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First description of genetic diversity for the genus *Metagonimus* using the complete *cox1* gene sequence

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ABSTRACT

The complete *cox1* gene sequence was analysed for *Metagonimus suifunensis* from eight localities in the Russian southern Far East, and the level of variability was compared with that of *Clonorchis sinensis* from the same territory of Russia. These species belong to the superfamily Opisthorchioidea, have a similar distribution in the Russian southern Far East and share second intermediate and definitive hosts, but are distinguished by their first intermediate hosts belonging to different orders of caenogastropods. The data obtained showed that the nucleotide sequence variability of the *M. suifunensis cox1* gene was significantly lower. This fact is considered in connection with a recent bottleneck passage for the *M. suifunensis* population, in contrast to *C. sinensis*, which could be due to the features of the *Metagonimus* life cycle under seasonal freezing temperatures, as well as historical geological and climatic changes in the Russian Far East. These factors could influence the microevolutionary processes and lead to a decrease in the level of variability in the *M. suifunensis* population. Based on the combination of genetic data and historical geo-processes in the region, the probable route of *M. suifunensis* expansion from the northern part of its current area in the Amur River basin to the southern territories of the Russian Far East is justified.

1. Introduction

Trematodes are parasites that make a significant contribution to food-borne infectious diseases, which are a worldwide health problem. Among food-borne parasites, *Metagonimus* spp. (Trematoda: Opisthorchioidea) were included in a list of the most important trematodes in the world (Fürst et al., 2012): they cause severe gastrointestinal disorders in humans and have high epidemiological significance (Chai et al., 2005). In eastern and south-eastern Asia, 10 species of the genus *Metagonimus* have been described (Chai and Jang, 2017; Tatonova et al., 2018). Five of the species, including *Metagonimus yokogawai*, *Metagonimus takahashii*, *Metagonimus miyatai*, *Metagonimus minutes* and *Metagonimus katsuradai*, have been recorded in humans (Chai and Jang, 2017). *Metagonimus yokogawai* has the greatest importance among representatives of this genus. In different regions, the number of people infected with this parasite depends on the food habits of the population and can reach 70% (Chai et al., 2005). This species also found in humans in the territory of the Russian Far East (Miropolskaya and Molochny, 2014; Solovyeva et al., 2015; Revutskaya and Polyakov, 2016). In 2017, based on the ITS2 region and 28S gene of nuclear ribosomal DNA, it was established that the trematoda known as *M. yokogawai* in the Russian southern Far East belongs to another species, *Metagonimus suifunensis* (Shumenko et al., 2017). This conclusion was confirmed by data based on the sequences of the *cox1* gene of mitochondrial DNA (mtDNA) (Tatonova et al., 2018). At the same time, genetic data of the flukes from different localities of the Russian southern Far East did not reveal any *Metagonimus* specimens corresponding to *M. yokogawai*, according to molecular markers.

In addition to clarifying the phylogenetic status of cryptic species, we compared 64 sequences of the complete ITS1 region of rDNA for *M. suifunensis* from six localities, but only one non-parsimony informative substitution was found. However, despite the low detected variability, the first known phylogeographic data for this species was obtained (Tatonova et al., 2018). The study of microevolutionary processes that reflect population origins and the causes of parasite spread (Criscione et al., 2005) can be useful in assessing risks and controlling various invasions. For example, based on the data of 119 full-length mitochondrial genomes of *Schistosoma japonicum* from different countries, analysis of results

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revealed that the evolutionary processes within the parasite population are related to the global historical process, the transition of human communities from hunting and gathering to agrarian activity (Yin et al., 2015).

Since mitochondrial markers are more sensitive and accumulate mutations faster, many researchers use the nucleotide sequences of mtDNA genes to describe the phylogeographic information of parasites. The *cox1* mtDNA gene is one of the most widely used markers for studies of genetic diversity of different parasites (Falla et al., 2015; Lagrue et al., 2015) including the species of the superfamily Opisthorchioidea (Brusentsov et al., 2013; Dung et al., 2013). However, it should be noted that partial sequences of the *cox1* gene were used in these studies, while data on the complete sequences of mtDNA genes may provide more information for analysis. For example, despite the absence of a statistically significant differentiation between *Clonorchis sinensis* samples from Russia and Vietnam, a clear geographic vector (from south to north) is revealed in the distribution of haplotype frequencies and nucleotide diversity along the complete sequences of the *cox1* gene, indicating local parasite adaptations to environmental conditions (Chelomina et al., 2014; Solodovnik et al., 2018). Therefore, the use of full-length sequences is of particular use for the studied parasite. The aim of this study was to analyse the complete *cox1* gene sequences for *M. suifunensis* from eight localities in the Russian southern Far East and compare the level of variability with that of *C. sinensis* from the same territory of Russia (Chelomina et al., 2014 and additional sequences obtained in this study). We used the data for *C. sinensis* because both of these species belong to the superfamily Opisthorchioidea, have a similar distribution in the Russian southern Far East and share second intermediate and definitive hosts (Posokhov, 2004). Comparison of these species variabilities will assist understanding of the historical processes occurring in this area and to predict the possible spread of the parasites.

