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GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

Population genetic analysis of trematode *Parasaccocoelium mugili* Zhukov, 1971 (Haploporidae Nicoll, 1914) from the Russian Far East and Vietnam based on ribosomal ITS and mitochondrial COI gene partial sequence data

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Abstract

Intraspecific variation of *Parasaccocoelium mugili* collected from mullet fish of the south of Russian Far East and Vietnam has previously been estimated on the basis of two molecular markers: ribosomal internal transcribed spacer 1 (ITS1) rDNA and mitochondrial cytochrome oxidase I (COI) gene sequences. In the present study, molecular identification of this species from the Kievka River, Primorye and from Vietnam was performed by analysis of 28S rDNA sequences. Analysis of ITS1 rDNA sequences variation revealed two highly differentiated main groups, representing trematode specimens from the two regions. Genetic variation within each region was relatively low. Mitochondrial COI gene sequence data analysis revealed fixed nucleotide and amino acid substitutions, and supported the existence of two genetically different groups associated with geographical origin. Analysis of the COI gene fragments showed extremely high variation within Russian and Vietnamese *P. mugili* samples. Our results for *P. mugili* most probably represent a case of initial step of allopotric speciation for this trematode, caused by living strategy of its definitive host at evolutionary scale. Mitochondrial DNA sequence data show that existence of gene flow between local populations of *P. mugili* in the Primorye Region caused by definitive hosts can be proposed.

Keywords Mullet fish · *P. mugili* · Sequence data · Haploporidae · COI

Introduction

The topic of phylogenetic interrelationships of trematodes of the family Haploporidae Nicoll, 1914 is an actively discussed problem at the present time (Besprozvannykh et al. 2017a, 2017b; Blasco-Costa et al. 2009; Pulis and Overstreet 2013; Pulis et al. 2013; Andres et al. 2014, 2015, 2018; Andres et al. 2016). Recent studies using complex morphological and molecular approaches have resulted in the identification of several new species, genera and subfamilies within the family, along with leading to some taxonomical rearrangements (Pulis et al. 2013; Besprozvannykh et al. 2015a, 2017a, b; Andres et al. 2015; Curran et al. 2018; Andres et al. 2018). Most studies of haploporid trematodes have focused on high taxa, thus omitting the micro evolutionary processes which are an important factor during speciation. Population genetic studies are useful for evaluating these processes through the analysis of intraspecific polymorphism and differentiation using molecular data.

The first investigation into these features was performed previously for the haploporid species *Parasaccocoelium mugili* Zhukov, 1971. Trematodes of the genus *Parasaccocoelium* Zhukov, 1971 (Haploporidae, Waretrematinae) are common intestinal parasites of the redlip

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Table 1 List of taxa incorporated into molecular analysis. *N*, number of sequences (28S/ITS1/COI). P (or C) and V, Primorye and Vietnam, respectively

Species	*N*	Host	Locality	Reference	№ of samples/accession numbers				
					28S rDNA		ITS1 rDNA		COI gene, mtDNA
Parasaccocoelium mugili	8/53/77	*Planiliza haematocheila*	Kievka River, Primorye, Russian Far East	Present study	P01-P08	MK128572-MK128579	P01-P12,P25,P27-29,P35,P38,P41,P43-P62,P66-P69,P71-P72	MK128519-MK128563	P01-P73, C01-C04	MK128586-MK128662
P. mugili	8/8/6	*Mugil cephalus*	Cat Ba, Vietnam	Present study	V01-V08	MK128564-MK128571	V01-V06,V08,V10	MK128511-MK128518-	V02-V03, V05-V08	MK128580-MK128585
P. mugili	3/–/–	*Planiliza haematocheila*	Razdolnaya River, Primorye, Russian Far East	Besprozvannykh et al. 2015a	P.mug2-2,P.mug1-1, P.mug2-1	HF548468, HF548470, HF548473	–	–	–	–
P. haematochelium	2/–/–	*Planiliza haematocheila*	Razdolnaya River, Primorye, Russian Far East	Besprozvannykh et al. 2015a	P.sp.n.1-1., Par7-4	HF548461, HF548467	–	–	–	–
P. polyovum	2/–/–	*Planiliza haematocheila*	Razdolnaya River, Primorye, Russian Far East	Besprozvannykh et al. 2015a	Par4-1, Par5-3	HF548474, HF548478	–	–	–	–

