

## Population structure of *Opisthorchis felineus* (Trematoda) and its second intermediate hosts – cyprinid fishes in the Ob-Irtysh focus of opisthorchiasis, based on allozyme data

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### Summary

Genetic variability of West Siberian populations of *Opisthorchis felineus* and two species of cyprinid fish, its second intermediate hosts, was studied by isozyme analysis. Low levels of allozyme variation and genetic differentiation in *O. felineus* from the Ob-Irtysh focus of opisthorchiasis were detected. The proportion of polymorphic loci was 21.1 %, the average observed heterozygosity (*Hobs*) was 0.008, and expected heterozygosity (*Hexp*) was 0.052. For most loci in *O. felineus* deficit of heterozygotes (*FIS* = 0.7424) was observed. A comparison of population genetic structure of fish and parasites showed they were not congruent. Estimates of genetic differentiation of the parasite were smaller than for the fish – its intermediate host. Migration and population structure of the second intermediate hosts do not play an important role in formation of the population-genetic structure of *O. felineus* in the Ob-Irtysh focus of opisthorchiasis.

**Keywords:** *Opisthorchis felineus*; cyprinid fishes; Ob-Irtysh river basin; genetic structure of populations; isozymes

### Introduction

In recent years, the population genetic studies of parasites have attracted much attention of researchers. This is not only a practical interest, but also to a large extent the development of new methods of studying the genetic structure of microscopic organisms with poorly studied genome. Significant progress has been made in studying the genetics of trematodes – parasites with complex life cycles, and parthenogenetic reproduction of the larval stages. For some species of trematodes the full sequencing of the mitochondrial genome (Shekhovtsov *et al.*, 2010) and more than 85 % of the transcriptome (Young *et al.*, 2010) are completed. In some cases, the life cycles of trematodes were a model for studying fundamental biological pro-

cesses, such as the restructuring of the genome during the passage of different stages and mitotic recombination (Grevelding, 1999; Bayne & Grevelding, 2003; Semyonova *et al.*, 2005; Be'er, 2005).

Particular attention is paid to trematodes of the family Opisthorchidae, which are pathogenic for humans. Opisthorchiasis is generally caused by *O. felineus* and *O. viverrini* trematodes in Southeast Asia, Central and Eastern Europe. It is estimated that worldwide over 23 million people are infected with *O. felineus* and more than 16 million people with *O. viverrini*, with prevalence rates of 40 to 95 percent in some areas (WHO Report, 1995; Andrews *et al.*, 2008). Large natural focus of opisthorchiasis exists in the Ob-Irtysh basin in West Siberia. Prevalence of this infestation in the local human populations in some areas reaches 80 %. The source of infection is known to be the cyprinid fishes, from which the roach, dace, and especially ide play the leading role. At the center of natural focus 100 % of ides infested by metacercariae of *O. felineus*, with the intensity of infection exceeding 1000 larvae per individual fish.

Genetic variability of *O. felineus* was studied using the methods of sequencing the sites of nuclear and mitochondrial genome (Katokhin *et al.*, 2008; Shekhovtsov *et al.*, 2009), polymerase chain reaction (Pauly *et al.*, 2003), and karyotyping (Polyakov *et al.*, 2010). The authors found a low level of polymorphism in the West-Siberian populations of *O. felineus*, although other species of this genus are known to have a wide genetic variability (Sithithaworn *et al.*, 2007; Saijuntha *et al.*, 2007; 2008a, b), influencing the epidemiological situation (Andrews *et al.*, 2008). To study the population structure of *O. viverrini* microsatellites (Laoprom *et al.*, 2010), RAPD-markers (Sithithaworn *et al.*, 2007) and isozyme markers (Saijuntha *et al.*, 2006 a, b) were used. Despite some difficulties in the interpretation of isozyme profiles of parasites (Rannala, 1991; Vilas *et al.*, 2002), isozyme analysis is a valuable method for re-

solving disputes of systematics and population genetics of parasites (Andrews & Chilton, 1999; Šnábel *et al.*, 2006). The study of population structure of parasites is not possible without data on genetic subdivision of their hosts. Snails play a major role for parasitism of trematodes, but in some cases, there is the effect of population structure and migrations of the second intermediate hosts for the parasite population structure (Keeney *et al.*, 2007). The aim of this research is a comparative study of population genetic structure of *O. felineus* and their intermediate hosts, the cyprinid fishes, in the Ob-Irtysh focus of opisthorchiasis.

