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MOLECULAR PHYLOGENETICS

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Molecular Genetic Characterization of the Far Eastern Trematode *Skrjabinolecithum spasskii*, Belous, 1954 (Digenea: Haploporidae), a Parasite of Mullets

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Abstract—Intraspecific genetic differentiation of the trematode *Skrjabinolecithum spasskii* and its phylogenetic relationships with other species of the family Haploporidae were studied by comparing the nucleotide sequences of a part of the 28S rRNA gene and the ITS1-5.8S-ITS2 rDNA region. Trematodes were isolated from so-iuy mullet *Liza haematocheila* fishes collected in rivers of Primorye and flathead grey mullet *Mugil cephalus* fishes collected in water bodies of Vietnam (27 fishes in total). A phylogenetic analysis showed that *S. spasskii* is close to species of the genus *Capitimitta* of the subfamily Waretrematinae. By intraspecific variation of rDNA sequences, trematodes were divided into three groups with tree different genotypes, which had fixed nucleotide substitutions. Genotype I was found in trematodes from fishes collected in Primorye. Genotype II was detected in trematodes from *M. cephalus* fishes collected in the Tonkin Bay, Cat Ba Island, Vietnam. Genotype III was found in five trematodes from *L. haematocheila* collected in the Kievka River, Primorye. The genetic distances between genotypes I and III from Primorye were 0.4 and 0.65% by 28S and ITS rDNA sequences, respectively. The lowest genetic distances were observed between genotypes II (Vietnam) and III (Primorye), 0.1 and 0.33% by 28S and ITS rDNA sequences, respectively. Possible causes of genetic differentiation of *S. spasskii* from different geographic locations and different definitive host species are discussed.

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Keywords: trematodes, PCR, 28S rDNA, ITS rDNA, 5.8S rDNA, Haploporidae, Mugilidae

INTRODUCTION

The trematodes *Skrjabinolecithum* are typical digenetic flukes that infest various mullets in Primorye [1]. The group has been first isolated from the so-iuy mullet *Liza haematocheila* (Temmincket Schlegel, 1845, former *Mugilso-iuy*) of the Razdol'naya River (former Suifun) and ascribed the status of a new genus, *Skjrabinolecithum* (Belous, 1954), with *S. spasskii* as a type species [2, 3]. Trematodes of the species have recently been found in *L. haematocheila* from the Razdo'naya River [1] and other water bodies of Primorye and in the flathead grey mullet *Mugil cephalus* (Linnaeus, 1758) from Vietnam. The sizes of the body and organs of these trematodes fully agree with the description [2]. However, a comparison with trematodes described in [2] has revealed substantial differences in the morphology of the body and visceral organs, and a new description has consequently been provided for the species *S. spasskii* because the type individuals [2] were not preserved. Trematodes morphologically similar to the species have been detected in various mullets from different creeks of Primorye and a geographically distant region, the Tonkin Bay (Vietnam), posing a question of their genetic identity. We were the first to use several DNA markers to solve the problem.

The objective of this work was to study genetic diversity of the trematode *S. spasskii*, which infests various mullets in the regions in question.

EXPERIMENTAL

We examined 27 trematode *S. spasskii* adults isolated from *L. haematocheila* and *M. cephalus* collected in several creeks of Primorye and one *M. cephalus* fish captured in the Tonkin Bay near the Kat Ba Island, Vietnam (Table 1). Material was fixed with 96% ethanol. Total DNA was extracted by alkaline lysis [4].

A 28S rRNA gene fragment was amplified in the polymerase chain reaction (PCR) with two primers, forward DIGL2 (5'-AAGCATATCACTAAGCGG-3') and reverse 1500R (5'-GCTATCCTGAGGGAAACTTCG-3'). The optimal annealing temperature for the primer pair was 55°C [5].