mullet *Planiliza haematocheila* (Temminck & Schlegel, 1845) in the Russian Far East. Following the revision of the Haploporidae family, the genus *Parasaccocoelium* has recently been restored using morphological and molecular approaches (Besprozvannykh et al. 2015a). This species has also been detected in the intestines of mullet in coastal waters of Cat Ba Island, Vietnam. Molecular studies of another giant intestine trematode species of the Waretrematinae subfamily isolated from mullet from the Russian Far East and Vietnam, *Skrjabinolecithum spasskii* Belous, 1954, revealed three main variants of 28S rDNA and internal transcribed spacer (ITS) nucleotide sequences, which differed from each other by several fixed nucleotide substitutions (Besprozvannykh et al. 2015b; Atopkin et al. 2015). Two sequence variants have been detected in trematodes of the Kievka River and the third variant was found in Vietnam. Considering the results of these previous studies, we predicted that similar intraspecific molecular differences may exist in *P. mugili*, associated with geographical origin. To test this hypothesis, we analysed mitochondrial DNA sequence data in order to estimate the intraspecific variation of *P. mugili.* This is, to our knowledge, the first time that such a study has been carried out on Haploporidae. The aims of this study were (1) to identify *P. mugili* in the intestines of mullet fish of the Kievka River in Primorye, south of the Russian Far East, and from Cat Ba Island, Vietnam using 28S rRNA gene sequence data analysis and (2) to estimate molecular variation of *P. mugili* from different geographic regions and definitive host species. To this end, evaluation of ribosomal ITS1, and the mitochondrial cytochrome oxidase I gene (COI) and estimation of the molecular variation of *P. mugili* from these regions were carried out.

Material and methods

Adult trematode specimens were collected from the intestine of one representative of *Planiliza haematocheila* in 2016 during parasitological field work from the Kievka River in the south of the Russian Far East ($n = 75$), and from one specimen of *Osteomugil cunnesius* (Valenciennes, 1836) from the coastal waters of Cat Ba Island, Vietnam ($n = 8$). Before preservation and DNA extraction, all worms were examined with light microscopy and all of them were identical morphologically to *Parasaccocoelium mugili*, Holotype № 50.1-Tr, which is in depository of biological samples of FSC of Biodiversity FEB RAS. Morphological description of this sample is given in Besprozvannykh et al. (2015a). Trematodes were fixed in 96% ethanol for molecular analysis. Total DNA was extracted from flukes using a hotSHOT technique (Truett 2006). DNA samples can be obtained from the corresponding author upon request.

The polymerase chain reaction (PCR) was used to amplify 28S ribosomal DNA (rDNA) using the primers DIG12 (5′-

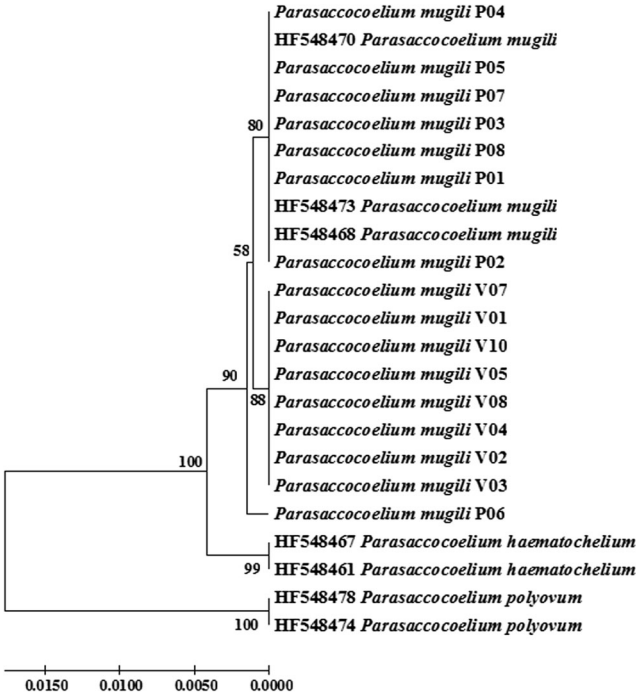

Fig. 1 UPGMA dendrogram of the genus *Parasaccocoelium* based on *p*-distance estimation using 28S rDNA partial sequences. Nodal numbers, values of bootstrap statistics (%); numerals, index number of specimen. P and V, Primorye and Vietnam, respectively