## Material and methods

Fish and *O. felineus* metacercariae were collected from 9 rivers of the Ob-Irtysh river basin, West Siberia, Russia, during the period November-January 2008 – 2010 (Fig. 1). The roach *Rutilus rutilus lacustris* Pallas, 1811 (Cypriniformes: Cyprinidae) was obtained from the 11 localities, the ide *Leuciscus idus* Linnaeus, 1758 (Cypriniformes: Cyprinidae) – from 16 localities. A total of 316 specimens of roach and 331 specimens of ide were surveyed (Table 1). Adult *O. felineus* were obtained by infection of the golden (Syrian) hamster *Mesocricetus auratus* in the standard vivarium conditions (Loskutova, 1980). A total of 30 hamsters were infected. We used muscles of the fish infected with metacercariae of *O. felineus*, which were collected from the same rivers (8 localities) as an invasive material. Muscle samples of fish, destined for infection, have been delivered to the laboratory in coolers with a

temperature of  $0 \pm 4^\circ\text{C}$ . They were then viewed under a microscope to assess the extent of infection, identify the trematodes found to a species level and determine their viability. Muscles containing viable larvae of *O. felineus*, were fed to hamsters, 10 g of tissue per animal. Three months later, adult flukes were recovered in the liver, washed several times in saline and then frozen in microtubes for subsequent electrophoretic study. The validity of *O. felineus* identification was confirmed by morphological study. There were collected 192 samples (each containing 1 – 5 individuals) of adult *O. felineus*. Additionally we took the samples of liver tissue and the blood of hamsters (infected and not infected) which were used as a control. Tissue samples were stored frozen at  $-40^\circ\text{C}$ . Proteins were extracted in a standard way using Tris-HCl buffer (pH 8.0) as follows. The sample was homogenized with a glass rod on the ice in an equal volume of buffer, frozen, thawed, and kept for half an hour at  $-4^\circ\text{C}$ , then the homogenate was centrifuged at 3000 g per minute 30 minutes at  $4^\circ\text{C}$ . The supernatant was mixed with a solution for the application of proteins on the gel (40 % sucrose solution, mixed with the bromophenol blue dye).

Vertical electrophoresis in 7.5 % polyacrylamide gel (Maurer, 1971) in a continuous Tris-borate-EDTA buffer system (Peacock & Dingman, 1967) was used for protein separation. Electrophoresis was performed in the following mode: 80 mA, 10 – 12 V/cm for 2.5 hours. Histochemical identification of proteins was carried out in accordance with the guidelines (Korochkin *et al.*, 1977; Richardson, 1986). Six enzymes: malic enzyme (ME, 1.1.1.40), lactate

