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A new subfamily, Pseudohaploporinae subfam. n. (Digenea: Haploporidae), with morphometric and molecular analyses of two new species: *Pseudohaploporus vietnamensis* n. g., sp. n. and *Pseudohaploporus planilizum* n. g., sp. n. from Vietnamese mullet

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ABSTRACT

Adult specimens representing *Pseudohaploporus vietnamensis* n. g., sp. n., *Pseudohaploporus planilizum* n. g., sp. n., and *Pseudohaploporus* sp. were collected from the intestines of *Osteomugil engeli* (Bleeker, 1858), *Moolgarda seheli* (Forsskål, 1775), and *Planiliza subviridis* (Valenciennes, 1836) from Vietnam. The new genus *Pseudohaploporus* n. g. is similar to the genus *Haploporus* Looss, 1902 in morphology and host-specificity. Species of *Pseudohaploporus* and *Haploporus* both possess an elongated body, a long oesophagus, an intestinal bifurcation behind the ventral sucker, a large hermaphroditic bursa, vitellarium consisting of two compact accumulations, concretions in the excretory vesicle, and numerous small eggs that contain miracidia with a pigment eye, and both infect mullet. In contrast to *Haploporus*, both species of the genus *Pseudohaploporus* possess two testes. These species differ from each other morphometrically. *Pseudohaploporus planilizum* sp. n., in comparison with *P. vietnamensis* sp. n., possess a larger maximum and mean size for the body length, oral and ventral suckers, pharynx, oesophagus length, hermaphrodite bursa, and egg width. Molecular data (ITS and 28S rDNA sequences) support the validity of the new genus and the two new species and indicate the presence of another representative of this genus, *Pseudohaploporus* sp. Phylogenetic reconstructions show that *Pseudohaploporus* formed a well-supported clade within a large clade that represents the Haploporidae Nicoll, 1914. Based on morphological and molecular data for *P. vietnamensis* sp. n. and *P. planilizum* sp. n., we erected a new subfamily, Pseudohaploporinae subfam. n., which appears to be a sister taxon to Haploporinae Nicoll, 1914. Molecular data show that *Pseudohaploporus* differ from other haploporids at a level similar to that between other subfamilies.

1. Introduction

The Haploporidae Nicoll, 1914 includes five subfamilies: Haploporinae Nicoll, 1914; Waretrematinae Srivastava, 1937; Forticulcitinae Blasco-Costa, Balbuena, Kostadinova & Olson, 2009; Chalcinotrematinae Overstreet & Curran, 2005; and Megasoleninae Manter, 1935. Members of this family parasitise the intestine of marine, estuarine, and freshwater fish species [1,2]. Among these, representatives of Haploporinae and Waretrematinae have been detected in mullet hosts (Mugilidae Jarocki, 1822) in the Western Pacific region, including mullet of Vietnam and the south of the Russian Far East [3–6]. In the present study, morphological and molecular data for two new trematode species within the Haploporidae from Vietnamese mullet are presented.

2. Materials and methods

2.1. Collection of trematodes

Adult worms were collected from the intestines of mullet (Mugilidae) from the coastal waters near Cat Ba Island, Halong Bay, Vietnam (20°84′N, 106°59′E). Worms from the fish, previously identified under a microscope, were rinsed in saline, killed in hot distilled water, and preserved in 70% ethanol. Following fixation, the worms were 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 xylene. The xylene treatment was followed by mounting the specimens in Canada balsam under a coverslip on a glass slide.

All measurements are given in micrometers (μm).

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Table 1
List of taxa used for molecular analysis.