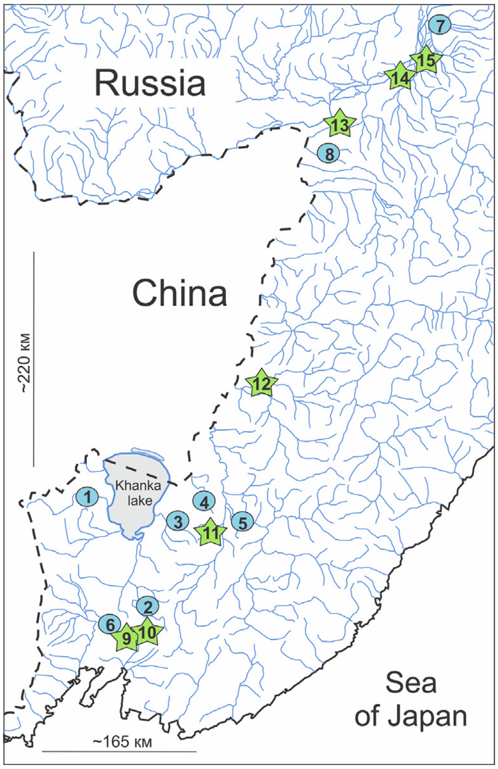

Fig. 1. Sampling locations in the Russian Far East. Circles and stars denote the sampling locations for *Metagonimus suifensis* (this study) and *Clonorchis sinensis* (this study, Chelomina et al., 2014 and specimen FJ381664 from GenBank), respectively. 1 – Komissarovka, 2 – Ilystaya, 3 – Odarka, 4 – Sorochevka, 5 – Arsenjevka, 6 – Komarovka, 7 – Anuj, 8 – Odyr, 9 – Kondratenovka, 10 – Soldatskoe, 11 – Kronshtadtka, 12 – Magdikovoe, 13 – Khabarovsk, 14 – Sindinskoe, 15 – Gassi.

2. Materials and methods

2.1. Sample collection

Adult worms of *M. suifunensis* from eight localities in the Russian southern Far East were used in this study. Adult flukes from all localities (the Komarovka, Komissarovka, Ilystaya, Odarka, Sorochevka and Arsenjevka Rivers in Primorsky Region) were obtained by feeding infected cyprinid fish to laboratory rats (independent experiments for each locality) (Shumenko et al., 2017). In addition to the samples of *M. suifunensis* from the Primorsky Region, samples of this species was obtained by feeding infected *Phoxinus phoxinus* that were caught on the Anuj and Odyr Rivers (Khabarovsk Region) to laboratory rats.

Clonorchis. sinensis samples were collected from four localities in the southern Far East of Russia: the Komarovka River (Kondratenovka), Soldatskoe Lake (Primorsky Region), Sindinskoe Lake and Gassi Lake (Khabarovsk Region). Information about the collection of the parasitological material are given in Chelomina et al. (2014). The map of sampling locations is shown in Fig. 1. Liver flukes were stored in 96% ethanol. Euthanasia, maintenance and use of the laboratory animals in the experiments were carried out in accordance with the Committee on the Ethics of Animal Experiments of the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Russia (Permit Number: 3 of 02.06.2011).

2.2. DNA extraction, amplification and sequencing

DNA was extracted from individual flukes of *M. suifunenis* and *C. sinensis* using the HotSHOT technique (Truett et al., 2000). The complete mitochondrial *cox1* gene of *M. suifunenis* was amplified by PCR using the following primers: forward MPF26 (5′-CTG-TCC-GTC-TTC-AAA-ATG-GG-3′) and reverse McR4 (5′-CTC-ACC-TCT-TCC-TAC-AAC-TCT-C-3′), designed with online software OligoAnalyzer version 3.1 (https://eu.idtdna.com/calc/analyzer) based on the sequence of *M. yokogawai* (GenBank accession number KC330755). PCR was performed in a total volume of 20 μl and contained 0.5 mM of each primer, 10 μl of GoTaq® Master Mix (Promega, USA), and 6 μl of DNA. PCR was performed in a GeneAmp 2720 Thermal Cycler (Applied Biosystems, USA) using the following cycling conditions: a 1 min initial denaturation step at 95 °C; 35 cycles of 30 s at 94 °C, 1 min at 57 °C, and 2 min at 72 °C; and a 7 min extension at 72 °C. Negative and positive controls with both primers were included. The following newly designed primers were used for sequencing: MPF26, McR4, CF2, CF7, CR1, CR7 and MPR30 (Table 1). For *C. sinensis*, the primers for amplification and sequencing, the composition of the reaction mixture, and PCR cycling conditions are reported in Chelomina et al. (2014). PCR

Table 1
Nucleotide sequences of primers used in this study.