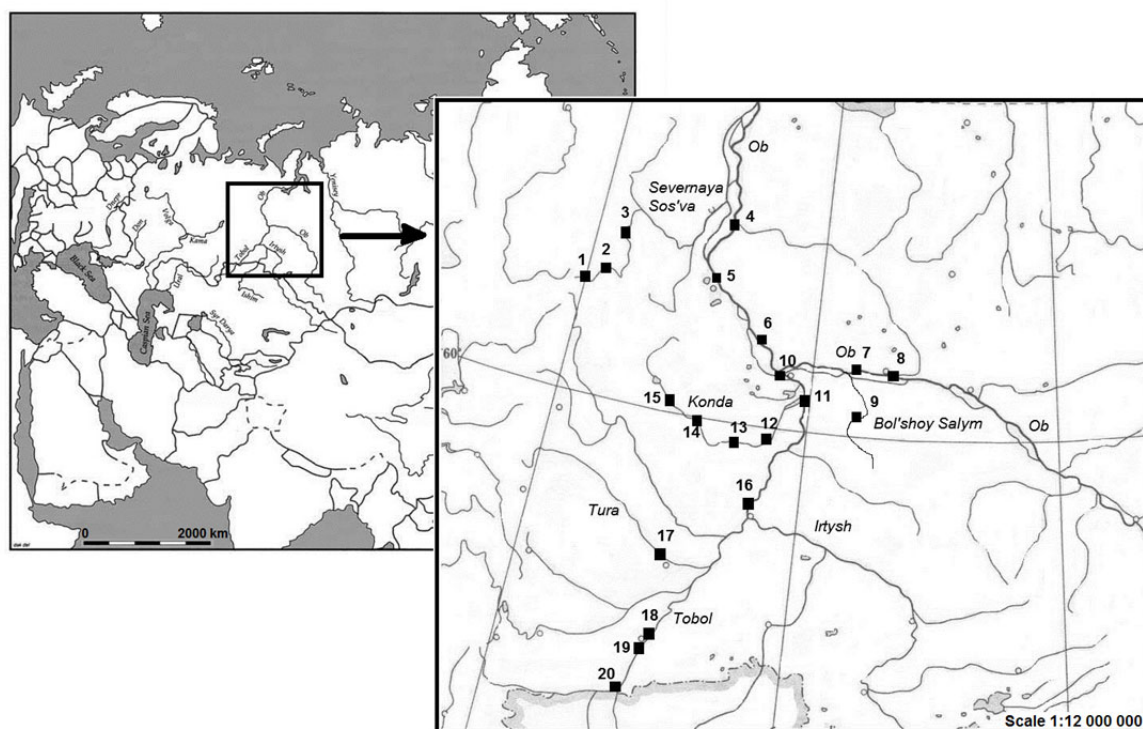


Fig. 1. Places of sample collection: 1 – SSp, 2 – SSs, 3 – SSH, 4 – Okm, 5 – Ob, 6 – Ok, 7 – On, 8 – Os, 9 – BSI, 10 – Ihm, 11 – Ik, 12 – Kb, 13 – Kk, 14 – Km, 15 – Kl, 16 – It, 17 – Tus, 18 – Tk, 19 – Ti, 20 – Tu  
Geographical localities codes listed in Table 1.

Table 1. Places of sample collection and the amount of material

Places of sample collection	Code	Amount of material		
		Ide	Roach	<i>O. felineus</i>
Severnaja Sosva River (Village Pugory)	SSp	34	31	
Severnaja Sosva River (Village Sosva)	SSs	34	34	20
Severnaja Sosva River (Village Khulimsunt)	SSh	30	15	
Ob River (Village Kazym Mys)	Okm	8		21
Ob River (Village Belogorje)	Ob	22		
Ob River (Village Kedrovii)	Ok	22	8	
Ob River (Nefteyugansk)	On	8	17	
Ob River (Sutgut)	Os	14		
Bolshoj Salym River (Village Lyampino)	BSI	31	32	
Irtys River (Khanty-Mansijsk)	Ihm			57
Irtys River (estuary of Konda River)	Ik	18		
Irtys River (Village Bolchary)	Kb	30	30	
Konda River (Village Lugovoj)	Kl			15
Konda River (Village Kondinskoje)	Kk		30	10
Konda River (Village Mezhdurechensk)	Km	31	30	15
Irtys River (Tobolsk)	It	18		20
Tura River (Village Sazonovo)	Tus	10		34
Tobol River (Kurgan)	Tk	12	38	
Ik River (tributary of the Tobol River in Kurgan area)	Ti		51	
Uj River (tributary of the Tobol River in Kurgan area)	Tu	9		
<b>Total</b>	<b>20</b>	<b>331</b>	<b>316</b>	<b>192</b>

dehydrogenase (LDH, 1.1.1.27), aspartate aminotransferase (GOT, 2.6.1.1), superoxide dismutase (SOD, 1.15.1.1), nonspecific esterase (EST, 3.1.1.n) and myogenes, were studied both in the parasites and the hosts.

According to the results of electrophoretic analysis the allele frequencies, proportion of polymorphic loci (*P*), indicators of the mean observed (*Hobs*) and expected heterozygosity (*Hexp*), the indices of Nei's genetic identity (*INei*) and Nei's genetic distance (*DNei*) (Nei, 1972), F-statistics was calculated using the PopGen32 software (Yeh *et al.*, 1999). Dendrograms were constructed on the basis of Nei's genetic distance using Unweighed Pair-Group method with the Arithmetic average (UPGMA) and the same software. A Mantel test (Mantel, 1967) was used to estimate the correlation between geographic and genetic distance matrices within each species. A nonparametric one-tailed Wilcoxon test was used to compare interpopulation genetic distances of fish and *O. felineus*.