Species	N (28S/ ITS)	Definitive host	Authors	Accession number in the NCBI	
				28S	ITS1-5.8S-ITS2
Haploporoidae					
Pseudohaploporinae subfam. n.					
Pseudohaploporus vietnamensis sp.n.	6/8	*Osteomugil engeli*	Present study	MF774420- MF774421, MF774423- MF774426	MF774427- MF774429; MF774436- MF774440
Pseudohaploporus vietnamensis sp.n.	1/1	*Moolgarda seheli*	Present study	MF774422	MF774431
Pseudohaploporus planilizum sp.n.	3/3	*Planiliza subviridis*	Present study	MF774417- MF774419	MF774433- MF774435
Pseudohaploporus sp.	2/2	*Moolgarda seheli*	Present study	MH986037, MH986038	MF774430, MF774432
Haploporinae					
Saccocoelium brayi	1/1	*Liza saliens*	[2]	FJ211234	FJ211244
S. cephali	1/1	*Mugil cephalus*	[2]	FJ211233	FJ211243
S. obesum	2/2	*Liza ramada*	[2]	FJ211259- FJ211260	FJ211265- FJ211266
S. tensum	2/2	*Liza ramada*	[2]	FJ211257- FJ211258	FJ211263- FJ211264
Dicrogaster contracta	1/1	*Liza aurata*	[2]	FJ211261	FJ211267
D. perpusilla	1/1	*Liza ramada*	[2]	FJ211238	FJ211248
Lecithobotrys putrescen	1/1	*Liza saliens*	[2]	FJ211236	FJ211246
Litosaccus_brisbanensis	1/1	*Mugil cephalus*	[19]	KM253765	
Haploporus benedeni	1/1	*Liza ramado*	[2]	FJ211237	FJ211247
Ragaia lizae	1/1	*Liza aurata*	[2]	FJ211235	FJ211245
Waretremadinae					
S. pyriforme	1/1	*Liza haematocheila*	[6]	HE806359	LN864990
S. spasskii	1/1	*Liza haematocheila*	[3]	LN614538	LK022754
Parasaccocoelium mugili	1/1	*Liza haematocheila*	Present study*	MG098882	MG098884
Spiritestis herveyensis	1/1	*Moolgarda seheli*	[14]	KC206500	
Capitimitta costata	1/1	*Selenotoca multifasciata*	[14]	KC206497	
Capitimitta darwinensis	1/1	*Selenotoca multifasciata*	[14]	KC206498	
Forticulcitinae					
Forticulcita gibsoni	1/1	*Mugil cephalus*	[2]	FJ211239	FJ211249
F. apiensis	1/1	*Mugil cephalus*	[15]	KP761087	
F. platana	1/1	*Mugil liza*	[15]	KP761086	
Xiha fastigata	1/1	*Mugil cephalus*	[15]	KP761088	
Chalcinotrematinae					
Saccocoelioides sp.	1/1	Unidentified molly (Poecilidae)	[17]	EF032696	–
Saccocoelioides beauforti	1/1	*Mugil cephalus*	[18]	MG925104	MG925103
Saccocoelioides elongatus	1/1	*Prochilodus lineatus*	[18]	MG925108	MG925107
Saccocoelioides magnus	1/1	*Cyphocharax voga*	[18]	MG925112	MG925111
Saccocoelioides nanii	1/1	*Prochilodus lineatus*	[18]	MG925114	MG925113
Saccocoelioides orosiensis	1/1	*Poecilia gillii*	[18]	MG925118	MG925117
Saccocoelioides tkachi	1/1	*Astyanax aeneus*	[18]	MG925122	MG925121
Intromugil mugilicolus	1/1	*Mugil cephalus*	[13]	KC430096	
Intromugil alachuaensis	1/1	*Mugil cephalus*	[13]	KC430095	
Hapladeninae					
Hapladena acanthuri	1/1	*Acanthurus chirurgus*	[20]	MH244119	
Hapladena cf. *varia*	1/1	*Acanthurus chirurgus*	[20]	MH244120	
Megasoleninae					
Megasolena hysterospina	1/1	*Archosargus rhomboidalis*	[20]	MH244121	
Megasolena sp. m MA-2018	1/1	*Holacanthus ciliaris*	[20]	MH244122	
Cadenatelinae					
Cadenatella americana	1/1	*Kyphosus sectatrix*	[20]	MH244117	
Cadenatella floridae	1/1	*Kyphosus incisor*	[20]	MH244118	
Atractotrematidae					
Isorchis anomalus	1/1	*Chanos chanos*	[21]	KU873018	
Isorchis currani	1/1	*Selenotoca multifasciata*	[21]	KU873017	
Isorchis megas	1/1	*Selenotoca multifasciata*	[21]	KU873015	
Plagiorchiidae					
Brachycladium goliath	1/1	*Balaenoptera acutorostrata*	[22]	KR703279	
Paragonimidae					
Paragonimus westermani	1/1	unknown	Narain et al., 2006, direct submission	DQ836244	DQ836243
Monorchiidae					
Hurleytrematoides chaetodoni	1/1	*Chaetodon striatus*	[20]	MH244116	

n - number of sequences, * - Ribosomal ITS1–5.8S-ITS2 and 28S were sequences for new specimens of *P. mugili* to include this species into phylogenetic analysis based on combined data.

2.2. DNA extraction, amplification, and sequencing

Sixteen adult worms collected from *Osteomugil engeli* (Bleeker, 1858), *Moolgarda seheli* (Forsskål, 1775), and *Planiliza subviridis* (Valenciennes, 1836) were fixed in 96% ethanol and used for molecular analysis (Table 1). Total DNA was extracted from flukes using a hot-SHOT technique [7].

28S ribosomal DNA (rDNA) was amplified by polymerase chain reaction (PCR) using the Q5 HF polymerase (New England Biolabs, UK) and the primers DIG12 (5′-AAG CAT ATC ACT AAG CGG-3′) and 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) [8] with an annealing temperature of 55 °C. The ribosomal ITS1-5.8S-ITS2 fragment was amplified with the primers BD1 (5′-GTC GTA ACA AGG TTT CCG TA-3′) and BD2 (5′-TAT GCT TAA ATT CAG CGG GT-3′) [9] with an annealing temperature of 54 °C. Negative and positive controls using both primer pairs were included. PCR parameters were as follows: a 1-min denaturation at 98 °C, followed by 35 cycles of 10 s at 98 °C, 5 s at 54/55 °C, 30 s at 72 °C, and a 2-min extension at 72 °C.

PCR products were directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA), as recommended by the manufacturer, with the internal sequencing primers described in [8] for 28S rDNA and in [9] for ITS. PCR product sequences were analysed using an ABI 3130xl genetic analyser at the Department of Cell Biology and Genetics, Far Eastern Federal University. Sequences were submitted to the GenBank database (NCBI).