Primer name (direction)	Sequence 5′-3′	Position on the complete *cox1* gene, bp
MPF26 (forward)	CTGTCCGTCTTCAAAATGGG	external
McR4 (reverse)	CTCACCTCTTCCTACAACTCTC	external
CF2 (forward)	TTTTCTTCTTCTTGATGCCG	191–210
CF7 (forward)	GGTCGTATAGGACTGTGGTA	1136–1155
CR1 (reverse)	CAAAACCACCAATCAAGACC	210–229
CR7 (reverse)	ATGTCCAAAGAACCAAAACA	683–702
MPR30 (reverse)	TCCCCCAATCCTAGCCATAG	1340–1359

products were sequenced with the ABI 3130 Genetic Analyzer (Applied Biosystems, USA; at the Federal Scientific Center of the East Asia Terrestrial Biodiversity (FEB RAS), Russia) using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

2.3. Analysis of genetic data

The obtained sequences were visualised in FinchTV version 1.4.0, and were assembled and aligned in MEGA version 5 (Tamura et al., 2011). GenBank accession numbers of sequences used in this study are provided in Table 2. The nucleotide sequence of the complete *cox1* gene of *M. yokogawai* (KC330755) from the Republic of Korea was downloaded from GenBank to comparing our results with the closest *Metagonimus* species. In addition, we used 40 *cox1* sequences of *C. sinensis* from Russia, which were analysed by Chelomina et al. (2014) and added 23 new *cox1* gene sequences to the analysis.

Sequence polymorphisms, amino acid variability and genetic distances among samples were estimated using the MEGA programme. The number of haplotypes (*H*) and segregating sites (S), and the level of nucleotide (π) and haplotype (Hd) diversity were detected using DnaSP version 5.10 (Librado and Rozas, 2009). The coalescence process for estimating the confidence intervals of the values of haplotype and nucleotide diversity was modelled in the same programme (Hudson, 1990). For estimation of the differentiation values among geographical populations, *Fst* and Nei's mean number of pairwise differences were calculated in Arlequin version 3.11 (Nei and Li, 1979; Slatkin, 1995). Nucleotide distribution graphs were also constructed with DnaSP (Rogers and Harpending, 1992).

Minimum spanning trees (MST) (Rohlf, 1973) generated in Arlequin were used for reconstruction of intraspecific phylogeny for the total dataset of *M. suifunensis* and for 63 complete *cox1* sequences of *C. sinensis* from Russia.

Table 2
Complete mitochondrial *cox1* gene sequences analysed in this study.

Species/localities	Number of sequences	GenBank accession number
Metagonimus suifunensis		
Komissarovka, Primorsky Region, Russia	11	**MK736830–MK736840**
Komarovka, Primorsky Region	5	**MK736841–MK736845**
Sorochevka, Primorsky Region	1	**MK736846**
Arsenjevka, Primorsky Region	6	**MK736847–MK736852**
Odarka, Primorsky Region	9	**MK736853–MK736861**
Ilystaya, Primorsky Region	7	**MK736862–MK736868**
Odyr, Khabarovsk Region, Russia	13	**MN116480–MN116492**
Anuj, Khabarovsk Region	1	**MK736869**
Metagonimus yokogawai		
Korea	1	KC330755
Clonorchis sinensis		
Magdikovoe, Primorsky Region	13	KJ204560–KJ204572
Kronshtadtka, Primorsky Region	13	KJ204573–KJ204585
Kondratenovka, Primorsky Region	15	KJ204586–KJ204598, **MN116457, MN116458**
Soldatskoe, Primorsky Region	9	**MN116459–MN116467**
Sindinskoe, Khabarovsk Region	10	**MN116468–MN116477**
Gassi	2	**MN116478, MN116479**
Khabarovsk, Khabarovsk Region	1	FJ381664

Accession numbers in bold are newly determined sequences.

The mismatch distribution graph was used to demonstrate differences in substitution between pairs of sequences using DnaSP. The sum of square deviations (*SSD*) and the Harpending's raggedness index (Hri) with 1000 bootstrap replicates were used in Arlequin to test whether the observed distributions deviated from those expected under the population expansion model (Harpending, 1994). Fu's *Fs* (Fu, 1997) and Tajima's *D* (Tajima, 1989) were calculated in Arlequin for estimation of deviations from selective neutrality. Estimation of probabilities of the observed values of both statistics was carried out using a simulation-based coalescent process.