## Results

Such enzymes as LDH, ME and GOT in *O. felineus* appear on electrophoregrams in the form of an invariant band. These loci are monomorphic in *O. felineus*, whereas in *O. viverrini* they were found polymorphic (Saijuntha *et al.*, 2007). Myogenes presented six bands on electrophoregrams, all of them were also monomorphic in *O. felineus*. Nonspecific esterases showed 8 zones, 2 of them were polymorphic. Three alleles were recorded at the *EST-5* locus and rare electrophoretic variants at the *EST-2* locus. The remaining bands were completely invariant in all samples and regions. Superoxide dismutase on electrophoregrams was represented by two zones, differing in activity, and corresponds to the two loci. Locus *SOD-1* has lower activity. At this locus, two individuals had a unique electrophoretic mobility associated with the faster allele. The remaining samples were monomorphic and identical.

Table 2. Allele frequencies of polymorphic isozyme loci in *Opisthorchis felineus*

Locus	Allele	Geographical localities <sup>a</sup>							
		SS	Okm	Kk	Km	Kl	Tus	Ihm	It
<i>EST-2</i>	100	1.000	0.952	1.000	1.000	1.000	1.000	0.982	1.000
	112	0	0.047	0	0	0	0	0.018	0
<i>EST-5</i>	100	0.750	0.952	0.400	0.400	0.433	0.029	0.886	0.175
	92	0.250	0.048	0.550	0.500	0.567	0.971	0.114	0.825
	115	0	0	0.050	0.100	0	0	0	0
<i>SOD-1</i>	100	1.000	0.952	1.000	1.000	1.000	1.000	1.000	0.950
	150	0	0.048	0	0	0	0	0	0.050
<i>SOD-2</i>	100	1.000	1.000	1.000	0.400	0.667	0.721	1.000	1.000
	95	0	0	0	0.600	0.333	0.279	0	0

<sup>a</sup> Geographical localities codes listed in Table 1

At the polymorphic SOD-2 locus, two alleles were detected in three of 8 surveyed localities.

We identified 19 loci in *O. felineus*, from them 15 (*EST-1*, *EST-3*, *EST-4*, *EST-6*, *EST-7*, *EST-8*, *GOT*, *LDH*, *ME*, 6 of myogenes) were monomorphic and uniform in all studied samples. Allele frequencies of polymorphic isozyme loci of *O. felineus* from different rivers of the Ob-Irtysh basin are presented in Table 2. The most polymorphic sample

was from the Ob River, where variability was found in 3 loci (*EST-2*, *EST-5*, *SOD-1*). The samples from the rivers Severnaya Sosva and Konda had only one polymorphic locus (*EST-5*). In the remaining samples the variability was found in 2 loci: *EST-5* and *SOD-2* or *SOD-1*. Genetic features of *O. felineus* from the Ob River are the presence of rare alleles at loci *EST-2* and *SOD-1*. These alleles have a limited distribution and, beside the Ob River, are found