2.3. Alignments and phylogenetic analysis

Ribosomal DNA sequences were assembled using the SeqScape v.2.6 software provided by Applied Biosystems. Alignments and estimations of the number of variable sites and sequence differences were performed using the MEGA 6.0 software [10]. The values for genetic p-distances were calculated for the 28S ribosomal DNA fragment. Phylogenetic relationships were obtained using a concatenated data set of partial sequences of the 28S rRNA gene and ITS1-5.8S-ITS2 rDNA. Phylogenetic analysis was performed using the Bayesian algorithm in the MrBayes v. 3.2.6 software [11]. The best nucleotide substitution model, a general time reversible model with estimates of invariant sites and gamma-distributed among-site variation (GTR + I + G), was estimated using the jModeltest v. 2.1.5 software [12] for combined ITS1-5.8S-ITS2-28S rDNA sequence data. 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 3,000,000 generations. The significance of the phylogenetic relationships was estimated using posterior probabilities [11]. Sequences of ITS1-5.8S-ITS2 rDNA and 28S rDNA of *Paragonimus westermani* Braun, 1899, *Brachycladium goliath* (van Beneden, 1858), and *Hurleytrematoides chaetodoni* (Manter, 1942) Yamaguti, 1954 from GenBank were used as the outgroup. Authors of these and other sequences from GenBank [2–4,6,13–22] and their accession numbers are given in Table 1.

3. Results

3.1. Morphological characterization of a new subfamily Pseudohaploporinae subfam. n.

3.1.1. Diagnosis

Body elongated; tegument spined; forebody and posterior end of body capable of retracting inside the body; eye-spot pigment dispersed between oral sucker and anterior border of ventral sucker or absent; oral sucker terminal, transversally oval, or round; ventral sucker round, equal to or larger in size to oral sucker, in posterior part of anterior third of body; prepharynx short; pharynx oval or elongated oval; oesophagus considerably longer than both prepharynx and pharynx; intestinal bifurcation posterior to ventral sucker, between ventral sucker and ovary; caeca two, short, saccular, end blindly in middle third of the body; testes two, entire, spherical or oval, tandem, contiguous, in middle of hindbody; external seminal vesicle saccular to elongated; hermaphroditic sac oval, sac-shaped, or elongated, antero-dorsal to and not extending or extending posterior to ventral sucker; proximal end of hermaphroditic sac with muscular sphincters; internal seminal vesicle thin-walled, oval, can occupy more than half of hermaphroditic sac; pars prostatica indistinct, tubular, prostatic cells large; hermaphroditic duct unarmed, with numerous large diverticula and surrounded by numerous glandular cells; genital pore median, immediately anterior to ventral sucker; ovary spherical, adjacent to anterior of testis or at level anterior margin of anterior testis; seminal receptacle uterine; uterus extending to posterior end of body; metraterm short with muscular walls; eggs numerous, operculate, containing miracidia developed with two fused eye-spots; vitellarium two, symmetrical, separated compact masses at level of ovary to front edge of anterior testis; excretory vesicle short, I-shaped, with concretion; excretory pore terminal; in intestine of Mugilidae; type genus *Pseudohaploporus* n.g.

3.2. Morphological characterization of a new genus Pseudohaploporus n. g.

3.2.1. Diagnosis

Body elongated with spines; forebody and posterior end of body capable of retracting inside the body; eye-spot pigment diffuse or absent; oral sucker terminal; ventral sucker equal to or larger than oral sucker; prepharynx short, pharynx oval or elongated oval; oesophagus considerably longer than prepharynx; caeca two, short, saccular; hermaphroditic sac oval or sac-shaped; hermaphroditic duct thick-walled, muscular, unarmed, with diverticula; external seminal vesicle saccular or elongated; testes two, entire, tandem, contiguous, in middle of hindbody; ovary, adjacent to anterior of testis or at level anterior margin of anterior testis; metraterm short with muscular walls; uterus extending to posterior end of body; eggs numerous, operculate, containing miracidia with two fused eye-spots; vitellarium two, symmetrical, separated compact masses at level of ovary or front edge of anterior testis; excretory vesicle shot, I-shaped, with concretions, pore terminal; in intestine of Mugilidae; type-species *Pseudohaploporus vietnamensis* sp. n.

3.2.2. Etymology

The genus was named *Pseudohaploporus* for its morphological similarity to *Haploporus* Looss, 1902.

3.3. Taxonomic summary of Pseudohaploporus vietnamensis sp. n.

Type host: *Osteomugil engeli* (Bleeker, 1858).
Other host: *Moolgarda seheli* (Forsskål, 1775).
Type-locality: Coastal water of Cat Ba Island, Tonkin Bay, northern Vietnam (20°84′N, 106°59′E).
Site: Intestine.
Material investigated: 30 flukes.
Type-deposition: Type No. 118-Tr, paratype No. 119–127-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: 2015.29.07.

3.3.1. Etymology

This species was named with respect to the country from which it originated.

Morphological description (Table 2, Fig. 1a-e). Body elongated; tegument spined in forebody; forebody capable of retracting inside the body; eye-spot pigment dispersed between oral sucker and anterior border of ventral sucker or absent; oral sucker terminal, transversally oval; ventral sucker round, at posterior part of anterior third of body; prepharynx short; pharynx oval; oesophagus long, bifurcating posterior to ventral sucker; caeca two, short, saccular, extending between

Table 2
Measurements (μm) of adult worms *Pseudohaploporus* n. g. and *Haploporus*.