AAG CAT ATC ACT AAG CGG-3′) and 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) as previously described (Tkach et al. 2003). The initial PCR reaction was performed in a total volume of 25 μl and contained 0.25 mM of each primer, approximately 10 ng of total DNA in water, 1 unit of Q5 High Fidelity polymerase (New England Biolabs, Ipswich, MA, USA) with 10× Q5 HF buffer, 1.25 mM dNTPs and GC-Enhancer. Amplification of a 1200-base pair (bp) fragment of 28S rDNA was performed using a GeneAmp 9700 (Applied Biosystems, MA, USA) with the following program: 3 min denaturation at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 55 °C and 2 min at 72 °C and a 7 min extension at 72 °C. Negative and positive controls using both primers were included.

Ribosomal ITS1-5.8S-ITS2 fragments were amplified using primers BD1 (5′-GTC GTA ACA AGG TTT CCG TA-3′) and BD2 (5′-TAT GCT TAA ATT CAG CGG GT-3′) (Luton et al. 1992) with an annealing temperature of 54 °C. Negative and positive controls using both primers were included. The COI gene fragments were amplified and directly sequenced using the primers Trema-cox1/F (5′-TTC GGT CAT CCT GAG GTT TAT GTT-3′) and Trema-cox1/R (5′-CAG CAA ATC ATG ATG CAA AAG GTA-3′) with an annealing temperature of 50 °C (Atopkin et al. 2018).

The PCR products were directly sequenced using the ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, MA, USA), following the manufacturer's instructions, with the internal sequencing primers described by Tkach et al. (2003) for 28S rDNA, and those described by Luton et al. (1992) for the ITS2 rDNA fragment. The PCR products were analysed using an ABI 3130xl genetic analyser at the Department of Cell Biology, Far Eastern Federal University. Sequences were submitted to GenBank of the NCBI database with the accession numbers detailed in Table 1.

Nucleotide sequences were assembled with SeqScape v. 2.6 software (Applied Biosystems, MA, USA). Alignments, estimation of the number of variable sites and sequence differences and unweighted pair group method with arithmetic mean (UPGMA) analysis were performed using MEGA 7.0 (Kumar et al. 2016). The UPGMA phylogenetic relationship significance was estimated using a bootstrap analysis (Felsenstein 1985) with 1000 replications.

Calculation of gene and nucleotide diversity indices (Hd and Pi, respectively), the differentiation index (Fst) and number of gene flow (Nm) were performed with DNASp v.6 software (Rozas et al. 2017). Genetic differentiation indexes were calculated according to Hudson et al. (1992). Relationships of nucleotide sequence variants were reconstructed with a median-joining algorithm using Network v.5 software (Flexus Technology Ltd., Suffolk, UK).

Results

Molecular identification of adult worms

Species identification was performed for eight specimens from the Russian Far East and eight specimens from Vietnam using 28S rDNA partial sequences which were 956 bp in length. Of these, four nucleotide positions were variable, including two fixed substitutions between the Russian Far Eastern and Vietnamese sequences. The 28SrDNA-based UPGMA dendrogram demonstrated that

Table 2 Fixed substitutions of 540 bp ITS1 rDNA nucleotide sequences between *Parasaccocoelium mugili* Russian Far Eastern and Vietnamese samples

№ of site	54	113	114	162	240	248	261	299	331	429
P. mugili Vietnam	G	T	G	C	T	T	C	T	G	G
P. mugili Primorye	A	C	A	A	C	C	T	C	A	A

Table 3 Polymorphism indexes for *Parasaccocoelium mugili* samples calculated on the basis of ribosomal ITS1 and mitochondrial COI gene (*N*, a number of sequences; *h*, a number of sequence variants/haplotypes; Hd, gene diversity; Pi, nucleotide diversity).

Sequence data	Samples	*N*	*h*	Hd ± St.err.	Pi ± St.err.
ITS1 rDNA	Overall	53	9	0.329 ± 0.077	0.00577 ± 0.0015
	Primorye	45	7	0.088 ± 0.057	0.00017 ± 0.00011
	Vietnam	8	2	0.250 ± 0.18	0.00047 ± 0.00034
COI gene mtDNA	Overall	83	45	0.949 ± 0.015	0.01505 ± 0.0036
	Primorye	77	39	0.94 ± 0.018	0.00538 ± 0.0015
	Vietnam	6	6	1 ± 0.096	0.00628 ± 0.0015

trematodes, collected from the Russian Far East and Vietnam in the present study, were genetically close to *P. mugili*, rather than with *P. haematochelium* or *P. polyovum* (Fig. 1). Thus, the trematodes collected in the present study were concluded to be *P. mugili*.