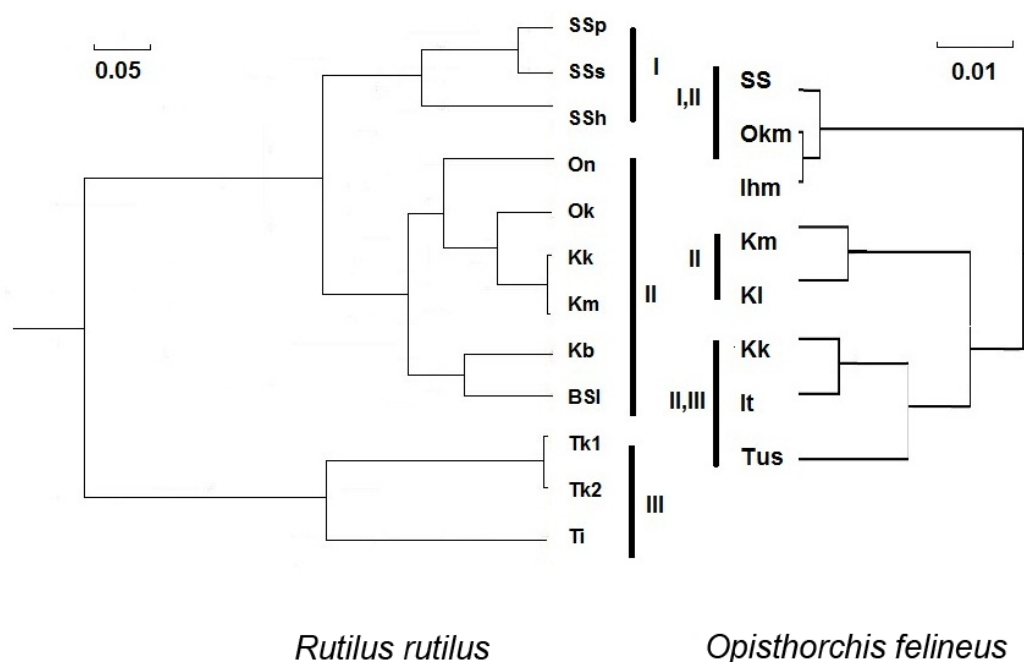


Fig. 2. Tree obtained by UPGMA clustering of Nei (1978) genetic distances for roach (a) and *Opisthorchis felineus* (b) from different geographical localities of the Ob-Irtysh basin. Geographical localities codes listed in Table 1.

only in the trematodes from the Irtysh River, where they are rare too. Genetic characteristics of *O. felineus* from the river Konda is a polymorphism at the locus *SOD-2*, the presence of 3 alleles at the locus *EST-5* and other allele frequencies in comparison with other areas. The fast allele was dominating at this locus in extreme northern areas, whilst a slow allele was prevailing in the south. However in the river Konda the frequency of fast and slow alleles were almost equal.

Low levels of genetic variation were detected in *O. felineus*. Only two loci were polymorphic by 95 % criterion and 4 loci – 99 % criterion. The proportion of polymorphic loci was 21.1 % for the entire sample and ranged from 5.3 to 15.8 % in different localities. The average observed heterozygosity (*Hobs*) was 0.008, expected heterozygosity (*Hexp*) was 0.052. For most loci in *O. felineus* a low frequency of heterozygous genotypes (Table 3) and a deficit of heterozygotes ( $F_{IS} = 0.742 > F_{ST} = 0.337$ ) were observed. Low frequency of heterozygotes was found in the other species of trematodes (Vilas *et al.*, 2003; Laoprom *et al.*, 2010). This can be explained by the Wahlund effect resulting from mixing the genetically heterogeneous groups of fish infested with parasites, as well as a consequence of reproductive patterns involving selfing.

Calculation of Nei's genetic distances showed a low level of genetic differentiation in *O. felineus* brought from different

localities of the Ob-Irtysh river basin (Table 4). The indices of genetic identity ranged from 0.931 to 0.999. The flukes from geographically remote sampling sites, such as Kazym Mys and the Khanty-Mansiysk, were almost identical genetically ( $INei = 0.9995$ ). Genetic distance between the most distant points studied (Kazym Mys and Tura) was 7 %. Cluster analysis based on allele frequencies allozyme loci allowed us to describe the population structure of *O. felineus* in the studied part of its area. The dendrogram shows the division of *O. felineus* populations into two groups (Fig. 2). The first cluster includes the three northern isolates (Severnaya Sosva river, Ob river, Irtysh river at the merging point with the Ob river). Genetic differentiation between these isolates is very poorly expressed, despite the considerable geographical distances. The second cluster includes the central part of the Ob-Irtysh focus of opisthorchiasis (Tobolsk) and the river Konda. This cluster includes two clusters differentiated from each other at the level of interpopulation differences. One of them consists of two isolates from the upper Konda river, the second one embraces the lower Konda river and Irtysh river. Thus, there are three *O. felineus* populations in the studied part of the area: the northern, upper-Konda and Kondo-Irtysh.

