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RESEARCH PAPER

Molecular Diversity of Far-Eastern Trematodes of the Genus *Crepidsotomum* (Allocreadiidae) by Means of Nuclear 28S rRNA and Mitochondrial COI Gene Sequences

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Abstract

The ribosomal 28S rRNA and mitochondrial cytochrome oxidase subunit I (COI) gene fragments variation of several Crepidostomum species, collected from different fish species in Japan and south of Russian Far East, was estimated. On the basis of these data, taxonomical and population genetic structure conclusions for these trematodes were studied. Results of the analysis of the COI gene-based median-joining network indicate the presence of at least five groups of haplotypes that can be distinguished on the basis of definitive host-specificity. Genetic differentiation between these groups has been estimated. Occurrence of C. metoecus and C. farionis and the presence of at least one new Crepidostomum species in Japanese parasite fauna have been confirmed. Ribosomal 28S rDNA-based median-joining analysis indicates low diversity of several Crepidostomum species, reported in latest studies. Critical comments about the evidence of validity of the genus Stephanophiala and species Crepidostomum nemachilus were provided.

Introduction

Trematodes of the genus *Crepidostomum* Braun, 1900 from the family Allocreadiidae Looss, 1902 are intestinal parasites of a wide range of semi-anadromous and freshwater fish species. The life cycle of these trematodes occur through bivalves and freshwater arthropods, which represent first and second intermediate hosts, respectively. Species of the genus *Crepidostomum* are characterized by different host-specificity and distribution around the Holarctic region. The most widely distributed species of this genus are *Crepidostomum farionis* (Muller, 1784) and *C. metoecus* Braun, 1900. These well-known intestinal parasites from Europe, the European part of Russia, and the Russian Far East are actively studied with different approaches (Thomas et al., 1957; Awachie, 1968; Skrjabin & Koval, 1966; Caira & Bogea, 2005; Moravec et al., 2002; Atopkin, Shedko, 2014; Greani et al., 2016; Shimazu, 2016; Soldánová et al., 2017; Petkevičiūtė et al., 2018; Faltýnková et al., 2020). However, there are controversies about co-specificity of the European and Far Eastern *C. farionis* and *C. metoecus* species used for complex morphological and molecular analysis (Shimazu et al., 2016; Soldánová et al., 2017; Petkevičiūtė et al., 2018; Faltýnková et al., 2020; Vainutis et al., 2021). Moreover, restoration the genus *Stephanophiala* and species *Crepidostomum nemachilus* Krotov, 1959 in the recent study on *Crepidostomum* species (Vainutis et al., 2021) is extremely questionable and need to be commented. Taxonomical questions within the genus *Crepidostomum* are not restricted with above-

mentioned examples. The problem of paraphyly of this genus is actively discussed in the recent studies (Soldánová et al., 2017; Atopkin et al., 2020; Faltýnková et al., 2020; Vainutis et al., 2021). Taxonomical status of North American, European and Asian *Crepidostomum* species that do not gather into monophyletic clade on molecular-based phylogenetic tree, is still questionable. Most probably, the taxonomical diversity within *Crepidostomum sensu lato* is deeper that it estimated at present. Taxonomical status of species *Crepidostomumn chaenogobii* Yamaguti & Matumura, 1942, a parasite of fish species of Gobiidae Cuvier, 1816 and Cottidae Bonaparte, 1831 from freshwater reservoirs of Japan and the Sakhalin Island, according to recent studies, should be also reconsidered.

The aim of the present study is to identify trematodes from different fish species, collected from Hokkaido, Japan, using molecular techniques.

Material and Methods

Samples Collection

Adults of the *Crepidostomum* flatworms from the intestine of fish species from Salmonidae Jarocki or Schinz, 1822, Nemacheliidae Regan, 1911 and Cottidae Bonaparte, 1831 families cached in different rivers of Hokkaido, Japan were collected by Prof. Takeshi Shimazu from April till August, 2011 (Table 1). The worms from the fish, previously identified under a microscope, were rinsed in saline, killed in hot distilled water and preserved in 70% ethanol. After fixation, the solution was replaced with 96% ethanol for molecular analysis.

DNA Extraction, Amplification and Sequencing

DNA extraction was performed from whole worms with silica technique as follows: 1. Trematode specimens were dried and homogenized in 3M Guanidine thyocyanate. 2. Homogenate was incubated for 10 minutes at 57°C. 3. Water suspension of silica (silicium dioxide) was added to homogenate (5 µl of 50% suspension per 2 µg of expected amount of DNA), mixed and incubated about 5 minutes at 57°C. 4. Silica/Guanidine solution was centrifuged for 15 sec. at 10 000 rpm, supernatant was elucidated. 5. Sediment was washed using cold washing buffer (20mM TRIS-HCl, pH = 7.4, 1 mM EDTA, 50mM NaCl, 50% ethanol) for four times by adding of 500 µl of the washing buffer, mixing and centrifuging as in previous step. 6. Sediment was dried in air for about one hour and then was resuspended in 100-150 µl of nuclease free water, mixed and incubated for 10 minutes at 57°C. 8. Water suspension was centrifuged for 2 minutes at 12 000 rpm, supernatant was transferred to the clean tubes and used for PCR.

A 28S rDNA fragment was amplified from the Russian specimens using the forward primer Dig12 (5'-AAG CAT ATC ACT AAG CGG-3') and reverse primer 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3') (Tkach et al., 2003). The initial PCR reaction was carried out in a total volume of 25 µl. Each reaction contained 0.25 mM of each primer pair, combined with 3 µl of aqueous solution of DNA and 2x GoTaq Green Master Mix (Promega, USA). Amplifications were performed under the following conditions: 3 min denaturation at 95°C, 30 cycles of 30 s at 95°C, 30 s at 54°C, 2 min 30s at 72°C, and a final extension step at 72°C for 7 min.

Mitochondrial COI gene fragment was amplified using primers MplatCOX1dF (5' – TGTAAAACGACGGCCAGTTTWCITTRGATCATAAG – 3') and MplatCOX1dR (5' – CAGGAAACAGCTATGACTGAAAYAAYAIIGGATCICCACC – 3') (Moszczynska et al., 2009). The initial PCR reaction was carried out in a total volume of 10 µl. Each reaction contained 0.25 mM of each primer pair, combined with 1.5 µl of aqueous solution of DNA and 2x GoTaq Green Master Mix (Promega, USA). PCR parameters began with a 1 min denaturation at 98ºC, followed by 35 cycles of 10 s at 98ºC, 5 s at 50ºC and 1 min at 72ºC, and concluded with a 5 min extension at 72ºC. Efficiency and contamination controls released using positive and negative PCR probes, respectively.

PCR products were directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Massachusetts, USA) as recommended by the manufacturer. The sequence reaction for COI gene was carry out using PCR primers. The internal sequencing primers for 28S rDNA, used in our work, are described in Tkach et al. (2003). PCR product sequences were analysed using an ABI 3500 genetic analyser at the Federal Scientific Center of Biodiversity FEB RAS. Sequences were submitted to the GenBank database (Table 1).

DNA Polymorphism, Differentiation and Median-joining Network Analyses

Nucleotide sequences were assembled with SeqScape v. 2.6 software (Applied Biosystems, Massachusetts, USA). Alignments, estimation of the number of variable sites, codon usage and p-distance estimation were performed using MEGA 7.1 software (Kumar et al., 2016).