Intraspecific variation of ITS rDNA nucleotide sequences of *P. mugili*

The intraspecific variation of 53 collected specimens of *P. mugili* (45 from the Russian Far East and 8 from Vietnam) was estimated using the 540 bp ITS1 rDNA. Of the base pairs in the fragment, 527 were found to be conservative and 13 were variable, including 11 parsimony-informative (PI). Of these, in 10 PI sites, there were fixed substitutions between Russian and Vietnamese samples (Table 2).

The observed *p*-distance between Russian Far Eastern and Vietnamese *P. mugili* specimens based on ITS1 sequences was 2.08 ± 0.57%. Within the Vietnamese samples, the genetic differentiation ranged from 0 to 0.19% (mean 0.05 ± 0.04%). Within *P. mugili* from the Russian Far East, the *p*-distance values ranged from 0 to 0.37% (mean 0.02 ± 0.01%).

The polymorphism parameter values of Russian Far Eastern *P. mugili* were lower than those of the Vietnamese specimens (Table 3). The differentiation index for the samples was relatively high (Fst = 0.925) and the gene flow was low (Nm = 0.02), indicating the high molecular-based divergence of the two samples.

The topology of the median-joining network showed two main groups of sequences, corresponding to the Russian Far Eastern and Vietnamese samples (Fig. 2), with 14 mutational steps between them. The sample from the Russian Far East contained seven sequence variants that differed from each other by one to five mutational steps. A central, single-shared sequence variant was detected for 39 specimens with a frequency of 86.7%. The Vietnamese group contained 2 sequence variants, which were separated from each other by one mutational step. The frequency of the main central sequence variant was 87.5%.

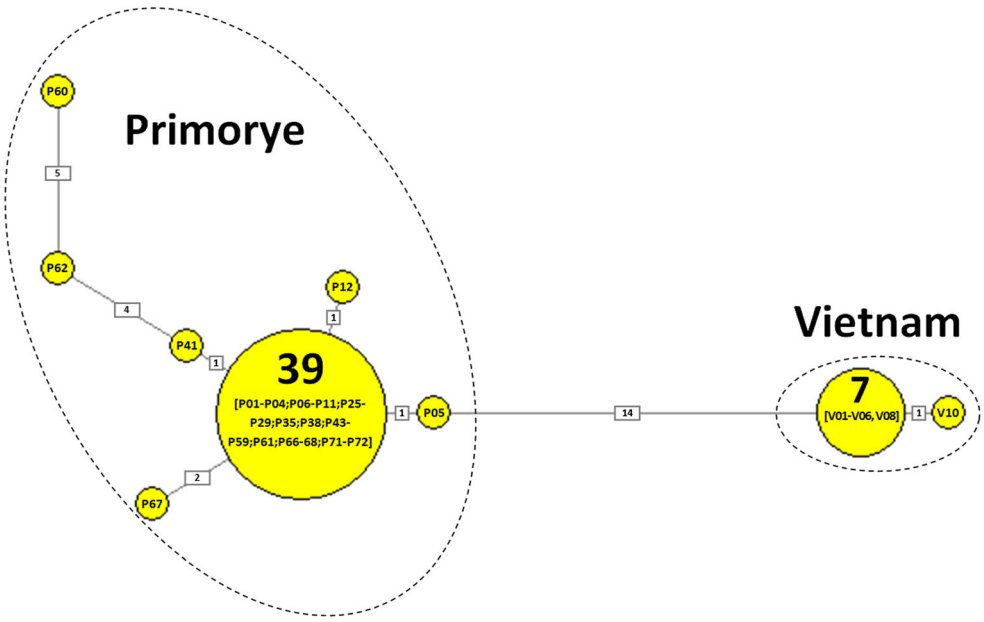

Fig. 2 Median-joining network of *Parasaccocoelium mugili* based on ITS1 rDNA sequences. Numbers within yellow rounds, amount of sequences within one sequence variant; numbers on lines, a number of mutational steps between sequence variants; numerals, index number of specimen. P and V, Primorye and Vietnam, respectively. Dashed ellipses indicate sequence groups of *P. mugili* from Primorye and Vietnam

Table 4 Fixed substitutions of COI gene partial sequences between *Parasaccocoelium mugili* Russian Far Eastern and Vietnamese samples.