Genetic structure of West Siberian populations of cyprinid fishes has been studied using the same isozyme markers previously (Zhigileva *et al.*, 2010; 2013).

Table 3. Genotype frequencies of polymorphic isozyme loci in *Opisthorchis felineus*

Locus	Genotype	Geographical localities <sup>a</sup>							
		SS	Okm	Kk	Km	Kl	Tus	Ihm	It
<i>EST-2</i>	100/100	1.000	0.905	1.000	1.000	1.000	1.000	0.982	1.000
	112/100	0	0.095	0	0	0	0	0	0
	112/112	0	0	0	0	0	0	0.018	0
<i>EST-5</i>	100/100	0.750	0.905	0.400	0.400	0.333	0	0.859	0.150
	100/92	0	0.095	0	0	0.200	0.059	0.053	0.050
	92/92	0.250	0	0.500	0.400	0.466	0.941	0.088	0.800
	115/92	0	0	0.100	0.200	0	0	0	0
<i>SOD-1</i>	100/100	1.000	0.952	1.000	1.000	1.000	1.000	1.000	0.950
	150/100	0	0.048	0	0	0	0	0	0
	150/150	0	0	0	0	0	0	0	0.050
<i>SOD-2</i>	100/100	1.000	1.000	1.000	0.400	0.667	0.647	1.000	1.000
	100/95	0	0	0	0	0	0.147	0	0
	95/95	0	0	0	0.600	0.333	0.206	0	0
<i>P, %</i> <sup>b</sup>		5.3	15.8	5.3	10.5	10.5	10.5	10.5	10.5
<i>Hobs (Hexp)</i> <sup>b</sup>		0.000	0.013	0.007	0.014	0.014	0.014	0.003	0.007
		(0.027)	(0.026)	(0.040)	(0.084)	(0.069)	(0.033)	(0.017)	(0.031)

<sup>a</sup> Geographical localities codes listed in Table 1.

<sup>b</sup> including other (monomorphic) loci (*LDH*, *ME*, *GOT*, 6 loci of *EST*, 6 loci of myogenes)