An ITS rDNA fragment, which included two internal transcribed spacers and the 5.8 rRNA gene (ITS1-5.8S-ITS2), was amplified with the primers BD1

Table 1. Trematode species used in this work (*n* is the number of individuals)

Species	*n*	Definitive host	Reference	GenBank accession no.	
				28S	ITS1-5.8S-ITS2
Haploporoidea					
Waretremadinae					
S. spasskii, Razdol'naya River, Primorye	4	*Liza haematocheila*	Our data	LK022754–LK022757	LK022759–LK022762
S. spasskii, Razdol'naya River, Primorye	1	*Mugil cephalus*	Our data	LK022758	LK022763
S. spasskii, Kievka River, Primorye	11	*Liza haematocheila*	Our data	HE806363–HE806376, LN614538–LN614539	HG530210–HG530219, LN614540–LN614541
S. spasskii, Karasik River, Primorye	4	*Liza haematocheila*	Our data	HE806377–HE806380	HG530220–HG530223
S. spasskii, Tonkin Bay, Kat Ba Island, Vietnam	7	*Mugil cephalus*	Our data	HG530224–HG530230	HG530203–HG530209
Intromugil mugilicolus	1	*Mugil cephalus*	[16]	KC430096	–
Intromugil alachuaensis	1	*Mugil cephalus*	[16]	KC430095	–
Spiritestis herveyensis	1	*Moolgarda seheli*	[16]	KC206500	–
Capitimitta costata	1	*Selenotoca multifasciata*	[16]	KC206497	–
Capitimitta darwinensis	1	*Selenotoca multifasciata*	[16]	KC206498	–
Haploporinae					
Saccocoelium brayi	1	*Liza saliens*	[14]	FJ211234	–
S. cephalic	1	*Mugil cephalus*	[14]	FJ211233	–
S. obesum	2	*Liza ramado*	[14]	FJ211259–FJ211260	–
S. tensum	2	*Liza ramado*	[14]	FJ211257–FJ211258	–
Dicrogaster contracta	2	*Liza aurata*	[14]	FJ211261–FJ211262	–
D. perpusilla	1	*Liza ramado*	[14]	FJ211238	–
Haploporus benedeni	1	*Liza ramado*	[14]	FJ211237	–
Lecithobotry sputrescen	1	*Liza saliens*	[14]	FJ211236	–
Chalcinotrematinae					
Saccocoelioides sp.	1	Unidentified molly	[25]	EF032696	–
Megasoleninae					
Hapladena nasonis	1	*Naso unicornis*	[26]	AY222265	–
Forticulcitinae					
Forticulcita gibsoni	1	*Mugil cephalus*	[14]	FJ211239	–
Atractorematidae					
Pseudomegasolena ishiga	1	*Scarus rivulatus*	[26]	AY222266	–
Atractotrema signai	1	*Siganu slineatus*	[26]	AY222267	–

(5'-GTCGTAACAAGGTTTCCGTA-3') and BD2 (5'-TATGCTTAAATTCAGCGGGT-3') and an optimal annealing temperature of 54°C [6]. The efficiency and specificity of PCR were checked using positive and negative controls, respectively.

Sequencing of the PCR products was carried out by synthesis with chain terminators, using a BigDye Terminator cycle sequencing kit (Life Technologies, United States) and a GA 3130 genetic analyzer (Institute of Biology and Soil Science FEB RAS). The 28S rDNA fragment was sequenced using the internal primers 300F, ECD2, 900F, and 1200R [5] and the amplification primers. The ITS1-5.8S-ITS2 fragment was sequenced using the internal primer 3S [6] and the original primer HaplRv2 (5'-CGTTCAAGATGTCGAT-3'). The sequences were deposited in the European Nucleotide Archive (ENA, Table 1).

Consensus sequences were aligned and analyzed using the programs SeqScape v. 2.6 and MEGA 6.06 [7]; the number of variable sites was determined; and the extent of sequence divergence was estimated (MEGA 6.06). The ancestral sequence was identified on the phylogenetic tree by the maximum likelihood algorithm, using the same software. Genotype networks were reconstructed using Arlequin 3.11 [8].

Phylogenetic relationships were reconstructed using the maximum likelihood algorithm of MEGA 6.06 and the Bayes algorithm of the program MrBayes v. 3.1.2 [9], using a reversible evolution model (with correction for among-site rate heterogeneity and due regard to the proportion of invariant sites [10]). The model was chosen as optimal by the program jModeltest 2 [11]. The Bayes algorithm performed two parallel analyses of ten million generations by four Markov chains. To reconstruct a consensus tree, the trees of the first million generations were rejected. The statistical significance of phylogenetic relationships was assessed by the bootstrap analysis [12] (in the case of the maximum likelihood algorithm) and the posterior probability method (in the case of the Bayes algorithm [9]).