№ of site	21	39	54	75	76	78	81	102	138	159	231	288	312
P.mugili Vietnam	T	C	G	C	C	G	T	G	A	G	A	A	G
P.mugili Primorye	A	T	A	A	T	A	C	T	G	T	T	T	A
№ of site	333	336	351	399	423	426	433	450	453	466	468	471	474
P.mugili Vietnam	C	G	G	A	G	G	G	T	G	T	G	G	T
P.mugili Primorye	T	A	A	G	A	A	A	A	A	C	A	A	A
№ of site	493	510	531	546	549	564	567	591	595	603	604	606	615
P.mugili Vietnam	G	C	A	A	T	T	C	T	G	T	G	T	G
P.mugili Primorye	A	T	G	G	G	C	T	C	A	A	A	A	A
№ of site	624	642	651	652	693	699	741	750					
P.mugili Vietnam	G	G	T	C	G	C	A	T					
P.mugili Primorye	A	A	C	T	A	T	T	C					

Intraspecific variation of mitochondrial COI nucleotide sequences of P. mugili

Variation of the 786 bp COI gene fragment was estimated for 83 specimens of *P. mugili* (77 from the Russian Far East and six from Vietnam). The mitochondrial COI gene fragment contained 681 conservative and 105 variable sites, including 81 parsimony-informative and 24 singleton sites. Samples from the two areas differed by 47 fixed nucleotide substitutions (Table 4). Within the Russian Far Eastern samples, there were two specimens that differed from the others by 30 fixed substitutions, and that differed from the Vietnamese sample by 53 fixed substitutions. Six amino acid substitutions were detected between translated COI sequences of samples from the Russian Far East and Vietnam (Table 5).

Genetic differentiation estimated through the calculation of the *p*-distance was found to be 7.33 ± 0.92% between the Russian Far Eastern and Vietnamese samples. The internal *p*-distance values ranged from 0 to 2.5 ± 0.5% and from 0.36 ± 0.1 to 2.87 ± 0.68% for the Russian Far Eastern and Vietnamese samples, respectively.

The polymorphism indices were relatively high for both samples (Table 3). This indicates a high number of haplotypes and high diversity within each of the regional samples.

The Fst and Nm between the Russian and Vietnamese samples were 0.923 and 0.02, respectively, indicating high molecular differentiation and extremely low gene exchange between the two samples.

Table 5 Fixed amino acid substitutions of COI partial sequences between *Parasaccocoelium mugili* Russian Far Eastern and Vietnamese samples

№ of Site	145	150	165	199	201	202
P.mugili Vietnam	V	S	V	V	S	V
P.mugili Primorye	I	R	I	I	R	I

The median-joining network revealed three genetically distant groups within *P. mugili* (Fig. 3). The first corresponds to the Russian sample and comprises 40 haplotypes which differ from each other by zero to two mutational steps. The main haplotype represented 14 in specimens, frequency 18.1% (contained sequences for haplotypes with 2 or more sequences; see in the legend in Fig. 3). The second group included two specimens from the Russian Far East that differed from other specimens of this region and from those of Vietnam by 26 and 53 mutational steps, respectively. The third group contained six haplotypes, corresponding to six Vietnamese samples that differed by one to five mutational steps. There were 9 specimens within a sample from Primorye, which are identical by both ITS1 and COI gene sequence data, namely P03, P07, P35, P44, P49, P55, P58, P61 and P72. Within Vietnamese, sample there were no identical samples by COI gene sequence data.

Discussion

The results of the present study indicate that *P. mugili* possesses a high molecular differentiation of distant geographical isolates. The mitochondrial COI gene fragment was analysed with high resolution for intraspecific variation studies of *P. mugili* compared with the ITS1 rDNA sequences. The polymorphism indices, including haplotype and nucleotide diversity for Russian and Vietnamese samples, indicated that the populations of the species were stable within each of the studied geographical regions. Both the ITS1 rDNA and mitochondrial COI gene analyses indicated high divergence of the Russian and Vietnamese populations of *P. mugili*. This result was supported by analysis of the differentiation indices, fixed substitutions and median-joining network topologies.