	Pseudohaploporus vietnamensis sp. n.			*Pseudohaploporus planilizum* sp. n.			*H. benedeni* (Blasco-Costa et al., 2009)	*H. pacificus* (Manter, 1963)	*H. indicus* (Rekharani et Madchavi, 1985)	*H. pseudoindicus* (Rekharani et Madchavi, 1985)	*H. spinosus* (Machida, 1996)	*H. magnisaccus* (Machida, 1996)	*H. musculosaccus* (Machida, 2003)	*H. mugilis* (Liu et Yang, 2002)
	Holotype	Range $n = 10$	Mean	Holotype	Range $n = 8$	Mean								
Body length	1080	724–1432	1040	1386	1080–2000	1463	1007–1298	1986–2000	752–1120	1280	1403–3040	1900–2500	1620–3260	1320–2320
Body width	262	169–554	268	447	246–462	400	344–516	490–493	162–208	320	310–510	580–680	400–660	290–520
Body length/ width %	24.3	22.0–42.5	25.8	32.2	20.0–37.0	27.3	32–44	–	–	–	–	–	–	–
Forebody length	366	212–424	330	462	366–685	520	320–447	593–670	–	400	–	–	–	–
Body/forebody length ratio %	33.9	26.8–38.1	31.7	33.3	25.6–43.0	35.5	30–37	–	30%	–	17–30	22–35	20–41	–
Oral sucker length	62	58–85	71	131	81–139	107	94–145	–	39–58	80	50–130	80–110	170–230	66–104
Oral sucker width	96	65–150	90	131	81–139	107	119–147	198–205	58–89	128	90–170	130–180	170–290	108–170
Ventral sucker length	69	69–116	88	146	96–146	118	114–132		39–58	64	100–160	140–15 0	100–160	88–134
Ventral sucker width	85	62–119	88	146	96–146	118	111–137	158–174	39–46	80	120–130	140–170	130–170	108–136
Ventral/oral sucker length ratio	1:0.90	1:0.59–1.16	1:0.81	1.11	0.91–1.36	1.10	1:0.88–1.21	–	–	–	–	–	–	–
Ventral/oral sucker width ratio	1:1.13	1:0.80–1.29	1:1.02	1.11	0.91–1.36	1.10	1:0.83–1.01	1:0.77–0.88	1:0.5–0.8	1:0.63	1:1.0–1.4	1:0.9–1.2	1:0.5–0.7	1:0.88–1:1.21
Prepharynx	7	0–23	9	50	8–50	25	5–81	134–174			10–50	0–60	0–30	10–46
Pharynx length	62	39–77	54	85	50–85	67	83–102	144–154	31–36	48	50–100	80–100	60–90	62–90
Pharynx width	54	46–73	55	69	42–69	53	82–122	108–117	27–29	80	50–110	70–100	60–110	40–90
Oesophagus length	350	193–470	343	732	450–1059	696	178	–	98–112	280	340–740	900	170–600	320–744
Ovary length	96	46–154	75	127	58–173	122	56–102	–	54–62	96	90–200	110–150	100–200	78–116
Ovary width	96	42–123	77	135	65–139	112	68–99	–	54–62	96	100–180	140–200	110–180	80–120
Anterior testis length	173	104–212	147	127	104–270	167	124–231 x	–	89–136 x	272 x	230-530x	340–640 x	420–1040 x	288–536 x
Anterior testis width	154	96–193	139	200	100–200	151	132–224		78–136	192	70–150	200–270	150–300	100–188
Posterior testis length	135	116–281	150	193	116–193	151								
Posterior testis width	131	85–235	135	212	97–227	150								
Hermaphroditic sac length	208	142–385	244	443	281–443	343	119–241	308–324	136–195	214	390–570	400–480	–	224–336
Hermaphroditic sac width	185	112–235	160	212	135–212	162	86.5–140	150–182	54–97	125	120–220	240–290	–	144–272
Vitellaria length	77	50–135	74	96, 92	58–135	93	43–70	–	39–46	64	–	–	70–110	060–104
Vitellaria width	54	42–123	59	77, 62	39–85	68	46–59	–	39–46	64	–	–	60–110	52–76
Post-testicular length	250	0–316	160	158	116–323	223	367–508	–	260–340	224	6–20%	16–21%	16–40%	224–520
Arm hermaphroditic duct	–	–	–				–	–	–	-(+) (Blasco-Costa et al., 2009)	+	–	–	+
Eggs length	27–39	27–39	–	35–39	35–39		38–43	58–63	31–35	19	34–42	34–42	45–54	39–44
Eggs width	15–19	15–19	–	23–27	23–27		24–27	27–31	11–15	15	18–23	18–26	17–23	19–22