DNA polymorphism estimation, differentiation indexes calculation and mismatch distribution analysis were performed with DNASp v.6 software (Rozas et al., 2017). Relationships of nucleotide sequence variants were reconstructed with a median-joining (MJ) algorithm using Network v.10.2.0.0 software, developed by Flexus Technology Ltd., Suffolk, UK (https://www.fluxus-engineering.com/sharenet.htm).

Median-joining network analysis based on 28S rDNA sequences was performed using original data and nucleotide sequences from the GenBank database (Tables 1 and 2).

Table 1. List of taxa with original nucleotide sequence data, incorporated into analysis (n – number of sequences of 28S rDNA/COI gene)

Species	n (28S/COI)	Location	Host	Accession number	
				28S	COI
Crepidostomum farionis	1/1	Nishibetsu River at Nijibestu, Shibecha Town, Hokkaido	*Salvelinus fontinalis*	MW566616	OL763881
Crepidostomum metoecus	5/6	Nishibetsu River at Nijibestu, Shibecha Town, Hokkaido	*Salvelinus fontinalis*	MW566614, MW566617-20	OL763877–80, OL763882 - 83
C. farionis	2/3	Mamachi River at Aoba-ku, Chitose City, Hokkaido	*Oncorhynchus masou*	MW566622-23	OL763847–48, OL763852
C. metoecus	3/3	Mamachi River at Aoba-ku, Chitose City, Hokkaido	*Oncorhynchus masou*	MW566624-25, MW566606	OL763855–56, OL763846
C. metoecus	5/5	Mamachi River at Aoba-ku, Chitose City, Hokkaido	*Salmo trutta*	MW566627, MW566629, MW566632, MW566634	OL763857-61
C. metoecus	5/5	Mamachi River at Aoba-ku, Chitose City, Hokkaido	*Salmo trutta*	MW566640, MW566642 - 44	OL763862 66
Crepidostomum. cf. nemachilus	2/2	Mamachi River at Aoba-ku, Chitose City, Hokkaido	*Barbatula toni*	MW566647-48	OL763870-71
C. cf. nemachilus	5/5	Mamachi River at Aoba-ku, Chitose City, Hokkaido	*Barbatula toni*	MW566653 - 57	OL763872-76
C. farionis	1/3	Tawaramappu River, a branch of the Shibetsu River (the next river on the north of the Nishibetsu River), at Nakashibetsu Town, Hokkaido	*Oncorhynchus masou*	MW566658	OL763868, OL763849-50
C. farionis	1/0	Tawaramappu River at Nakashibetsu Town, Hokkaido	*Salvelinus leucomaenis*	MW566609	-
C. metoecus	3/3	Tawaramappu River at Nakashibetsu Town, Hokkaido	*Salvelinus leucomaenis*	MW566659 - 61	OL763884, OL763891-92
C. metoecus	8/9	Tawaramappu River at Nakashibetsu Town, Hokkaido	*Salvelinus malma kraschenninnikovi*	MW566662 - 69	OL763885–90, OL763893-95
Crepidostomum cf. chaenogobii	1/1	Tobestu River at Tobestu, Hokuto City, Hokkaido	*Cottus amblystomopsis*	MW566670	OL763869
C. farionis	3/1	Mamachi River at Aoba-ku, Chitose City, Hokkaido	*Oncorhynchus masou*	MW566611 - 13	OL763851
C. farionis	5/5	Kedrovaya River, Primorye, Russia	*Oncorhynchus masou*	OL763306 - 10	OL763841-45
C. metoecus	1/1	Kedrovaya River, Primorye, Russia	*Salvelinus curilus*	OL763312	OL763854
C. metoecus	1/1	Steklannaya River, Primorye, Russia	*Barbatula toni*	OL763313	OL763867
C. farionis	1/1	Amgu River, Primorye, Russia	*Oncorhynchus masou*	OL763311	OL763853

Table 2. List of taxa with 28S rDNA nucleotide sequence data from the GenBank database, incorporated into analysis (n – number of sequences)

Accession numbers	n	Location	Host	Reference
		Crepidstomum metoecus		
MT196323 - 24	2	Mamachi River at Aoba-ku, Chitose City, Hokkaido	*Oncorhynchus masou*	Vainutis et al., 2021
MT196325 - 29	5	Mamachi River at Aoba-ku, Chitose City, Hokkaido	*Salmo trutta*	Vainutis et al., 2021
MT196330 - 33	4	Mamachi River at Aoba-ku, Chitose City, Hokkaido	*Salmo trutta*	Vainutis et al., 2021
MT196334 - MT196338	5	Mamachi River at Aoba-ku, Chitose City, Hokkaido	*Barbatula toni*	Vainutis et al., 2021
MT196339 - MT196344	6	Mamachi River at Aoba-ku, Chitose City, Hokkaido	*Barbatula toni*	Vainutis et al., 2021
MT196345 - 47	3	Tawaramappu River at Nakashibetsu Town, Hokkaido	*Salvelinus leucomaenis*	Vainutis et al., 2021
MT196348 - 54	7	Tawaramappu River at Nakashibetsu Town, Hokkaido	*Salvelinus malma krascheninnikovi*	Vainutis et al., 2021
MK818602	1	Kedrovaya River, Primorye, Russia	*Salvelinus curilus*	Vainutis et al., 2021
MK818603	1	Steklannaya River, Primorye, Russia	*Barbatula toni*	Vainutis et al., 2021
FR821405	1	Maximovka River, Primorye, Russia	*Cottus szanaga*	Atopkin, Shedko, 2014
FR821406- 07	2	Kuznetsovka River, Primorye, Russia	*Salvelinus leucomaenis*	Atopkin, Shedko, 2014
MK818593	1	Sakhalin Island	*Salvelinus curilus*	Vainutis et al., 2021
MK818594, 96-97, 99 – 601	5	Sakhalin Island	*Pungitius tymensis*	Vainutis et al., 2021
MK818595	1	Sakhalin Island	*Gammarus* sp.	Vainutis et al., 2021
MK818598	1	Sakhalin Island	*Barbatula toni*	Vainutis et al., 2021
MK818610-16	7	Muravyinka River, Primorye, Russia	*Gammarus* sp.	Vainutis et al., 2021
MT196355 - 58	4	Artyomovka River, Primorye, Russia	*Barbatula toni*	Vainutis et al., 2021
MT217146	1	Artyomovka River, Primorye, Russia	*Barbatula toni*	Vainutis et al., 2021
KY513140	1	Lake Takwatn, Norway	*Pisidium casertanum*	Soldanova et al., 2017
KY513141 - 148	8	Lake Takwatn, Norway	*Gammarus lacustris*	Soldanova et al., 2017
KY513148	1	Lake Takwatn, Norway	*Salmo trutta*	Soldanova et al., 2017
		Crepidostomum farionis		
MW368678	1	Sakhalin Island	*Salvelinus leucomaenis*	Vainutis et al., 2021
MW368679, 82-88	8	Sakhalin Island	*Oncorhynchus masou*	Vainutis et al., 2021
MW368680-81	2	Sakhalin Island	*Salvelinus curilus*	Vainutis et al., 2021
MT217147	1	Sakhalin Island	*Salvelinus leucomaenis*	Vainutis et al., 2021
MT230576-77	2	Tawaramappu River, a branch of the Shibetsu River (the next river on the north of the Nishibetsu River), at Nakashibetsu Town, Hokkaido	*Oncorhynchus masou*	Vainutis et al., 2021
MT230578 - 80	3	Mamachi River at Aoba-ku, Chitose City, Hokkaido	*Oncorhynchus masou*	Vainutis et al., 2021
MW368672 - 76	5	Kedrovaya River, Primorye, Russia	*Oncorhynchus masou*	Vainutis et al., 2021
MW368677	1	Amgu River, Primorye, Russia	*Oncorhynchus masou*	Vainutis et al., 2021
FR821399 - 404	7	Maximovka River, Primorye, Russia	*Oncorhynchus masou*	Atopkin, Shedko, 2014
KY513134-35, 38-39	4	Lake Takwatn, Norway	*Pisidium casertanum*	Soldanova et al., 2017
KY513136-37	2	Lake Takwatn, Norway	*Sphaerium* sp.	Soldanova et al., 2017
MT080780, 83-85	1	Lake Hafravatn, Iceland	*Salvelinus alpinus*	Faltýnková et al., 2020
MT080781-82	2	Lake Hafravatn, Iceland	*Salmo trutta*	Faltýnková et al., 2020
		'*Crepidostomum nemachilus*'		
MK818622-28	7	Sakhalin Island	*Barbatula toni*	Vainutis et al., 2021
MK818617-21	5	Komissarovka River, Primorye, Russia	*Barbatula toni*	Vainutis et al., 2021
FR821408	1	Maximovka River, Primorye, Russia	*Barbatula toni*	Atopkin, Shedko, 2014
FR821408	1	Klyuch River, Primorye, Russia	*Barbatula toni*	Atopkin,Shedko,2014
		Crepidostomum chaenogobii		
MK818589, 91-92	1	Sakhalin Island	*Gammarus koreanus*	Vainutis et al.,2021
MK818591-92	2	Sakhalin Island	*Pungitius tymensis*	Vainutis et al.,2021
MN826678-79	2	Sakhalin Island	*Pungitius sinensis*	Atopkin et al.,2020