Our results agree with previous data for the trematode *S. spasskii* Belous, 1954 (Besprozvannykh et al. 2015b; Atopkin et al. 2015), which revealed high molecular

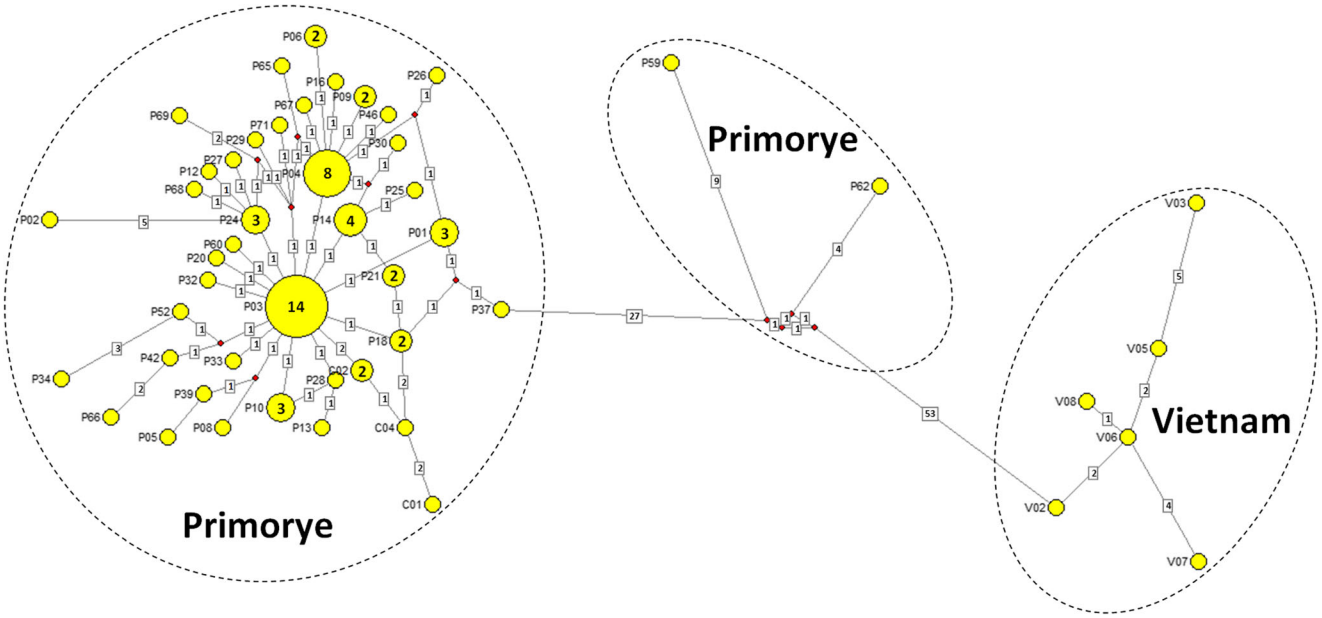

Fig. 3 Median-joining network of *P. mugili* based on mitochondrial COI gene partial sequences. Numbers within yellow rounds, amount of sequences within one haplotype; numbers on lines, a number of mutational steps between haplotypes; numerals, index number of specimen. Dashed ellipses indicate sequence groups of *P. mugili* from Primorye and Vietnam. P (or C) and V, Primorye and Vietnam, respectively. Haplotype P03 (14 sequences) [P03;P07;P15;P17;P35-P36;P40-P41;P44;P49;P55;P58;P61;P72]; Haplotype P04 (8 sequences) [P11;P23;P31;P53-P54;P63-P64]; Haplotype P14 (4 sequences) [P14;P38;P43;P50]; Haplotype P01 (3 sequences) [P01;P19;P56]; Haplotypes P10 (3 sequences) [P10;P22;P51]; Haplotype P24 (3 sequences) [P24;P73;P47]; Haplotype P09 (2 sequences) [P09;P48]; Haplotype P06 (2 sequences) [P06;P70]; Haplotype P21 (2 sequences) [P21;P45]; Haplotype P18 (2 sequences) [P18;P57]; Haplotypes C02 (2 sequences) [C02;C03]

differentiation between populations from Primorye and Vietnam by both 28S and ITS sequences. Authors provided a detailed discussion of this result and hypothesised that differentiation and geographical distribution of *S. spasskii* is caused by distribution of one of its definitive host, *Mugil cephalus*, along Far Eastern coastal waters. Deep genetic differentiation of *P. mugili* from the Russian Far East and Vietnam, revealed in our study, support this hypothesis, because of *Mugil cephalus* also has been reported for *P. mugili* as definitive host (Besprozvannykh et al. 2015a), as so as for other haploporids (Overstreet, Curran, 2005; Andres et al. 2018). Molecular differentiation of *P. mugili* specimens from Primorye and Vietnam by ITS1 rDNA corresponds to the intraspecific level of haploporids (Blasco-Costa et al. 2009). Thus, our results most probably represent a case of initial step allopatric speciation for *P. mugili*, caused by living strategy of its definitive host at evolutionary scale.