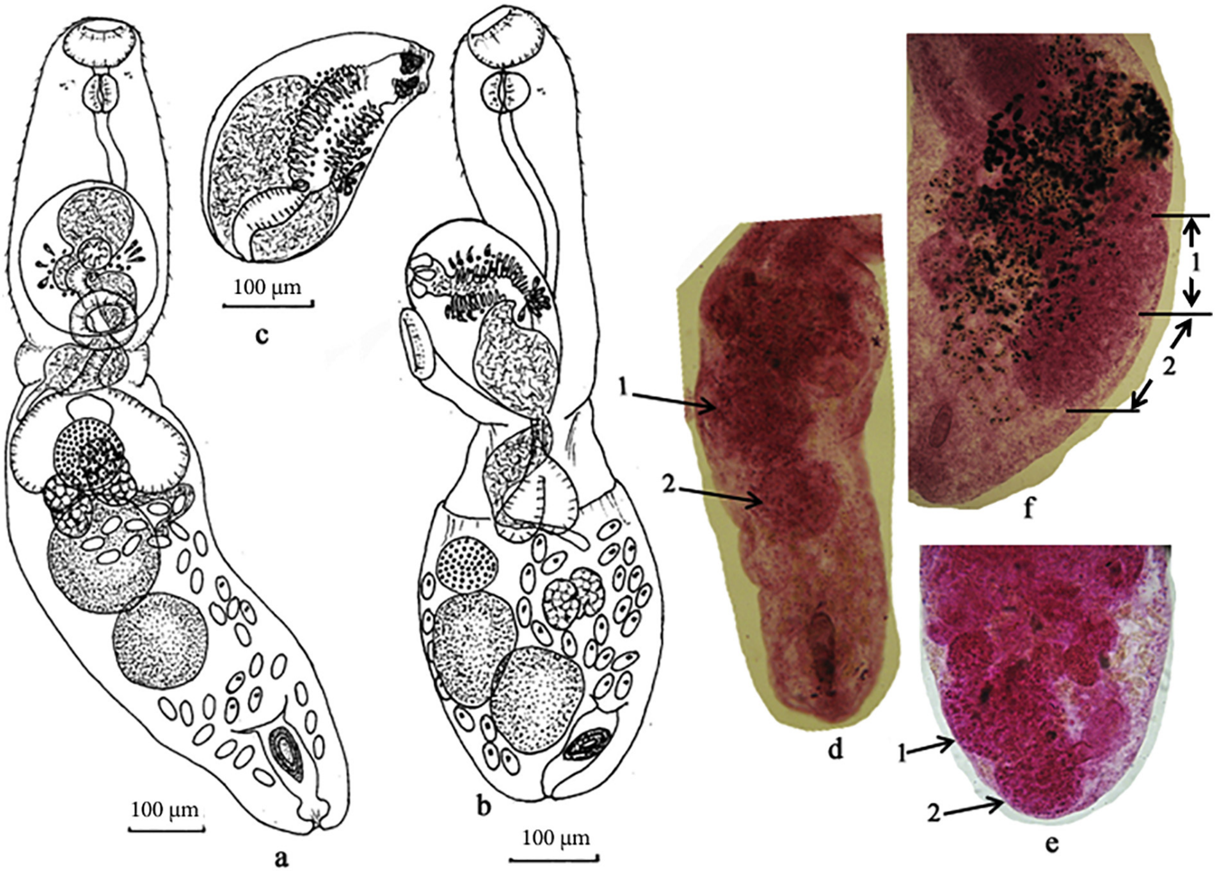

Fig. 1. *Pseudohaploporus vietnamensis* sp. n.: a - adult worm, b - adult mature worm with a partially retracted body, c - hermaphroditic sac, d,e - well differentiated testes, f - contiguous testes (1– anterior testis, 2 - posterior testis).

acetabulum and anterior testis; testes two, entire, spherical or oval, tandem, contiguous or overlapping dorso-ventrally, in middle of hindbody; external seminal vesicle saccular or elongated; hermaphroditic sac oval or sac-shaped, dorsally from ventral sucker; posterior end of hermaphroditic sac does or does not overlap with border of posterior margin of ventral sucker; proximal end of hermaphroditic sac with muscular sphincters; internal seminal vesicle oval, size depends on fullness of genital products; pars prostatica small with few large prostatic cells; hermaphroditic duct unarmed, with numerous large diverticula and surrounded by numerous glandular cells; genital pore median, immediately before ventral sucker; ovary spherical, adjacent to anterior of testis or at level of anterior margin of anterior testis; seminal receptacle uterine; uterus extending to posterior end of body; metraterm short with muscular walls; vitellarium two, symmetrical, coalesced, separated masses at level of ovary to front edge of anterior testis; eggs numerous, operculate, embryonated; miracidia with two fused eye-spots; excretory vesicle short, I-shaped, with single concretion; excretory pore terminal.

3.3.2. Molecular data

For four specimens of *P. vietnamensis* sp. n. collected from *O. engeli* and *M. seheli*, there were 1023 and 934 alignable characters available for analysis in the 28S rRNA gene and ITS1–5.8S-ITS2 rDNA fragment datasets, respectively. The 28S rRNA gene nucleotide sequences of different specimens of *P. vietnamensis* sp. n. had no variable sites. Ribosomal ITS1–5.8S-ITS2 rDNA sequences contained one single variable site. The sequences were submitted to the NCBI database with accession numbers, as provided in Table 1.

3.4. Taxonomic summary of *Pseudohaploporus planilizum* sp. n.

Type host: *Planiliza subviridis* (Valenciennes, 1836).
Type-locality: Coastal water of Cat Ba Island, Tonkin Bay, northern Vietnam (20°84′N, 106°59′E).
Site: Intestine.
Material investigated: 12 flukes.
Type-deposition: Type No. 128-Tr, paratype No. 129–139-Tr. This material is held in the collection of the Zoological Museum (Federal Scientific Centre of Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia); *E*-mail: petrova@ibss.dvo.ru. Deposited: 2016.20.06.

3.4.1. Etymology

Species was named in concordance with generic name of definitive host species – *Planiliza subviridis*.