Results

Mitochondrial COI Gene-based Analysis

The mitochondrial COI gene fragment, 660 bp in length, was successfully amplified and sequenced for 56 individuals, firstly identified as *C. farionis*, *C. metoecus*, *Crepidostomum cf. nemachilus* Krotov, 1959, and *Crepidostomum cf. chaenogobii* Yamaguti & Matumura, 1942. Overall, 149 (22.5%) variable and 129 (19.5%) parsimony-informative nucleotide positions were observed. Interspecific p-distance values ranged from 0.56 ± 0.26% (*C. metoecus*/*Crepidostomum cf. nemachilus*) to 25.2 ± 2.5% (*C. metoecus*/*C. farionis*), whereas intraspecific values ranged from 0.58 ± 0.25% to 25.2 ± 2.5% for *C. farionis* and from 0.12 ± 0.08 to 8.21 ± 1.1% for *C. metoecus*. Maximal intraspecific p-distance values for both *C. farionis* and *C. metoecus* were extremely high, indicating a presence of at least two species within each sample and/or reflecting geographical isolation between samples within each species.

Median-joining network topology for the overall sample indicated the presence at least five large groups of haplotypes that can be distinguished on the basis of definitive host-specificity (Figure 1). These groups contain a different number of specimens and are characterized by different heterogeneity. The '*oncorhynchus*' group consists of trematodes identified as *C. farionis* ex *Oncorhynchus masou* (Brevoort, 1856) from different Japanese and Russian Far Eastern territories. The '*salvelinus*' group contains specimens of *C. metoecus* ex *Salvelinus* spp. from different rivers of Japan. Also within this group, a specimen, briefly identified as *C. farionis* ex *Salvelinus fontinalis* (Mitchill, 1814) from Nishibetsu River, Hokkaido, has been spanned with three mutational steps. This result explained high intraspecific maximal p-distance values within the whole sample of *C. farionis*; indeed, this trematode belongs to *C. metoecus*. The next 'mixed host' group was directly related to the '*salvelinus*' group and contained specimens of *Crepidostomum cf. nemachilus* and *C. metoecus* from different host species, including *Oncorhynchus masou, Salmo trutta* Linnaeus, 1758, and *Barbatula toni* (Dybowksi, 1869). Within this group, eight haplotypes that differ from each other mainly by one or two mutational steps have been observed. The proposed '*cottus* + *barbatula*' group consists of two specimens, identified as *Crepidostomum cf. chaenogobii* ex Japanese *Cottus amblystomopsis* Schmidt, 1804 and *C. metoecus* ex *Barbatula toni* from the Russian Far East. The median-joining tree showed this Japanese trematode as closely related with the 'mixed host' group, which included *Crepidostomum cf. nemachilus* and *C. metoecus* from different hosts in Japan. This result indicates that Japanese trematode ex

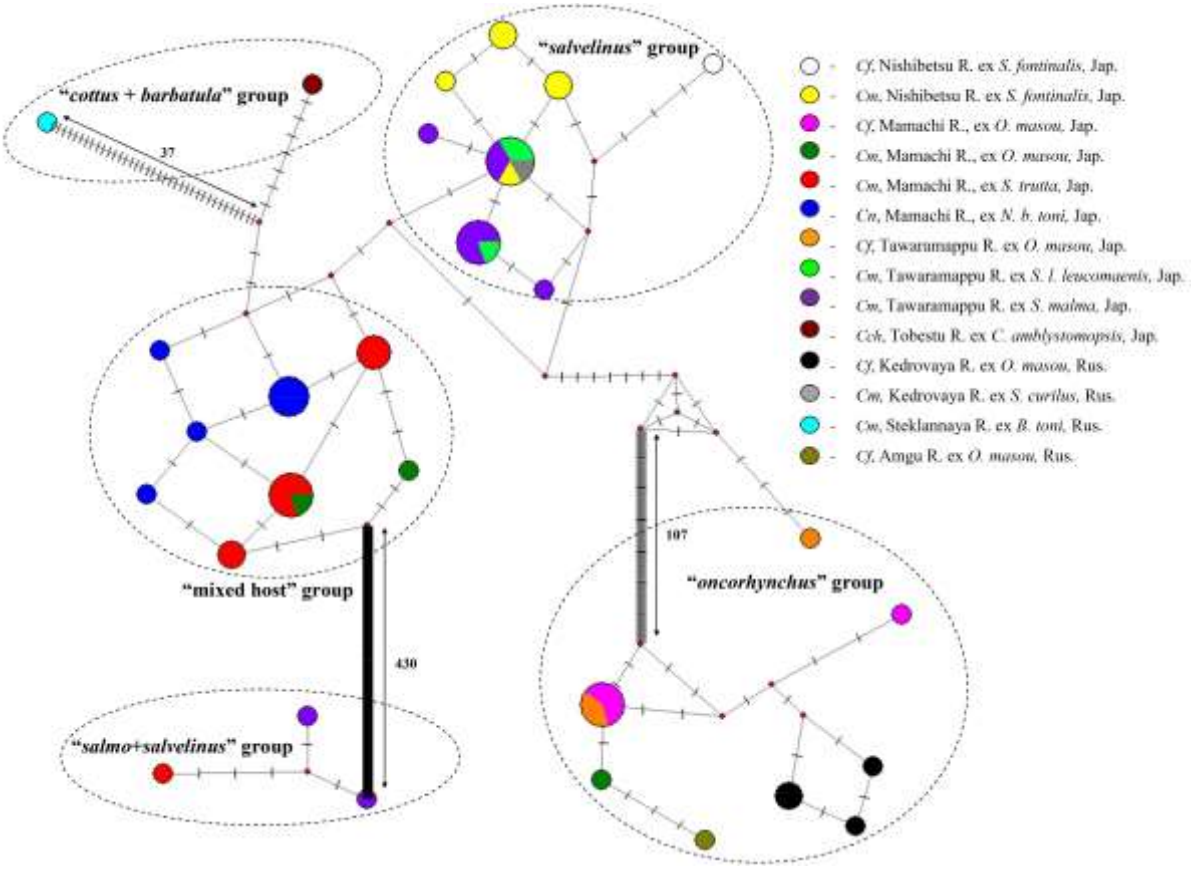

Figure 1. Median-Joining network of *Crepidostomum* species based on 660-bp fragment of mitochondrial COI gene sequences. Short lines on branches represent a number of mutational steps between sequence variants; red points represent median vectors.