For *S. spasskii*, considerable molecular differentiation within the sample from the same biotope of Primorye (Kievka River) has been observed (Besprozvannykh et al. 2015b; Atopkin et al. 2015). This result has been interpreted to be a consequence of the physical isolation of trematodes that infect different species of gastropods (the first intermediate hosts) and a role of the definitive host has not been excluded (Besprozvannykh et al. 2015b; Atopkin et al. 2015). Our results indicate that no deep intrapopulational molecular differentiation exists in *P. mugili* from the Primorye Region. However, results, based on COI gene sequence data, revealed high variation of the trematode in this region. Accepting that all trematodes were from single specimen of *P. haematocheila*, most probable explanation is that molecular variation revealed with COI gene sequence data represents intraspecific variation of *P. mugili* across feeding area of the mullet. Intraspecific molecular diversity of different isolates of *Paragonimus westermanii*, for example by COI gene partial sequence data (approximately 400 bp) ranged from 0.25% for close isolates to 11.5% for distant isolates (Blair et al. 1997; Iwagami et al. 2000). In the light of these data, our results on intrapopulational COI gene variation of *P. mugili* most probably indicate that representatives of isolates of this trematode from different geographical localities, restricted by Primorye Region area, presented in the intestine of definitive host. Following this, existence of gene flow between local populations of *P. mugili* within this area caused by definitive host, which can be revealed with mitochondrial DNA sequence data, can be proposed. This hypothesis can be applied to explain the absence of deep molecular differentiation within population of *P. mugili* by ITS1 rDNA, in spite of *S. spasskii*, from Kievka River, Primorye.

In conclusion, using mitochondrial COI gene sequence data seems to allow uncovering high variation of local populations of haploporid trematode species. This molecular marker, therefore, is useful in comparing populations of other haploporid species.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interest.

References

Andres MJ, Pulis EE, Cribb TH, Overstreet RM (2014) Erection of the haloporid genus *Litosaccus* n.g. and its phylogenetic relationship within the Haloporidae Nicoll, 1914. Syst Parasitol 89:185–194. https://doi.org/10.1007/s11230-014-9521-4

Andres MJ, Curran SS, Fayton TJ, Pulis EE, Overstreet RM (2015) An additional genus and two additional species of Forticulcitinae (Digenea: Haloporidae). Folia Parasitol 62:025. https://doi.org/10.14411/fp.2015.025

Andres MJ, Pulis EE, Overstreet RM (2016) Description of three species of Isorchis (Digenea: Atractotrematidae) from Australia. Acta Parasitol 61:590–601

Andres MJ, Pulis EP, Curran SS, Overstreet RM (2018) On the systematic of some marine haloporids (Trematoda) with the description of a new species of *Megasolena* Linton, 1910. Par Int 67:805–815. https://doi.org/10.1016/j.parint.2018.08.002

Atopkin DM, Beloded AY, Ngo HD (2015) Molecular genetic characterization of the far eastern trematode *Skrjabinolecithum spasskii* Belous, 1954, (Digenea, Haloporidae), a parasite of mullets. Rus J Mol Biol 49:373–379. https://doi.org/10.1134/S0026893315030024

Atopkin DM, Nakao M, Besprozvannykh VV, Ha ND, Nguyen HV, Sasaki M (2018) Morphological and molecular data for species of *Lecithaster* Lühe, 1901 and *Hysterolecithoides* Yamaguti, 1934 (Digenea: Lecithasteridae) from fish of East Asia and phylogenetic relationships within the Hemiuroidea Looss, 1899. J Helminthol. In press

Besprozvannykh VV, .Atopkin DM, Ermolenko, AV, Nikitenko AYu (2015a) Restoration of the genus *Parasaccocoelium* Zhukov, 1971 (Digenea: Haloporidae) and a description of two new species from mugilid fish in the Far East of Russia. J Helminthol 89:565–576. https://doi.org/10.1017/S0022149X14000443