Morphological description (Table 2, Fig. 2a-c). Body elongated, with spines in forebody; forebody and posterior end of body capable of retracting inside the body; eye-spot pigment dispersed between oral sucker and anterior border of ventral sucker or absent; oral sucker terminal, round; ventral sucker round, in posterior part of anterior third of body; prepharynx short; pharynx elongated, oval; oesophagus long, bifurcating posterior to ventral sucker; caeca two, short, saccular, extending between acetabulum and ovary; testes two, entire, tandem, contiguous or slightly overlapping dorso-ventrally, in the posterior portion of hindbody; external seminal vesicle elongated; germaphroditic sac oval or sac-shaped, dorsal to ventral sucker; posterior end of hermaphroditic sac overlaps with border of posterior margin of ventral sucker; proximal end of hermaphroditic sac with muscular sphincters; internal seminal vesicle oval, size depends on fullness of genital products; pars prostatica small, with few large prostatic cells;

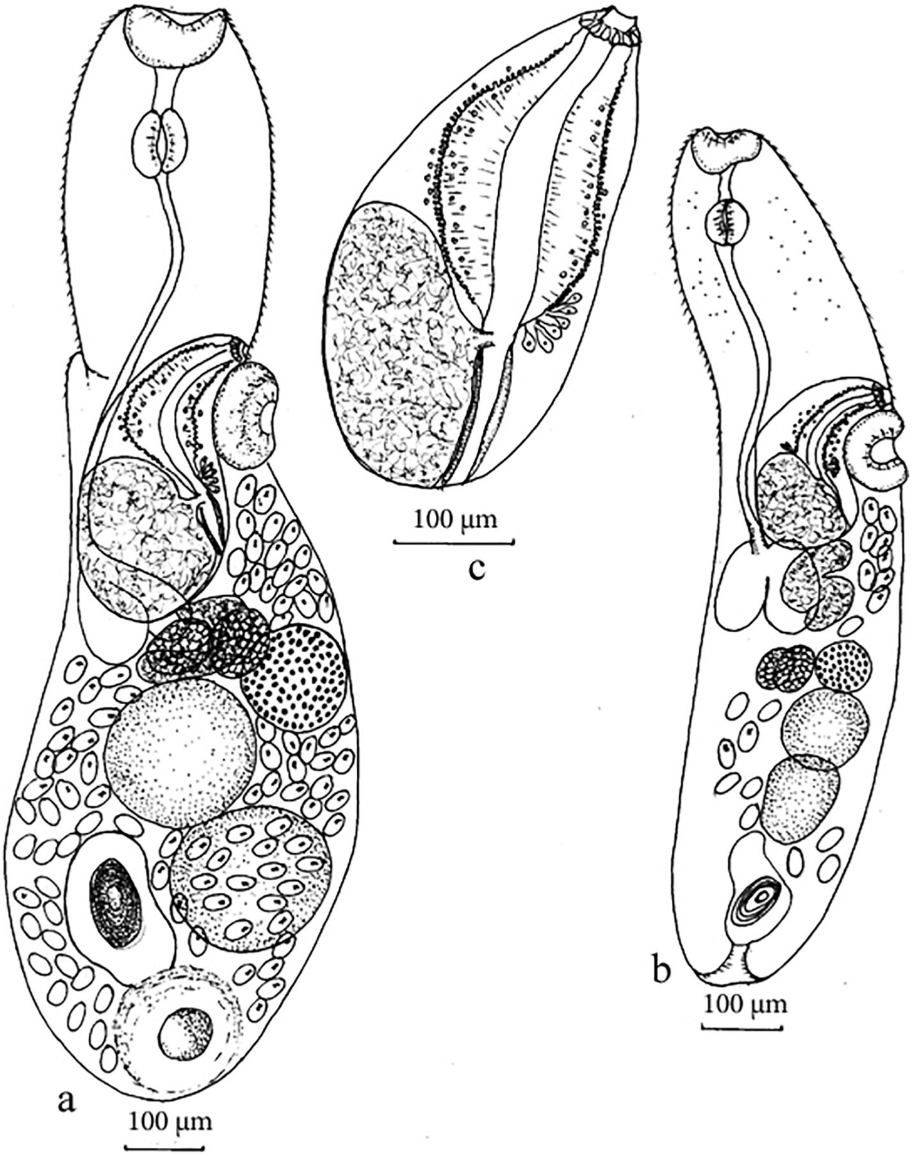

Fig. 2. *Pseudohaploporus planilizum* sp. n.: a - adult mature worm, b – young mature worm, c - hermaphroditic sac.

hermaphroditic duct unarmed, with small diverticula, surrounded by numerous glandular cells; genital pore median, immediately anterior to ventral sucker; ovary spherical, anterior of testes or at level anterior margin of anterior testis; seminal receptacle uterine; uterus extending to posterior end of the body; metraterm short with muscular walls; vitellarium two, symmetrical, coalesced, separated masses level of ovary or front edge of with anterior testis; miracidia with two fused eyespots; excretory bladder short, I-shaped, with single concretion; excretory pore terminal.

3.4.2. Molecular data

For *P. planilizum* sp. n., there were 1021 and 934 alignable characters available for analysis in the 28S rRNA gene and ITS1–5.8S-ITS2 rDNA fragment datasets, respectively. Neither the 28S rRNA gene nor ITS1–5.8S-ITS2 rDNA fragments of *P. planilizum* sp. n. contained variable sites. The sequences were submitted to the NCBI database with accession numbers MF774417–MF774419 for the 28S rRNA gene and MF774433–MF774435 for the ITS1–5.8S-ITS2 rDNA.