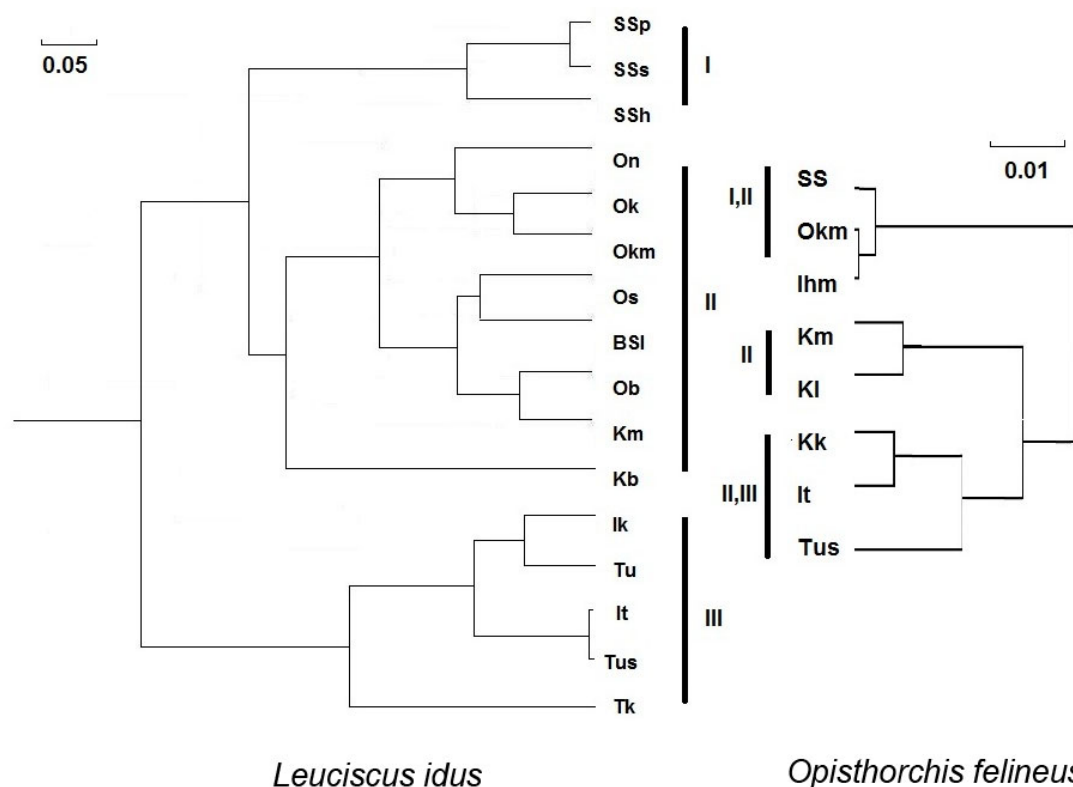


Fig.3. Tree obtained by UPGMA clustering of Nei (1978) genetic distances for *Leuciscus idus* (a) and *Opisthorchis felineus* (b) from different geographical localities of the Ob-Irtysh basin. Geographical localities codes listed in Table 1.

Dendrogram based on Nei's genetic distances in the populations of roach shows that this species can be divided into three groups - the southern (Tobolsk), central (middle stream of the Ob river, Konda river and Bolshoy Salym river) and northern (Severnaya Sosva river), which have considerable specificity of their genetic structure (Fig. 2). Moreover, the southern group of roach (Tobol River and Ik River) is distinct from the others significantly ( $INei = 0.65$ ). The high level of divergence may indicate a different origin of these population groups of roach. The highest rates of genetic identity in Kurgan population of roaches is

observed with other samples from the river Tobol, but an index of genetic identity ( $INei = 0.83 - 0.86$ ) is too low to classify them as one population.

Ide, as well as roach, is divided into three groups of populations - the southern including the Tobol river, Tura and Irtysh rivers, and the central one, including the river Ob (middle stream), Konda river and Bolshoy Salym river, as well as the northern one that includes Severnaya Sosva river. The central group of populations genetically closer to the north than to the south, forming with it a common cluster, but with a rather low level of genetic identity

The matrix of genetic identity and distance <sup>a</sup> in *Opisthorchis felineus* from different geographical localities of the Ob-Irtysh basin

Geographical localities <sup>b</sup>	Okm	SS	Ihm	Kk	Km	Kl	It	Tus
Okm	-	0.9968	0.9995	0.9795	0.9554	0.9620	0.9555	0.9313
SS	0.0032	-	0.9989	0.9928	0.9681	0.9851	0.9758	0.9563
Ihm	0.0005	0.0011	-	0.9848	0.9608	0.9774	0.9630	0.9407
Kk	0.0207	0.0073	0.0153	-	0.9753	0.9927	0.9957	0.9831
Km	0.0456	0.0324	0.0400	0.0250	-	0.9967	0.9689	0.9808
Kl	0.0284	0.0150	0.0229	0.0073	0.0033	-	0.9874	0.9884
It	0.0455	0.0245	0.0377	0.0043	0.0316	0.0127	-	0.9928
Tus	0.0711	0.0447	0.0611	0.0170	0.0194	0.0117	0.0073	-

<sup>a</sup> Above the diagonal – indices of Nei's genetic identity ( $INei$ ), below the diagonal – Nei's genetic distance ( $DNei$ ).

<sup>b</sup> Geographical localities codes listed in Table 1.

(Fig. 3). Surgut ide is genetically close to ide from the Bolshoy Salym river, which is due to geographical proximity of these items. Ide groups from the rivers Tobol and Tura were genetically very similar, the rate of genetic identity ( $INei = 0.99$ ) indicates that they belong to one population, despite the fact that they live in different rivers. Together with ides from the river Uy, they form a common cluster in the dendrogram, which includes the remaining samples from the rivers Irtysh and Tobol. In each cluster the isolates from different parts of a river are united not due to geography, which indicates a significant role of interpopulation exchange within population groups of fish. Values of Mantel correlation coefficient ( $R_M$ ) were low for all of investigated species: in the ide – 0.017, in the roach – 0.012, in *O. felineus* – 0.058. Paired testing of interpopulation genetic distances showed that  $DNei$  for the ide were significantly greater than  $DNei$  for *O. felineus* based on a nonparametric one-tailed Wilcoxon test ( $Z = 3.98$ ,  $P = 0.00007$ ).