Besprozvannykh VV, Atopkin DM, Ngo HD, Beloded AY, Ermolenko AV, Ha NV, Tang NV (2015b) *Skrjabinolecithum spasskii* Belous, 1954 (Digenea: Haloporidae), parasite of mullets (Mugilidae) from Primorsky Region (Far East of Russia) and Tonkin Bay (North Vietnam): morphology and molecular data. Rus J Mar Biol 41:267–275. https://doi.org/10.1134/S1063074015040021

Besprozvannykh VV, Atopkin DM, Ermolenko AV Beloded AY (2017a) Morphometric and molecular analyses for a new species *Skrjabinolecithum pyriforme* n. sp. (Digenea: Haloporidae) in mullet fish from the Primorsky Region, Russia. J Helminthol 91:625–632. https://doi.org/10.1017/S0022149X16000626

Besprozvannykh VV, Atopkin DM, Ngo HD, Ha NV, Tang NV, Beloded AY (2017b) Morphometric and molecular analyses of two digenean species from the mullet: *Skrjabinolecithum spinosum* n. sp. from the Russian southern Far East and *Unisaccus tonkini* n. sp. from Vietnam. J Helminthol 92:713–724. https://doi.org/10.1017/S0022149X17000943

Blair D, Agatsuma T, Watanobe T, Okamoto M, Ito A (1997) Geographical genetic structure within the human lung fluke, *Paragonimus westermanii*, detected from DNA sequences. Parasitology 115:411–417

Blasco-Costa I, Balbuena JA, Kostadinova A, Olson PD (2009) Interrelationships of the Haploporinae (Digenea: Haloporidae): a molecular test of the taxonomic framework based on morphology. Parasitol Int 58:263–269. https://doi.org/10.1016/j.parint.2009.03.006

Curran SS, Pulis EE, Andres MJ, Overstreet RM (2018) Two new species of *Saccocoelioides* (Digenea: Haloporidae) with phylogenetic analysis of the family, including species of *Saccocoelioides* from North, Middle and South America. J Parasitol 104:221–239. https://doi.org/10.1645/17-189

Felsenstein J (1985) Confidence limits on phylogenies: an approach using bootstrap. Evolution 39:783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x

Hudson RR, Slatkin M, Maddison WP (1992) Estimation of levels of gene flow from DNA sequence data. Genetics 132:583–589

Iwagami M, Ho LY, Su K, Lai PF, Fukushima M, Nakano M, Blair D, Kawashima K, Agatsuma T (2000) Molecular phylogeographic studies on *Pragonimus westermanii* in Asia. J Helminthol 74:315–322. https://doi.org/10.1017/S0022149X00000469

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0. Mol Biol Evol 33:1870–1874. https://doi.org/10.1093/molbev/msw054

Luton K, Walker D, Blair D (1992) Comparisons of ribosomal internal transcribed spacers from two congeneric species of flukes (Platyhelminthes: Trematoda: Digenea). Mol Biochem Parasitol 56:323–327. https://doi.org/10.1016/0166-6851(92)90181-I

Pulis E, Overstreet R (2013) Review of haloporid (Trematoda) genera with ornate muscularisation in the region of the oral sucker, including four new species and a new genus. Syst Parasitol 84:167–191. https://doi.org/10.1007/s11230-012-9401-8

Pulis E, Fayton T, Curran S, Overstreet R (2013) A new species of *Intromugil* (Digenea: Haloporidae) and redescription of *Intromugil mugilicolus*. J Parasitol 99:501–508. https://doi.org/10.1645/12-106.1

Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, Sánchez-Gracia A (2017) DnaSP 6: DNA sequence polymorphism analysis of large data sets. Mol Biol Evol 34:3299–3302. https://doi.org/10.1093/molbev/msx248

Tkach VV, Littlewood DTJ, Olson PD, Kinsella JM, Swiderski Z (2003) Molecular phylogenetic analysis of the Microphalloidea Ward, 1901 (Trematoda: Digenea). Syst Parasitol 56:1–15

Truett GE (2006) Preparation of genomic DNA from animal tissues. In: Kieleczawa J (ed) The DNA book: protocols and procedures for the modern molecular biology. Jones & Bartlett Publisher, MA, pp 33–46