RESULTS

Trematode nucleotide sequences of a 28S rRNA gene fragment (1047 bp) and the ITS1-5.8S-ITS2 rDNA fragment (1511 bp) were obtained by amplification and sequencing and aligned. Of the total fragment lengths, 1041 (99.4%) and 1494 (98.8%) bp proved to be conserved, and 5 (0.5%) and 14 (0.9%) were phylogenetically informative in the 28S rRNA gene and the ITS1-5.8S-ITS2 rDNA fragment, respectively. The 28S rDNA fragment sequences were used to reconstruct the phylogenetic relationships of the trematode with other members of the family Haploporidae (Nicoll, 1914) (Fig. 1). The result showed that *S. spasskii* is close to trematodes of the genus *Capitimitta* (Pulis, Overstreet, 2013) of the subfamily Waretrematinae (Srivastava, 1937), to which *S. spasskii* has initially been ascribed [1, 13]. However, other members of the subfamily, including the genera *Intromugil* (Overstreet, Curran, 2005) and *Spiritestis* (Nagaty, 1948), are far less close to the cluster (*S. spasskii* + *Capitimitta*). Species of the genus *Intromugil* cluster with an unidentified species of the genus *Saccocoelioides* (Szidat, 1954) of the subfamily Chalcinotrematinae, while the species *Spiritestis herveyensis* (Pulis, Overstreet, 2013) forms a separate branch. Species of the genera *Intromugil*, *Spiritestis*, and *Saccocoelioides* cluster with the species *Forticulcita gibsoni* (Blasco-Costa, Balbuena, Kotadinova et Olson, 2009), which has been ascribed the status of a subfamily, Forticulcitinae (Blasco-Costa, Balbuena, Kotadinova et Olson, 2009 [14]).

Table 2. Fixed nucleotide substitutions of the 28S rDNA fragment in different *S. spasskii* genotypes and the results of the ancestral sequence identification test

Site	530	543	564	569	662
Genotype I	C	C	T	C	T
Genotype III	C	T	C	T	A
Genotype II	T	T	C	T	A
Ancestral sequence identification test					
Substitution, $p > 90\%$	C → T	C → T	C → C	C → T	T → A

An intraspecific rDNA sequence variation was observed for *S. spasskii*: trematodes were divided by genotype into three groups, which differed by fixed nucleotide substitutions. Genotype I was characteristic of the trematodes that were isolated mostly from *L. haematocheila* in various Primorye creeks and from one *M. cephalus* fish from the Razdol'naya River. Genotype II was observed in the trematodes that were isolated from *M. cephalus* captured in the Tonkin Bay near the Kat Ba Island (Vietnam). Genotype III was detected in the five trematodes that were isolated from *L. haematocheila* of the Kievka River (Primorye). The differences between Primorye genotypes I and III by the number of differentiating mutations of the 28S and ITS sequences were 0.4 and 0.65%, respectively. The lowest differences (0.1 and 0.33%, respectively) were observed between genotypes II (Vietnam) and III (Primorye). The 28S rDNA sequences of the most differentiated genotypes I and II differ in five sites with fixed point substitutions (0.5%), of which four are T/C transitions and one is a T/A transversion (Table 2). The ITS1-5.8S-ITS2 sequences of genotypes I and II differ by 12 mutations (0.9%), of which nine are transitions (six T/C and three A/G substitutions). Transversions were found in three sites in a terminal region of the ITS1 fragment (Table 3). A sequence comparison showed that genotypes II and III are similar in four of out of the five variable sites of the 28S rDNA and eight out of the 12 variable sites of the ITS1-5.8S-ITS2 fragment (Tables 2, 3). A genotype network recon-