## Discussion

A study of eight isolates of *O. felineus* from different localities of the Ob-Irtysh basin shows that a vast part of the range of this species is characterized by a low genetic diversity at isozyme loci. Samples from geographically remote areas have low values of genetic distances. Nevertheless, there are three population groups of *O. felineus* in the part of the area surveyed. The northern group includes the lower reaches of the Ob river to the merging point with the Irtysh river and Severnaya Sosva river. It is characterized by the lowest values of genetic distances between isolates of *O. felineus* within this area and significant genetic differences from other groups. The second group, which is called the upper-Konda one, covers a limited area and includes two isolates from the upper Konda river. It is characterized by unique alleles at the locus *EST-5*, not encountered in other areas, and the unique distribution of allele frequencies of polymorphic loci. The third group includes the isolates from Tobolsk and the Irtysh-Konda confluence. Its genetic peculiarity is the presence of a rare allele at the locus *SOD-1* (as in the northern group) and a relatively high frequency of the slow allele at the locus *EST-5*, which is typical for the isolates from Konda. Thus, this group has a mixed ancestry.

Low levels of genetic variation and differentiation in *O. felineus* obtained from other genetic markers (ITS2, CO1, nuclear marker Pm-int9, ISSR) were noted by other authors (Katokhin *et al.*, 2008; Shekhovtsov *et al.*, 2009; Zhigileva *et al.*, 2013a). This may be either due to phylogeographic reasons (the relatively recent and rapid spread of *O. felineus* in the surveyed area from a single source), or environmental reasons – an intense mixing of the gene pool by migration of the hosts. The high frequency of host migration can negate the local adaptation of the parasite (Gandon & Michalakis, 2002). Low levels of genetic polymorphism of trematodes may be due to other causes

related to the characteristics of their population biology: the presence of parthenogenetic phase, contributing to genetic homogeneity, coupled with a sedentary first intermediate hosts, impeding a broad exchange of genes (Be'er, 2005; 1999; Zhigileva, 2007). At the same time, the closely related species *O. viverrini* in Southeast Asia revealed a very high level of genetic differentiation between the provinces, until the fixation of alternative alleles of isozyme loci that allow to suspect the presence of cryptic species (Saijuntha *et al.*, 2006 a, b; Saijuntha *et al.*, 2007). It is unlikely that in closely related species (*O. felineus* and *O. viverrini*), forming a similar parasitic system, the factors determining population differentiation would be different. Low genetic diversity in *O. felineus* can be explained by the Pleistocene glacial events and subsequent sudden population growth from a very limited group of founders (Brusentsov *et al.*, 2013).

Comparison of the population-genetic structures of parasite populations with the population subdivisions of the intermediate hosts, the cyprinid fishes, demonstrated that they were not congruent. The level of genetic differentiation of the parasite is much lower than that of the fish. Lack of strict correlation between genetic distances of the parasite and the hosts was found in other trematode species, the parasites of frogs (Rannala, 1992), of marine fishes (Cribb *et al.*, 2002) and in obligate parasitic unionoid mussel, the parasites of fish in North America (Zanatta & Wilson, 2011). The genetic population structures of these species were broadly, but not perfectly, congruent. This may be due to facts that host-parasite co-evolution might only partially contribute to the shaping of genetic structure and instead, populations might be often established via localized episodes of host-switching. A variety of historical and temporally independent events may result in alternate adaptive trajectories that differently influence host and parasite gene diversities, along with different rates of protein evolution in hosts and parasites. A strict congruence between the population genetic structure of host and parasite is mostly observed only in the case of highly specific parasites (Thompson, 1994). In our case, *O. felineus* can use several fish species as intermediate hosts. Consequently, we can conclude that migration and population structure of the second intermediate hosts (fish) does not play an important role in shaping the population genetic structure of *O. felineus* in the studied part of the Ob-Irtysh basin. A possible reason for the low differentiation of populations in *O. felineus* may affect its genetic structure of the final host – humans. Due to large population movements and transport of fishery products, there are the conditions for gene flow in populations of the parasite.

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