C. amblystomopsis represents a specimen of *C. metoecus* that differs from other worms of this species. Worms extracted from *B. toni* from the Russian Far East considerably differ from the Japanese specimens, as well as from the 'mixed host' group, proposing a different taxonomical status for this specimen. The '*salmo* + *salvelinus*' group contained three specimens, first identified as *C. metoecus* from *Salmo trutta* and *Salvelinus malma krascheninnikovi* Taranetz, 1933, collected in different rivers of Hokkaido, Japan. Each of the three specimens possessed its own unique haplotype that differs from each other from one to three mutational steps. This group was directly related to the 'mixed host' group and differs by 430 mutational steps, indicating obvious interspecific differentiation for these two groups.

On the basis of the obtained median-joining network and its interpretation, we estimated p-distance values within and between the revealed groups of haplotypes, and also differentiation index (Fst) and gene flow index (Nm) values between groups (Tables 3, 4 and 5). The '*oncorhynchus*' group considerably differs from the others; p-distance values ranged from 16.74 ± 1.27% to 17.35 ± 1.25% (see Table 3 for details). Genetic differentiation between the '*cottus* + *barbatula*' group and the three groups of salmonid trematodes ranged from 3.86 ± 0.52% to 3.9 ± 0.53%. The '*salmo* + *salvelinus*', '*salvelinus*', and 'mixed host' groups, which included *C. metoecus* and *C. cf. nemachilus*, differ from each other by 0.37 ± 0.13%–0.72 ± 0.28%.

Internal p-distance values (Table 4) were 0.51 ± 0.22% for '*salmo* + *salvelinus*', 0.25 ± 0.11% for '*salvelinus*', and 0.17 ± 0.09% for the 'mixed host' group. These values are compatible with intergroup values, indicating these specimens belong to the same species. The mean internal p-distance value for the '*oncorhynchus*' group was 2.9 ± 0.25%, which can also be interpreted as intraspecific. The internal value for the '*cottus* + *barbatula*' group was 6.67 ± 0.87%. This group includes one specimen of *C. metoecus* ex *Barbatula toni* from the south of the Russian Far East and one trematode specimen ex *C. amblystomopsis* from Japan primarily identified as *Crepidostomum cf. chaenogobii*. The p-distance values between these two specimens were relatively high and can be interpreted as interspecific. The specimen of *C. metoecus* ex *Barbatula toni* differs from the three salmonid groups witn 0.14% ('mixed host'), 0.41% ('*salmo+salvelinus'*) and 0.62% ('*salvelinus'*).

Genetic differentiation index values (Table 5) indicate trematodes from the '*salvelinus*' and '*salmo* + *salvelinus*' haplotype groups look the most genetically related to each other; the differentiation index, Fst, between these samples has minimal value, and the gene

Table 3. Genetic p-distances (%) between different group of haplotypes of trematodes of the genus *Crepidostomum* from Japan and south of Russian Far East.

		1	2	3	4	5
1	"*oncorhynchus*"		1.27	1.29	1.27	1.25
2	"*salvelinus*"	16.79		0.28	0.13	0.53
3	"*mixed*"	17.0	0.72		0.2	0.52
4	"*salmo_salv*"	16.74	0.37	0.53		0.52
5	"*cottus+barbatus*"	17.35	3.9	3.88	3.86	

Table 4. Genetic variation parameters for different groups of haplotypes of trematodes of the genus *Crepidostomum* from Japan and south of Russian Far East. N – number of samples, h – number of haplotypes, Hd – haplotype diversity, Pi – nucleotide diversity, k – average number of pairwise differences, d, % - genetic p-distances

	N	h	Hd	Pi	k	d, %
"*salvelinus*"	19	8	0.842±0.057	0.0025±0.0005	1.661	0.25±0.11
"mixed"	18	8	0.745±0.064	0.0017±0.0004	1.15	0.17±0.09
"*salmo+salvelinus*"	3	3	1.000±0.272	0.00505±0.0016	3.333	0.51±0.22
Overall salmonid trematodes	39	14	0.901±0.023	0.00473±0.00036	3.120	0.31±0.14
"*oncorhynchus*"	13	8	0.859±0.089	0.00497±0.0006	3.282	2.9±0.25

Table 5. Genetic differentiation indexes between group of haplotypes of trematodes of the genus *Crepidostomum* from Japan and south of Russian Far East (below diagonal – Fst, above diagonal - Nm)

		1	2	3	4	5
1	"*oncorhynchus*"		0.01	0.01	0.01	0.12
2	"*salvelinus*"	0.97912		0.21	30.50	3.89
3	"*mixed*"	0.98149	0.70581		0.90	3.72
4	"*salmo_salv*"	0.97196	0.01667	0.35607		6.45
5	"*cottus+barbatus*"	0.80493	0.11383	0.11854	0.07190	

flow index, Nm, is maximal (Table 4). The '*cottus* + *barbatula'* haplotype group is considerably different from all three salmonid trematode groups and; the values of Fst and Nm ranged from 0.07190 to 0.11854 and from 3.72 to 6.45, respectively, suggesting that gene flow occurs between *Crepidostomum* trematodes from different hosts. The '*oncorhynchus*' group is highly different from each of the four other samples by these indexes, as well as by p-distances, confirming high divergence of these trematodes at species level at least.

For detailed analysis of the molecular diversity of salmonid trematodes, parameters of genetic variation and differentiation were estimated (Table 2). Values of genetic variation parameters indicate that '*salmo* + *salvelinus*' is the most diverse salmonid trematode group; it contains three different haplotypes that considerably differ from each other. Moreover, within this group, the nucleotide diversity parameter is markedly higher in comparison to the other groups. The group of trematodes '*salvelinus*' are slightly more diverse in comparison to the 'mixed' group of trematodes. These results indicate that host-specificity has no marked effect on genetic variation of trematode communities. Alongside this, we estimated the genetic diversity within the '*oncorhynchus*' group. Haplotype diversity within this group was similar to that of the '*salvelinus*' and 'mixed' groups, whereas nucleotide diversity, as well as the average number of pairwise differences and internal genetic distance values, was comparable with that of the '*salmo* + *salvelinus*' group, indicating high nucleotide variation of certain variable sites.