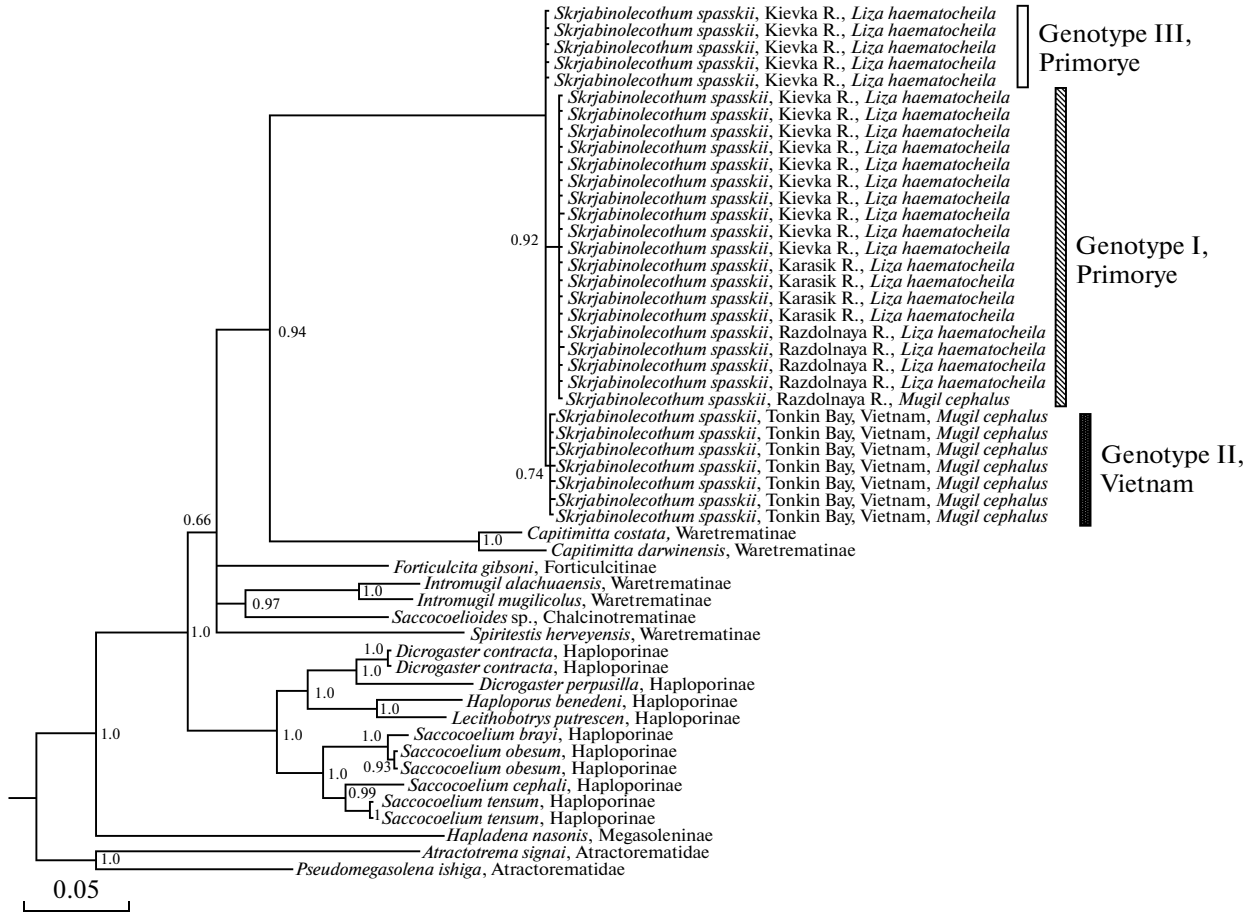

Fig. 1. Phylogenetic relationships in the family Haploporidae as reconstructed using the Bayes algorithm and the results of a partial sequencing of the 28S rRNA gene. The posterior probability, which reflects the significance of a phylogenetic node, is indicated.

structed for the trematode under study on the basis of pooled data showed that minor Primorye genotype III is closely related to Vietnamese genotype II, being divided by five mutation events (Fig. 2). On the other hand, minor Primorye genotype III is separated from major Primorye genotype I by 14 mutation events. Several branches originate from the major Primorye genotype and include the sequences that are characteristic of genotype I, but have additional single nucleotide substitutions.