4. Discussion

4.1. Remarks

Morphologically and genetically, the worms collected from mullet of Vietnam belong to the family Haploporidae. Within Haploporidae, only the species of *Megosalena* Linton, 1910, *Vitellibaculus* Montgomery, 1957, and *Metamegasolena* Yamaguti, 1970 from the subfamily Megasoleninae possess two tandem testes. However, representatives of these genera, in contrast to *Pseudohaploporus*, “possess vitellarium with numerous follicles throughout the hindbody, usually relatively small, occasionally larger and ellipsoidal or elongated, always confluent posteriorly, and the miracidium lacks eye-spots” [1]. According to the present available data, species of Megasoleninae were not detected in Mugilidae. Only representatives of Haploporinae and Waretrematinae were detected in mullet hosts (Mugilidae) in the Western Pacific Region, and there are no reports of worms with two testes. Of the other morphological parameters, specimens of *Pseudohaploporus* are similar to representatives of the genus *Haploporus* (Haploporinae), which possess an elongated body, a long oesophagus, an intestinal bifurcation posterior to the ventral sucker, a large hermaphrodite bursa, vitellarium

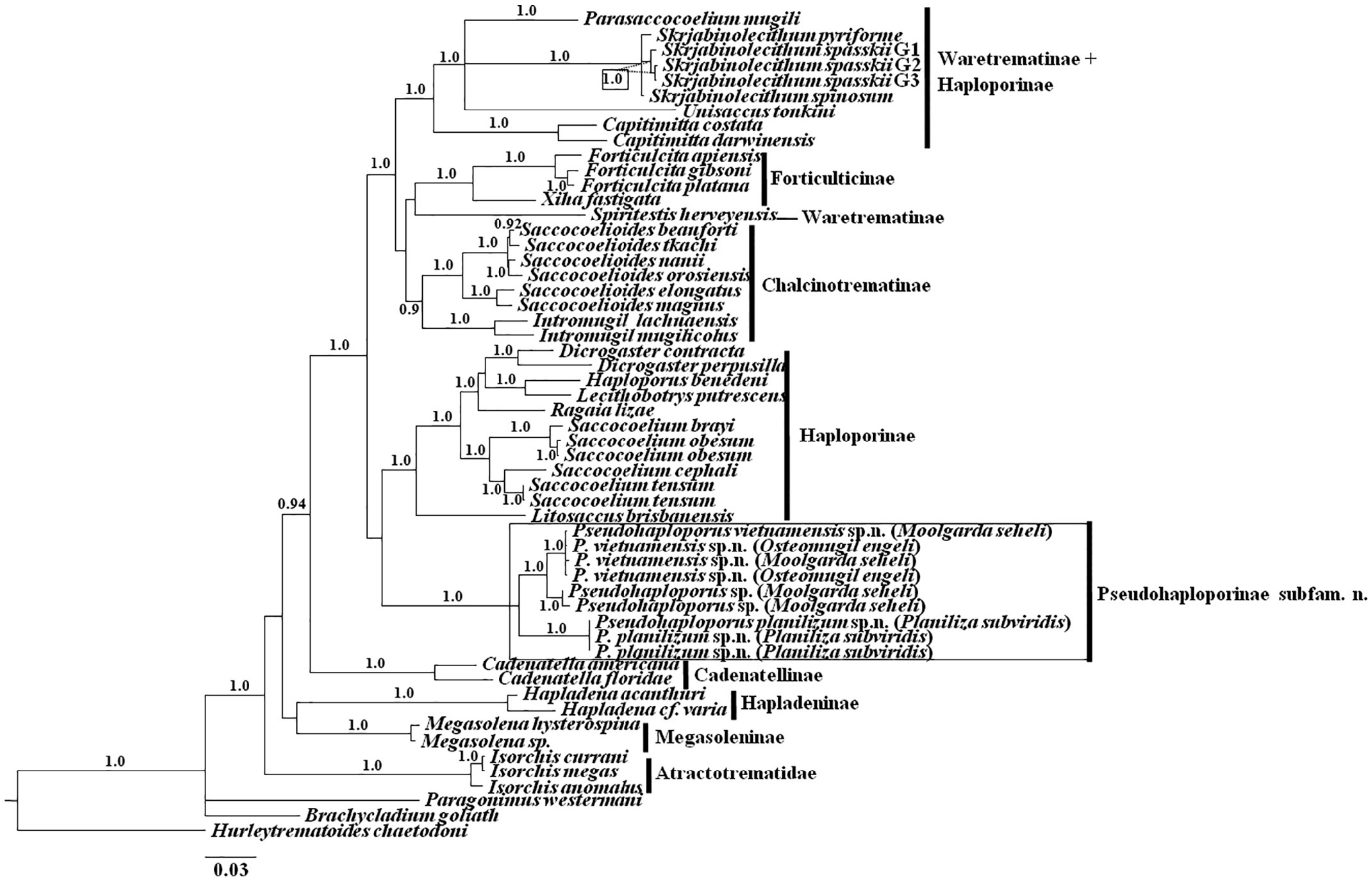

Fig. 3. Phylogenetic relationships of the family Haploporidae obtained with Bayesian algorithm based on concatenated nucleotide sequence data set of ITS2 rDNA and partial 28S rRNA gene (overall 1805 bp) including geographical interpretation. Nodal numbers - posterior probabilities that indicate statistical support of phylogenetic relationships, only significant values (0.9–1.0) are showed.

consisting of two compact accumulations, a concretion in the excretory vesicle and numerous small eggs containing miracidium with pigment eyes. Morphological investigation of numerous specimens of *P. vietnamensis* sp. n. shows that the two testes were overlapping and sometimes looked like a single testis (Fig. 1f). This fact allows the proposal that some *Haploporus* species for which a single testis was indicated may possess two testes that are closely adjoined.