Distribution of the pairwise differences was unimodal for '*salvelinus*' and 'mixed host' groups and corresponds to expected expansion model (Figures 2a, 2b, 3a, 3b). Bimodal distribution was observed for the '*salmo* + *salvelinus'* group (Figures 4a, b), indicating potential differentiation through isolation for members

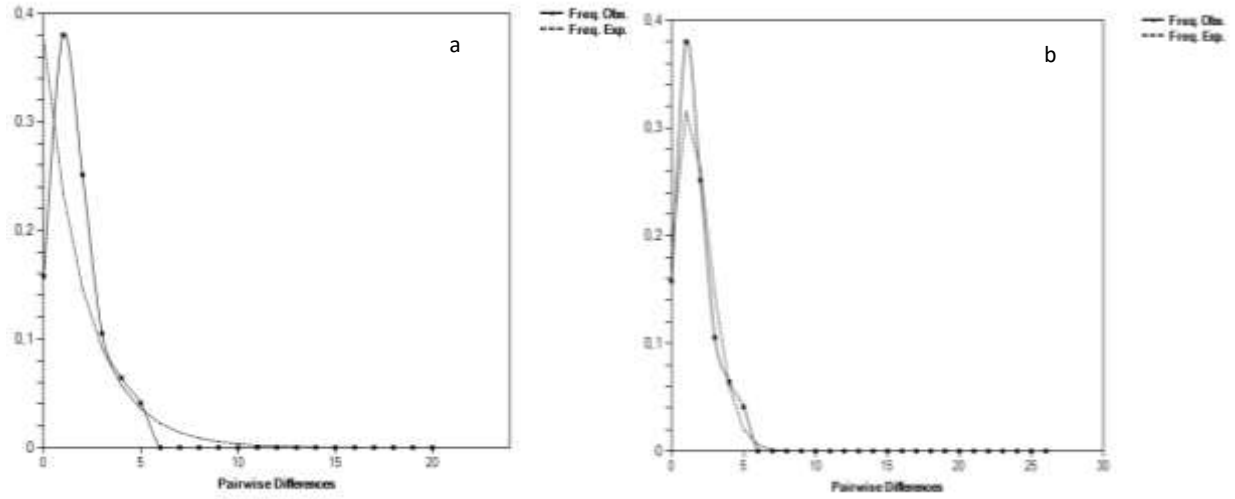

Figure 2. Mismatch distribution of 660-bp fragment of mitochondrial COI gene sequences of the '*salvelinus*' haplotype group: a – for constant population size model, b – for growth-decline population size model.

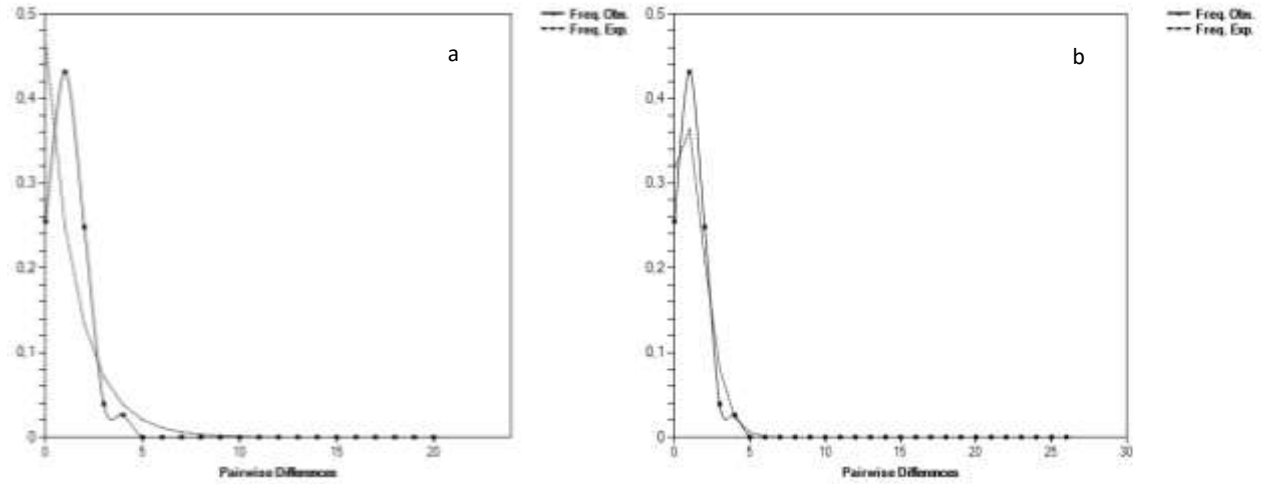

Figure 3. Mismatch distribution of 660-bp fragment of mitochondrial COI gene sequences of the 'mixed host' haplotype group: a – for constant population size model, b – for growth-decline population size model.

of '*salmo* + *salvelinus*'. These results indicate growth events for the '*salvelinus*' and 'mixed host' groups and constant population size for the '*salmo* + *salvelinus*' group. Mismatch distribution analysis for the three above-mentioned groups showed bimodal distribution of pairwise differences, which confirm a presence of considerable molecular differences within trematodes from salmonid fish species. Mismatch distribution analysis indicated an early bottleneck event for the '*oncorhynchus*' group; distribution of pairwise differences looks like hole with a following considerable peak (Figures 5a, b). In summary, results of the mismatch distribution analysis indicate recent expansion events for both trematodes from '*oncorhynchus*' group and worms from '*salvelinus*' and 'mixed host' groups. Taking into account geographical origin of samples studied (mainly Mamachi and Tawaramappu Rivers), it can be proposed that different species from different definitive hosts were undergo by same environmental factors, that realized in similar population-genetic structure of different trematode species; solitary specimens from Russian Far East fit to this interpretation well. Nevertheless, more representative analysis of molecular variation of trematodes from both Japan and Russian Far East must be performed to clarify phylogeographic events for *Crepidostomum* species in these regions.

Ribosomal 28S rDNA-based Median-joining Analysis

The median-joining network based on 28S rDNA sequence data (Figure 6) showed differentiation of *Crepidostomum* specimens from different hosts and location at four groups. Some nucleotide sequences of *C. farionis* and *C. metoecus* from the GenBank and our original data, collected from same hosts and localities of Russian Far East and Japan, were identical. First group has a star-like structure contained all adult specimens of

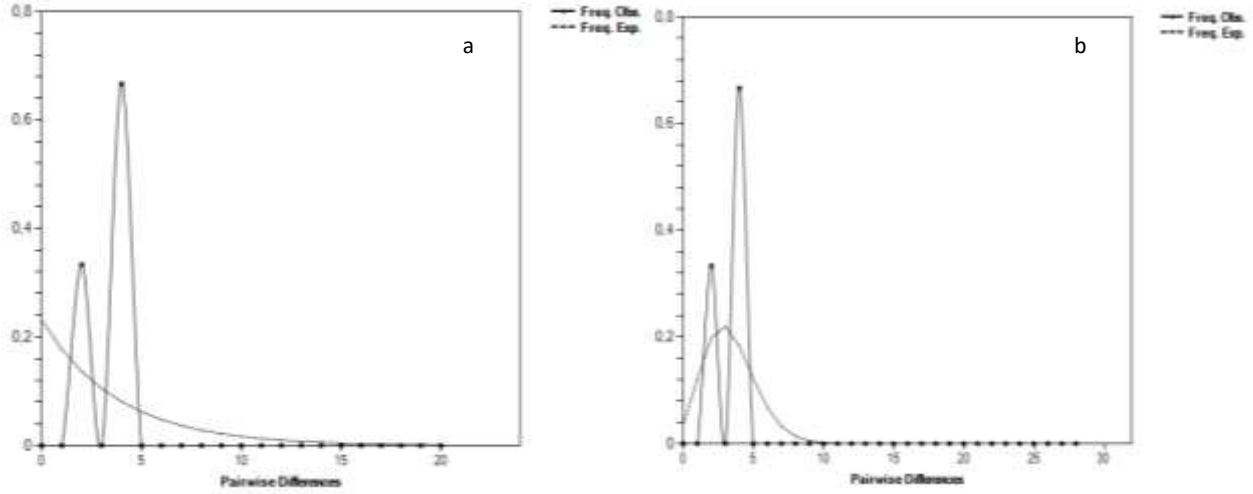

Figure 4. Mismatch distribution of 660-bp fragment of mitochondrial COI gene sequences of the '*salmo+salvelinus*' haplotype group: a – for constant population size model, b – for growth-decline population size model.