DISCUSSION

Our analysis of the phylogenetic relationships of *S. spasskii* within the family Haploporidae on the basis of the partial 28S rDNA sequence showed, on the one

Table 3. Fixed nucleotide substitutions of the ITS1-5.8S-ITS2 rDNA fragment in different *S. spasskii* genotypes and the results of the ancestral sequence identification test

Site	140	145	199	242	301	347	348	355	357	1206	1255	1256
Genotype I	C	G	T	C	T	C	C	T	A	T	T	C
Genotype III	C	G	T	T	T	A	T	A	G	C	C	T
Genotype II	T	A	C	T	G	A	T	A	G	C	C	T
Ancestral sequence identification test												
Substitution, $p > 90\%$	C → T	G → A	T → C	C → T	T → G	C → A	C → T	T → A	A → G	T → C	T → C	C → T

hand, that the species belongs to the subfamily Waretrematinae because it clusters with species of the genus *Capitimitta*. On the other hand, species of the genera *Spiritestis* and *Intromugil* of the same subfamily are rather distant from each other in the phylogenetic tree and significantly cluster with members of other subfamilies. Differentiation of the *S. spasskii* 28S rDNA sequences from their counterparts of the *Intromugil* and *Capitimitta* species is relatively high (11.6–12.9%), as is differentiation between *S. spasskii* and *Sp. herveyensis* (12.1%), although the two species belong to the same subfamily [2, 3, 15, 16]. The estimates are far greater than intergeneric differentiation within the subfamily Haploporinae (Nicoll, 1914) (4.8–8%) [14]. The same extent of differentiation is observed between the genera *Saccocoelioides* and *Intromugil* (7.9%). Thus, we suggest that the taxonomic positions of the genera *Spiritestis*, *Intromugil*, *Skjrabinolecithum*, *Capitimitta*, *Saccocoelioides*, and *Forticulcita* are still an open question and require additional morphological and molecular studies.

The rDNA nucleotide sequence analysis revealed substantial intraspecific genetic differentiation for *S. spasskii* (the highest estimates were 0.5 and 0.9% for the 28S and ITS1-5.8S-ITS2 rDNA sequences, respectively; Table 4). Yet the estimates do not exceed the minimal known level of interspecific genetic differentiation between species of the genus *Saccocoeilum* (Looss, 1902) (0.9 and 2.1% for 28S and ITS rDNA sequences, respectively [14]). The extent of differentiation of the rDNA sequences in *S. spasskii* and the topology of the genotype network indicate that Vietnamese genotype II is closely related to minor Primorye genotype III. On the other hand, major Primorye genotype I differs from genotype III to a greater extent, but the two genotypes are still related.

An ancestral sequence identification test showed that fixed substitutions arose in genotype I at a more than 90% probability, resulting in genotypes II and III (Tables 2, 3). This finding, along with the reconstructed genotype network, points to a certain order of differentiation between rDNA sequence variants, i.e., Primorye genotype III was the first to arise, while Vietnamese genotype II arose more recently. We think that genetic differentiation of *S. spasskii* is not associated with infestation of two different definitive host species because of two factors. First, a trematode with genotype I was isolated from an *M. cephalus* fish captured in the Razdol'naya River of Primorye; i.e., *M. cephalus* is not a host infested exclusively by trematodes with Vietnamese genotype II. Second, trematodes with genotypes I and II were found in the same *L. haematocheila* fishes from the Kievka River of Primorye, and this finding rules out the possibility of rDNA nucleotide sequence divergence due to trematode isolation in different definitive host species. The findings indicate that trematodes infest mullets at random. Yet clear differentiation of the *S. spasskii* rDNA sequences by fixed nucleotide substitutions suggests a long-term isolation for the corresponding trematode groups.

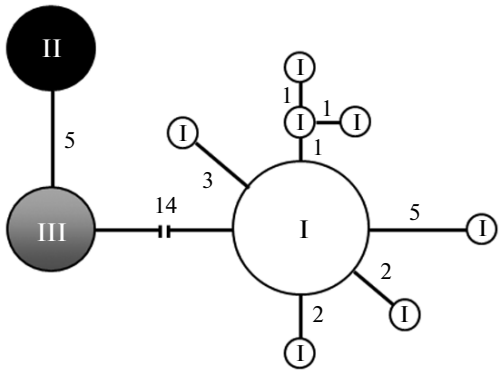

Fig. 2. *Skrjabinolecithum spasskii* genotype network reconstructed using the combined sequences of the 28S and ITS1-5.8S-ITS2 rDNA fragments and the maximum likelihood algorithm. The number of mutation events is shown with Arabic numerals; *S. spasskii* genotypes are indicated with Roman numerals.

