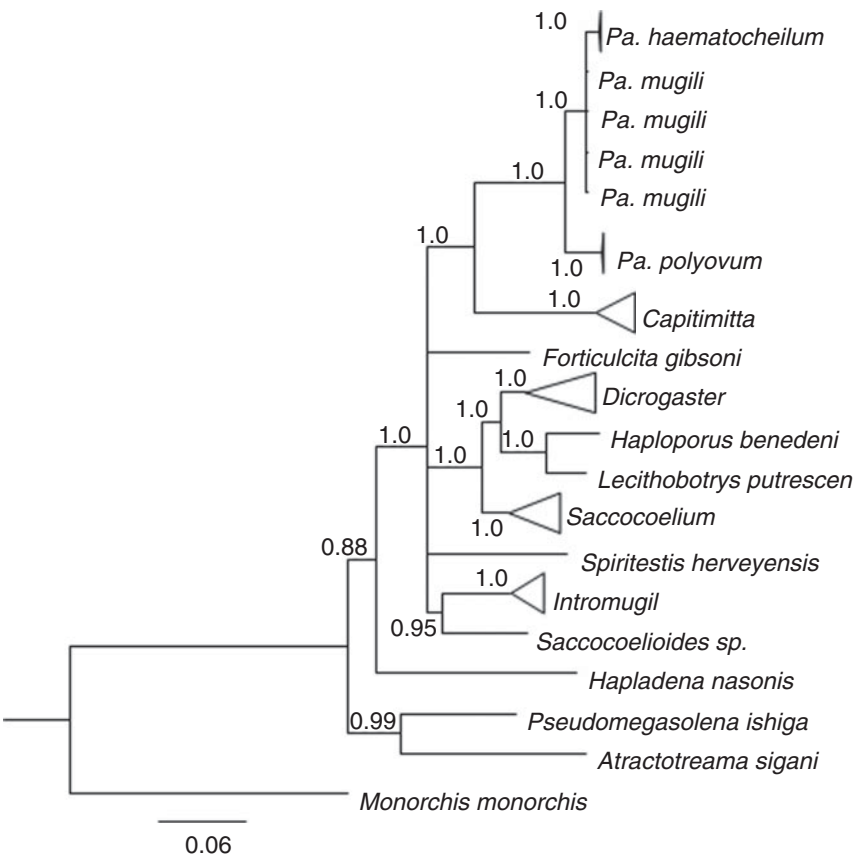

Fig. 3. Phylogenetic tree of different genera of the family Haploporidae based on the analysis of partial sequences of 28S rDNA using the Bayesian algorithm. Nodal numbers indicate posterior probabilities.

hermaphroditic sac and the middle of the testis. Eggs are operculate and possess a curved knob. Miracidia, each with an eye spot, develop only in water and not *in utero*.

Our molecular results supported the species designation for the specimens under investigation. The minimum p-distance values were obtained between *Pa. mugili* and *Pa. haematocheilum* n. sp., which were also considerably differentiated from each other by morphometrical data. Based on the interspecific variation of 28S rDNA sequences for haploporid trematodes, earlier estimates ranged from 0.9 to 4.6% for the genus *Saccocoelium* (Blasco-Costa *et al.*, 2009) and from 2.7 to 2.8% for the genera *Capitimitta* (Pulis *et al.*, 2013) and *Intromugil* (Pulis & Overstreet, 2013). Genetic differentiation between species of the genus *Parasaccocoelium* from our study ranged from 0.73 to 3.11%. These species have considerable morphological differences. We argue that such interspecific ranges of genetic differences in the genus *Parasaccocoelium* can be accepted, whereas intrageneric molecular data are lacking for the family Haploporidae. The 28S rDNA sequences of *Pa. polyovum* species are characterized by a four-nucleotide deletion between the positions 407 and 410, which we considered to be possible apomorphy (table 4). This conclusion is based on the results of the inferred ancestral sequences test using a maximum parsimony algorithm to re-construct a phylogenetic tree for the species of *Parasaccocoelium*. The results were statistically significant (probability of inference >90%) and showed that the four nucleotides between the 407 and 410 positions of the 28S rDNA fragment were deleted (table 4). This species, therefore, can be considered to be the younger species. Bayesian analysis of partial 28S rDNA sequences showed that species of *Parasaccocoelium* formed a monophyletic cluster (fig. 3). *Parasaccocoelium haematocheilum* n. sp. was closely related to *Pa. mugili*, and *Pa. polyovum* n. sp. appeared as a sister species to the cluster containing *Pa. mugili* and *Pa. haematocheilum* with 3.2% sequence differentiation. Species of the genus *Parasaccocoelium* clustered with species of genus *Capitimitta* (subfamily Waretrematinae). The results presented here support the taxonomical status of the genus *Parasaccocoelium* and the inclusion of this genus in the subfamily Waretrematinae.

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Conflict of interest

None.

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