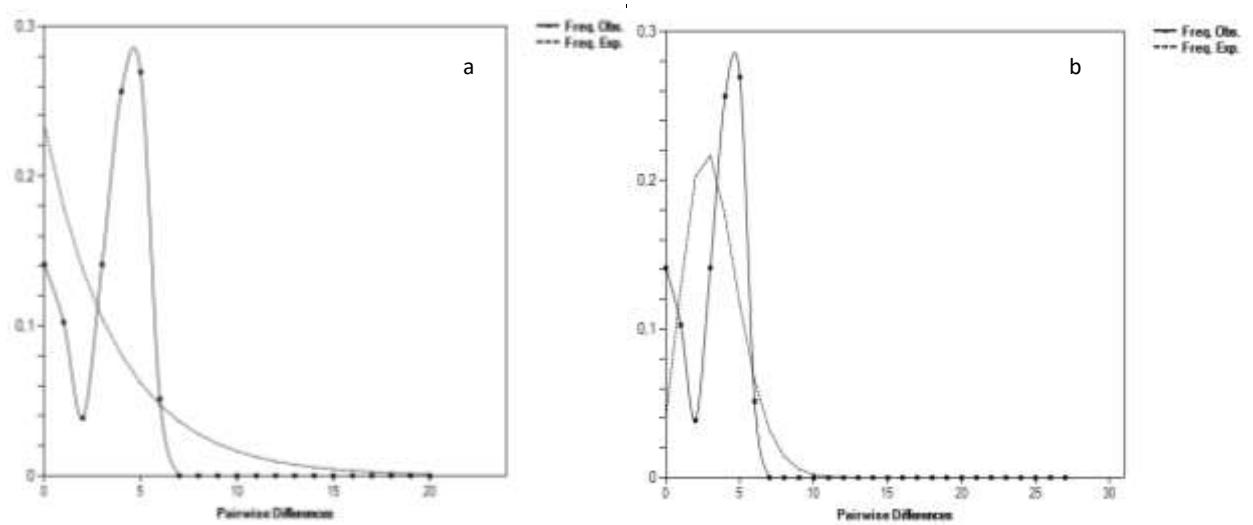

Figure 5. Mismatch distribution of 660-bp fragment of mitochondrial COI gene sequences of the '*oncorhynchus*' haplotype group: a – for constant population size model, b – for growth-decline population size model.

C. farionis ex *O. masou* from Russian Far East (including Sakhalin Island) and Japan, ex *Salvelinus alpinus* (Linnaeus, 1758) and *S. trutta* from Iceland, and cercariae ex molluscs Sphaeriidae Deshayes, 1855 from Norway. Most of listed specimens possess one shared 28S rDNA sequence variant that appears as ancestral (Figure 6, group 1(1)). One Japanese *C. farionis* specimen from Mamachi River (ex *O. masou*) has 28S rDNA sequence variants different from ancestral by one mutational step, as well as sequence variant of specimen ex *O. masou* from Maximovka River (south of Russian Far East) and ex *Salmo curilus* Pallas, 1814, Sakhalin Island (GenBank data). Single Japanese specimens firstly identified as *C. metoecus* from Mamachi River extracted from *O. masou*, appears within first group and differs from ancestral variant with two mutational steps. Most probably this specimen belongs to *C. farionis*.

The group 2 was most diverse and contained mainly specimens of *C. metoecus* from Japan, Russian Far East, Norway and Iceland, and specimens named *Crepidostomum nemachilus* ex *Barbatula toni* from Japan and Russian Far East, including Sakhalin Island (GenBank data). This group was directly related with the group 1 through 21 mutational steps and contained three main sequence variants, which were closely related to each other. Sequence variant #1 was observed for specimens of *C. metoecus* from Japanese Mamachi River (ex *Salmo trutta*) and from different rivers and hosts of Russian Far East. It is notably that same 28S rDNA sequence variant was observed for *C. metoecus* and *C. nemachilus* from *B. toni*, collected from rivers of Russian Far East and Japan and for one trematode specimen firstly identified as *Crepidostomum cf. chaenogobii* ex *C. amblystomopsis* from Japan. This last specimen has high differentiation from Japanese *C. metoecus* individuals by COI gene sequence data and formed separate haplotype group on the MJ network (see Figure 1).

Sequence variant #2 was closely related with first main variant and differs from that with one mutational step. This sequence variant was identified only for trematodes from Russian Far East, including *C. metoecus* ex *Salvelinus leucomaenis* (Pallas, 1814) and *B. toni* from Maximovka and Artyomovka Rivers, respectively, *C. nemachilus* ex *B. toni* from Klyuch River and *Crepidostomum akhmerovi* Vainutis, Voronova, Urabe, 2021, described recently from *B. toni*, Komissarovka River. Two specimens of this species possess unique sequence variants and differ from main with one mutational step.

Sequence variant #3 was unique for trematodes named *C. nemachilus* Krotov, 1959 ex *B. toni* from Sakhalin Island and it was closely related with variant #2 and differ from that with one mutational step. Other trematode specimens that differs from three main