Trematode specificity for different first intermediate host species (gastropods) seems to be the most likely cause of the genetic variation observed. The life cycle of the family Haploporidae is associated with two host species, first intermediate and definitive. The intermediate hosts of Haploporidae include freshwater gastropods, e.g., species of the genera *Stenothyra* (Benson, 1856) and *Posticobia* (Smith, 1882) [17, 18]. In contrast to the definite host (mullets), gastropods are infested as a result of high activity exerted by miracidia (first-stage larvae in the trematode life cycle) in searching for a first intermediate host [19, 20]. Moreover, gastropods are incapable of rapid long-distance migrations, and this circumstance might facilitate the formation of isolates that are genetically differentiated to a great extent or evolved into different species. The problem needs further investigation because the first intermediate host species are still unknown for *S. spasskii*.

The genetic variation observed in *S. spasskii* can be related to the fact that adult trematodes are occasionally transferred from one creek to another. For instance, North Pacific *M. cephalus* is known to occur in two forms, sedentary and migratory, which differ in reproductive behavior [21, 22]. The presence of migratory forms in the *M. cephalus* population structure

Table 4. *Skrjabinolecithum spasskii* genotype differentiation (%) by the results of a partial sequencing of the 28S (at the top of the diagonal) and ITS1-5.8S-ITS2 (below the diagonal) rDNA fragments

Genotype	1	2	3
1		0.90	0.65
2	0.50		0.33
3	0.40	0.10	

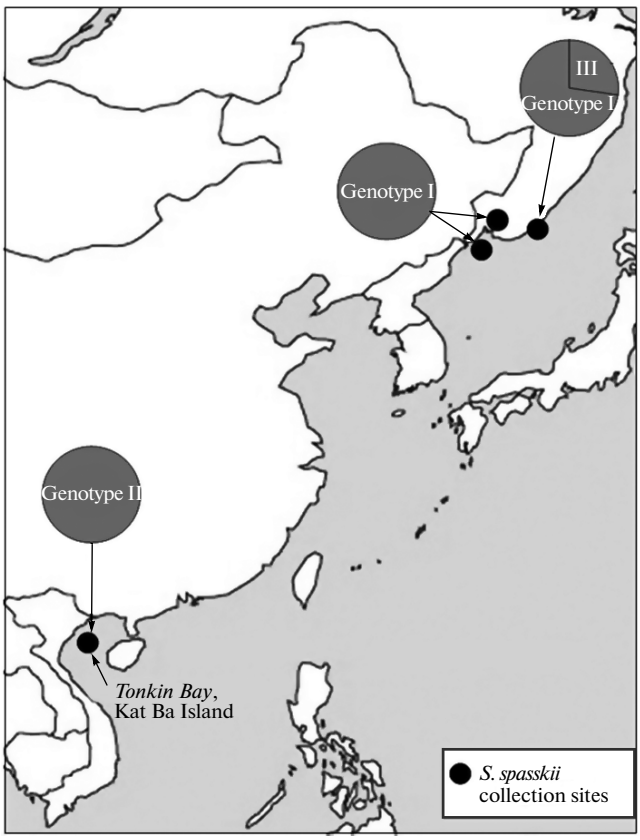

Fig. 3. Geographic locations of different *S. spasskii* genotypes.

does not preclude the possibility of fish migration from mesopopulations along the continental coastal line within the macropopulation area. The results of many studies (Fig. 3) make it possible to assume that differentiation and genotype geography of *S. spasskii*, which is an intestinal parasite of *M. cephalus* and *L. haematocheila*, are determined by the specifics of the phylogeny and life strategy of its definitive hosts [21–24].

Thus, our results suggest initial speciation for trematode *S. spasskii* hemipopulations. At the molecular level, the process is seen as divergence of rDNA sequences. While the process is likely to occur in accord with differentiation of the first intermediate host (gastropods), a role of the definitive hosts in spreading trematodes within their macroareas cannot be excluded.

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