Pseudohaploporus vietnamensis sp. n. and *P. planilizum* sp. n. differ from *Haploporus* species by a smaller (*H. pacificus, H. musculosaccus*) or larger (*H. pseudoindicus*) egg size, unarmed hermaphrodite duct (*H. mugilis, H. spinosus*), considerably larger oesophagus length, large pharynx (*H. benedeni*), larger pharynx and sucker sizes (*H. indicus*), smaller body size, ventral sucker and pharynx (*H. magnisaccus*), and other morphometric parameters, as presented in Table 2 [2,23–27]. There are no apparent morphological differences between *Pseudohaploporus vietnamensis* sp. n. and *P. planilizum* sp. n. These two species differ from each other by morphometric parameters. *Pseudohaploporus planilizum* sp. n., in comparison with *P. vietnamensis* sp. n., possesses a larger maximum and mean size of body length, oral and ventral suckers, pharynx, oesophagus length, hermaphrodite bursa, and egg width (Table 2).

4.2. Molecular analysis

Molecular data confirm that the new trematodes belong to the family Haploporidae and represent distinct species. Genetic differentiation between the two *Pseudohaploporus* species was 4.77 ± 0.6% for the 28S rDNA dataset and 5.0 ± 1.0% using the ITS1–5.8S-ITS2 dataset, which corresponds to the interspecific p-distance value ranges of Haploporidae for both molecular markers [2]. There were two trematode specimens from *M. seheli* that differ from *Pseudohaploporus vietnamensis* sp. n. by 19 (1.86%) and 34 (3.64%) variable sites using 28S rRNA gene and ITS1–5.8S-ITS2 rDNA sequences, respectively. Of these, 14 (1.37%) and 27 (2.89%) variable sites were parsimony informative, respectively. These values correspond to rDNA-based interspecific differentiation of Haploporidae. Blasco-Costa et al., (2009) reported a lower interspecific percentage of molecular variation for the genus *Saccocoelium* (Haploporinae) of 2.1% and 0.9% for ITS2 and 28S rDNA, respectively [2]. These values were obtained for two species of *Saccocoelium* Looss, 1902 that clearly differ from each other morphologically. On the other hand, intraspecific molecular variations of morphologically identical specimens were observed for *Dicrocoelium dendriticum* Rudilphi, 1819 [28] and *Skrjabinolecithum spasskii* Belous, 1954 [5]. Nevertheless, our molecular results suggest the presence of a third unnamed species, *Pseudohaploporus* sp.

Genetic p-distance values calculated between *Pseudohaploporus* and species of other haploporid subfamilies: Haploporinae, Waretrematinae, Forticulcitinae, Chalcinotrematinae, and Megasoleninae, ranged from 10 ± 0.78% to 15.37 ± 1.08% for the 28S rDNA dataset and from 16.2 ± 1.53% to 19.3 ± 1.58% using the ITS1–5.8S-ITS2 rDNA dataset. These values are within the p-distance range for different subfamilies. Bayesian phylogenetic tree topologies support morphological-based validity of the genus *Pseudohaploporus* within Haploporidae (Fig. 3). The phylogram based on the analysis of a concatenated nucleotide sequence dataset of ITS rDNA and partial 28S rRNA gene shows that *Pseudohaploporus* represents a separate clade, which is sister to Haploporinae with poor support, within a large group comprising Waretrematinae, Chalcinotrematinae, Forticulcitinae, and

Haploporinae. Representatives of *Cadenatella* Dollfus, 1946 (Cadenatellinae Gibson and Bray, 1982), *Hapladena* (Hapladeninae), and *Megasolena* (Megasoleninae) formed highly supported clades.

Our results show a single distinct morphological feature of the new specimens relative to most other haploporids, namely the existence of two testes. Analysis of rDNA sequence differentiation values and phylogenetic reconstructions shows that *Pseudohaploporus* differs from other haploporids at the same level as different subfamilies. The new genus can, therefore, be considered as a member of a new distinct subfamily; thus, based on the morphological and molecular data obtained for *P. vietnamensis* sp. n. and *P. planilizum* sp. n., we erected a new subfamily, Pseudohaploporinae subfam. n. In recent studies of molecular-based phylogenetic relationships, it was stated that Megasoleninae is restricted to haploporid species with two testes, and that this character represents a basal state for Haploporoidea [20]. For this reason, the authors suggested that Megasoleninae could be elevated to family level. The results of our study do not provide any basis for this suggestion, since *Pseudohaplporus* with two testes is closely related to "mugilid" haplporids, which possess a single testis. Nevertheless, we agree with the viewpoint reported in [20] that the revision of haploporid subfamilies is far from finalised.

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Conflict of interest

None.

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