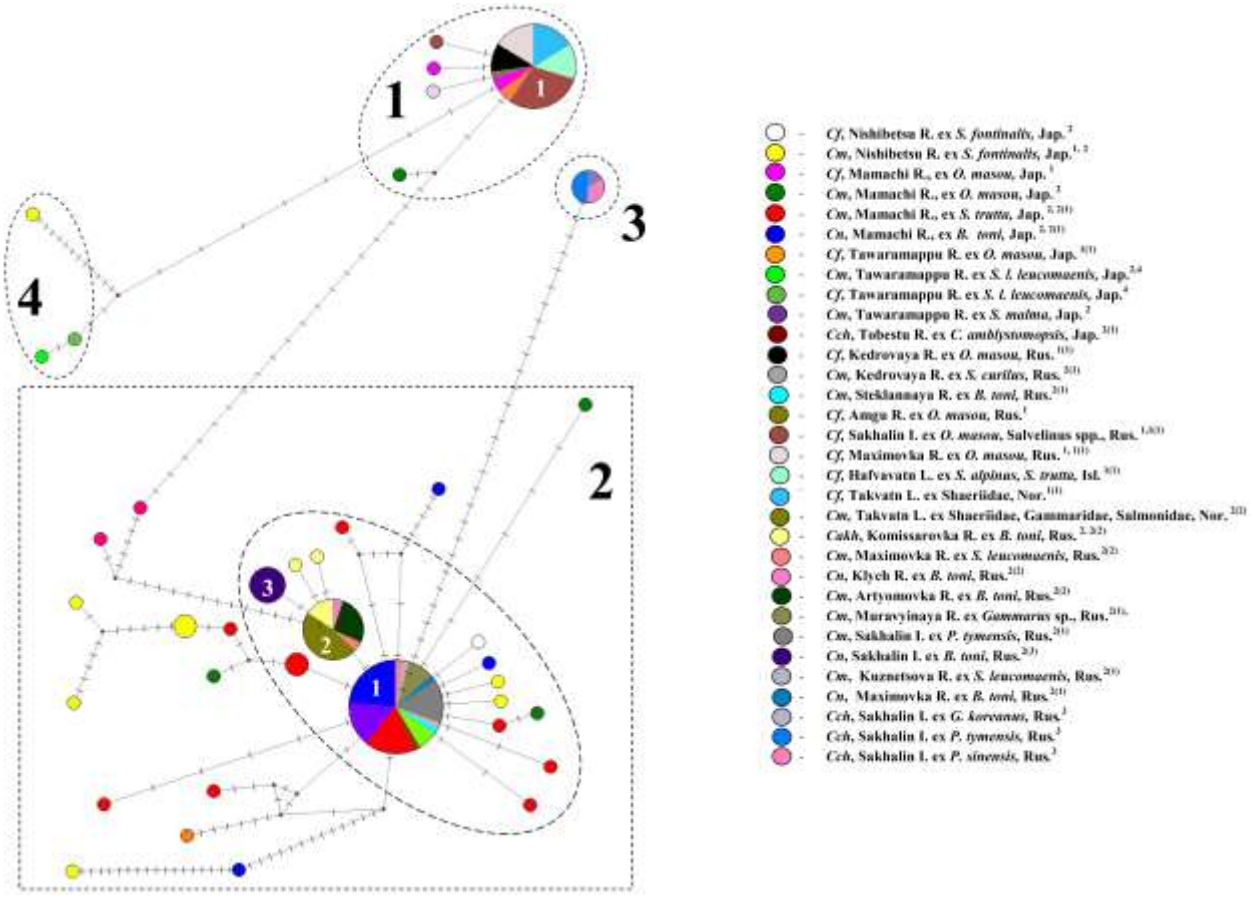

Figure 6. Median-Joining network of Crepidostomum species based on 885-bp fragment of ribosomal 28S rRNA gene sequences. Short lines on branches represent a number of mutational steps between sequence variants; red points represent median vectors.

sequence variants and directly related with variant #1, belongs to *C. metoecus* or *C. cf. nemachilus* from Japanese salmonids and *B. toni*, respectively. Single trematode specimen, firstly identified as *C. farionis* ex *Salvelinus fontinalis* (Mitchill, 1814) from Nishibetsu River, Japan, was within second group, related directly with variant #1 and differs from that with one mutational step. Most probably, this specimen belongs to *C. metoecus*.

Third group was formed by specimens of *Crepidostomum chaenogobii* ex *Gammarus koreanus* Uéno, 1940 and *Pungitius tymensis* (Nikolskii, 1889) from Sakhalin Island. This group was directly related with variant #1 of the second group and differs from it with 30 mutational steps. The fourth group contained three unique sequence variants of Japanese specimens firstly identified as *C. farionis* ex *Salvelinus l. leucomaenis* from Tawaramappu River, *C. metoecus* from the same host and locality and *C. metoecus* ex *Salvelinus fontinalis* from Nishibetsu River. The last specimen differs from two worms from Tawaramappu River with twelve mutational steps. In a whole, the group #4 was directly related with the group #1 through nine mutations.

Genetic p-distances between revealed sequence groups ranged from 1.72±0.38% (Gr.1 / Gr. 4) to 5.53±0.69% (Gr.1/Gr.3); internal p-distances for these groups ranged from 0 (Gr.3) to 1.13±0.27% (Gr.4). These values indicate that all specimens from groups #2 belong to same species in spite of relatively high its heterogeneity, observed on the median-joining network. On the other hand, Japanese specimens from the Group 4 can represent at least one separate species that closely related to *C. farionis*. Internal p-distance values for the Group 4 as well as 12 mutational steps between single worm from Nishibetsu River and two worms from Tawaramappu River indicate that these trematodes can represent different species.

Discussion

In the previous work on the analysis of 28S rDNA of Japanese *Crepidostomum* specimens, firstly identified as *C. farionis*, *C. metoecus* and *C. nemachilus*, indicated high intraspecific molecular diversity for both species (Vainutis et al., 2017). In the present study first detailed analysis of molecular diversity these Japanese trematodes along with specimens from other region was performed by means of ribosomal 28S rDNA and mitochondrial COI gene partial sequences. Obtained results indicate on presence in Far Eastern salmonid fish species of several trematode groups for that species status within the genus *Crepidostomum* can be assigned. On the other hand, our results indicate that some previous taxonimical conclusions, including establishment of new species and separate allocrediid genera, in recent studies (Vainutis et al., 2021) are extremely doubtful and speculative. Firstly, in our study, trematode specimens from Japan and Russian Far East identified as *C. farionis* formed the '*oncorhynchus*' haplotype group on the COI gene-based MJ network, which highly differs from other groups by both p-distance values and differentiation indexes (see tables 3, 4). These trematodes, alongside with specimens from Sakhalin Island, Norway and Iceland (GenBank data), formed the group #1 on the 28S rDNA-based MJ tree and differs from other trematodes with 1.72±0.38% – 5.53±0.69%, indicating on species differentiation level. The minimum of this range revealed between the group 1 and the group 4, which contained three Japanese trematode specimens and, possible, represents at least one additional species. This question should be clarified with additional morphological and molecular studies of Japanese fauna of *Crepidsotomum* species. Vainutis et al. (2021) on the basis of molecular-based phylogenetic analysis results conclude about validation of the genus *Stephanophala* Nicoll, 1909 for *C. farionis* specimens from these regions and from Iceland, Norway and Sakhalin Island. We believe this taxonomical conclusion is quite unreasonable, and the provided diagnosis for this genus in that study is speculative due to the absence of morphological evidence. The description of the genus *Stephanophiala,* as well as its synonymization with the genus *Crepidsotomum*, were performed on the basis of certain morphological characteristics that cannot be ignored for restoration of the genus *Stephanohpiala*. Moreover, there are obvious discrepancies in the comparative morphological analysis of *C. farionis* and *C. pseudofarionis* Faltýnková, Pantoja, Skírnisson & Kudlai, 2020, provided by Faltýnková et al. (2020). Morphological characteristics assigned to *C. pseudofarionis* in this study agree with the original description of *C. farionis* and follow detailed description by Slusarski (1958), whereas the general morphology of *C. farionis* corresponds with that provided by Shimazu (2016) for the ex *Salvelinus fontinalis* specimens from Japan. For these reasons, we conclude that the final taxonomical resolution for validation of *C. farionis* and *Stephanophiala* are far to be realized, and we will keep the name *Crepidostomun farionis* for Japanese trematodes ex *Oncorhynchus masou* and other specimens identical of highly similar to these worms by molecular data until detailed morphological analysis is performed. However, the high values of p-distances between '*oncorhynchus*' group and other groups revealed on COI gene-based MJ network (16 – 17%), obtained in our study, doesn't exclude possible generic status for *C. farionis*, at least in Japan and Russian Far East. These results agree with previous molecular studies (Atopkin et al., 2020) in proposing an erection of a separate genus for these specimens in future.

Molecular differentiation between the '*salvelinus*' and 'mixed host' haplotype groups correspond with differentiation within each one, which allow us to consider trematodes from these two groups as the same species. The results of 28S rDNA-based phylogenetic analyses showed that these Japanese specimens are very close to *C. metoecus* from other studies (Atopkin &

Shedko, 2014; Petkevičiūtė et al., 2018). Moreover, the '*salvelinus*' group includes one specimen of ex *Salvelinus curilus* from the south of Russian Far East, first identified morphologically as *C. metoecus*. Referring to these earlier studies, we recognize the specimens of the '*salvelinus*' and 'mixed host' haplotype groups as *C. metoecus*.

Special attention should be brought to the '*salmo* + *salvelinus*' haplotype group, which includes three Japanese specimens first identified as *C. metoecus* ex *Salmo trutta* and *Salvelinus malma krascheninnikovi.* These specimens are identical to Japanese *Crepidostomum* sp. ex *Cottus amblystomopsis* from the Steklyannaya River from '*cottus* + *barbatula*' by 28S rDNA sequence data (Vainutis et al., 2017). Differentiation of these specimens by mitochondrial COI gene sequence data ranged from 1.52 ± 0.45% to 1.82 ± 0.61%, which can be considered as intraspecific, suggesting a presence in the Japanese freshwater fauna of a potentially new *Crepidostomum* species, which can use salmonid and cottid fish species as definitive hosts. However, this assumption has to be confirmed with additional studies using extended samples and additional molecular markers.

Trematode specimen ex *Cottus amblystomopsis* from the '*cottus* + *barbatula*' group is highly diverse from *C. chaenogobii* in the studies of Atopkin et al. (2020) and Vainutis et al. (2021) and from *C. farionis* and *C. metoecus* in other studies (Atopkin & Shedko, 2014; Soldánová et al., 2017, Petkevičiūtė et al., 2018) by 28S rDNA sequence data. This specimen has direct relationships with the 'mixed host' group through seven mutational steps on the COI gene-based MJ tree and possesses 28S rDNA sequence variant #1 of the group 2 (Figure 2) in the present study. For this reason, we assigned this specimen as *Crepidostomum metoecus* until additional morphological and molecular studies are performed. Another trematode specimen from the '*cottus* + *barbatula*' group, first identified as *C. metoecus* ex *B. toni* from Steklyannaya River, Russian Far East, is highly diverse from other Japanese *C. metoecus* specimens by COI gene sequence data, whereas it identical by 28S rDNA. Hypothetically, this specimen could be recognized as *C. nemachilus* Krotov, 1957, described as the *B. toni* form from Sakhalin Island. Unfortunately, we cannot make comparative molecular analysis with COI gene sequence data of this specimen with trematodes, notified as *C. nemachilus* ex *B. toni* from Sakhalin Island available in the GenBank database from the study of Vainutis et al. (2021), due to the different COI gene region. However, we were able to perform comparative analysis of 28S rDNA sequence data of these worms. Molecular differentiation of these specimens with trematodes ex *B. toni* from the continental part of the Russian Far East by 28S rDNA can be recognized as intraspecific because of this specimen possesses 28S rDNA sequence variant #1 within the group 2 along with the other 45 specimens of *C. metoecus* from different regions and hosts. Moreover, '*C. nemachilus*' from Sakhalin Island, reported in Vainutis et al. (2021), differs from continental specimens of *C. metoecus* with one mutational step, indicating on intraspecific differentiation level. Vainutis et al. (2021) do not provide any morphological evidence for validation of these specimens as *C. nemachilus* described by Krotov (1959). Moreover, differentiation of continental and island trematode specimens from *B. toni,* revealed with mitochondrial COI gene sequence data in that study, also do not provide a basis for recognizing these specimens as *C. nemachilus* Krotov, 1959 without morphological analysis. In the present study we cannot recognize *C. nemachilus* as valid species until detailed comparative morphological analysis of new specimens from Sakhalin Island, continental samples and original material from Krotov (1959) will be performed. We believe that validation of *C. nemachilus* provided by Vainutis et al. (2021) is quite doubtful. For this reason, we omit using this species name for interpretation of our results and keep the name for specimens ex *B. toni* from Russian Far East and Japan as *Crepidostomum cf. metoecus*.

Species *C. akhmerovi* also cannot be recognized with our results of 28S rDNA-based genetic p-distance and median-joining analyses. Most of specimens of these trematodes possesses variant #2 within the group 2 of 28S rDNA sequences that presence in trematodes from Russian Far East, including *C. metoecus* from *S. leucomaenis* and *B. toni* from Maximovka and Artyomovka Rivers, respectively, and *Crepidostomum cf. nemachilus* ex *B. toni* from Klyuch River. Despite of morphological description and morphometric analysis for these worms, molecular data from both Vainutis et al. (2021) and our study do not confirm validity of this species. The 28S rDNA-based tree from Vainutis et al. (2021) showed *C. akhmerovi* located within large polytomic clade together with *C. metoecus* and '*C. nemachilus*'. On the mitochondrial COI gene-based tree, provided by Vainutis et al. (2021), *C. akhmerovi* and '*C. nemachilus*' formed two separate subclades as so as the *C. metoecus* specimens from sister clade. The obvious conclusion from this phylogenetic analysis is that *C. metoecus* possesses same molecular differentiation level as observed between *C. akhmerovi* and '*C. nemachilus*' that prejudice molecular-based delimitation of *C. akhmerovi* and '*C. nemachilus*', stated by Vainutis et al. (2021). Detailed analysis of morphological and morphometric data, provided by Vainutis et al. (2021) allow to consider it as not conclusive for delimitation of *C. metoecus* and *C. akhmerovi*. Firstly, drawing of *C. metoecus* markedly differs from original data and descriptions of other authors (Nybelin, 1932; Dinulescu, 1942; Skrjabin & Koval, 1966; Slusarski, 1958; Shimazu, 2016). On the contrary, general morphology of *C. akhmerovi* is agreed with all previous descriptions of *C. metoecus*, mentioned above. Secondly, there are no hiatus between morphometric parameter values for *C. akhmerovi* and original data for *C. metoecus*, provided in Vainutis et al.

(2021) that do not allow considering these trematodes as different species.

Conclusions

Summarizing our results, we can unambiguously say about presence of *C. metoecus*, *C. farionis* and at least one new *Crepidostomum* species in Japanese parasite fauna, whereas validity of *C. chaenogobii* for Japanese fishes is not confirmed with molecular tools. Validity of *C. nemachilus* and *C. akhmerovi* was not supported, as well as validity of the genus *Stephanophiala*. Results of the mismatch distribution analysis indicate recent expansion events for both trematodes from '*oncorhynchus*' group and worms from '*salvelinus*' and 'mixed host' groups of *Crepidostomum* species. Our results allow to propose that different species from different definitive hosts were undergo by same environmental factors, that realized in similar population-genetic structure of different trematode species.

Ethical Statement

Not applicable.

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Author Contribution

The author of this study perrofmed all experimental works, data analyses and writing the MS.

Conflict of Interest

The author(s) declare no conflict of interest and compliance with all relevant ethical standards. All original molecular data are verified and can be approved with protocols and raw data.

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