We used the nucleotide substitution rate of 2.5% per million years to estimate the time of species divergence (Attwood et al., 2008).

2.4. Data accessibility

Data are available in Mendeley Data: Tatonova, Yulia (2019), "MetagonimusClonorchis_cox1", Mendeley Data, v3, https://doi.org/10.17632/ttr6nz56c3.3.

3. Results

3.1. Sequence analysis

The length of the complete nucleotide sequences of the *M. suifunensis cox1* gene is 1539 bp, which is similar to that of the *M. yokogawai* complete sequence (KC330755). The stop-codons are TAG and TAA for *M. suifunensis* and *M. yokogawai*, respectively. The *p*-distance between these species is 0.1528 (±0.0086): 229 nucleotide substitutions (including 25 non-synonymous substitutions) were found between the species (Fig. 2). The approximate divergence time of the species was about 6.1million years ago. At the amino acid level, only two codons (UAA, GCC) were not found for the *cox1* gene of *M. suifunenis*, whereas for *M. yokogawai*, five codons (UAG, CUA, CAA, ACC and ACA) were not detected for *M. yokogawai*. The UUG/Leu, UUU/Phe, AUG/Met, GGU/Gly, AUU/Ile and GUG/Val codons comprise almost 30% of all *M. suifunenis* codons (listed in descending order of frequency of occurrence). For *M. yokogawai*, the list of the most used codons (29%) is slightly different: UUG/Leu, UUU/Phe, AUG/Met, GUG/Val, GGG/Gly, GGU/Gly. The AT-proportion of the *cox1* gene was estimated to be 1.3 (nucleotide frequencies were 17.9, 37.9, 28.3 and 15.9% for A, T, G, C, respectively) and 1.2 (17.4, 37.7, 28.6 and 16.3%) for *M. suifunensis* and *M. yokogawai*, respectively.

A low level of nucleotide diversity was obtained for the total *M. suifunensis* population ($\pi = 0.0006 \pm 0.0002$); 19 transitions and one transversion ($A \rightarrow C$) were observed. Three of those were parsimony informative. Two substitutions lead to amino acid substitutions in two samples from the Arsenjevka River, and two non-synonymous substitutions were detected in two samples from the Odyr River (Fig. 2). The haplotype diversity was higher for *M. suifunensis* (0.405 ± 0.087). The level of nucleotide and haplotype diversities was higher for the total population of *C. sinensis* ($\pi = 0.0037 \pm 0.0008$; Hd = 0.977 ± 0.008). *Fst* statistics for sequences from the Primorsky Region and the Odyr River (Khabarovsk Region) had a low value. However, the sequence from the Anuj River (Khabarovsk Region) differed from the other populations (Table 3). The presence of this highly distinguished sequence affects the *Fst* value for the total sample (*Fst* = 0.205), while excluding this sample from the analysis leads to a significant decrease in the parameter (*Fst* = 0.005). *Fst* value for *C. sinensis* was 0.205. Nei's pairwise differences were 0–2% and 0–2.6% between and within *M.*

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                                              1111111112234444445555555
                                           6678146667777571145558000011
                                           1996536890125162481579467912
n = 49 Metagonimus suifunensis             VMYGCFASEEMNHICITMIIMNECSSGR
n = 1  M. suifunensis  (Arsenjevka)        .................I..........
n = 1  M. suifunensis  (Arsenjevka)        .....................S......
n = 1  M. suifunensis  (Odyr)              .........................F..
n = 1  M. suifunensis  (Odyr)              ...........................W
KC330755 M. yokogawai  (Korea)             MTFNYLVGNQLSYMYM.VVVV.DFG.KE
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Fig. 2. Variable sites of amino acid sequences of the complete *cox1* gene for *Metagonimus* spp. *n* is number of sequences in the haplotype.

Table 3
Fixation index *Fst* between samples of *Metagonimus suifunensis* from eight localities in Russia.

	Komissarovka	Komarovka	Sorochevka	Arsenjevka	Odarka	Ilystaya	Odyr
Komarovka	0.16981						
Sorochevka	0.00000	−1.00000					
Arsenjevka	0.10811	−0.02595	−1.00000				
Odarka	0.02343[a]	−0.00501	−1.00000	0.02216			
Ilystaya	0.00000	0.07285	0.00000	0.02778[a]	−0.03067		
Odyr	0.04950	−0.01417	−0.86667	0.04470	0.01553	0.00137	
Anuj	1.00000	0.92308	1.00000	0.57895	0.91489	1.00000	0.72947

[a] Significant Fst, $P < 0.05$.

suifunensis population, respectively, with the exception of the sample from the Anuj River, which differs from other populations by 5–6%. Population average pairwise differences of Nei were higher for *C. sinensis*, but did not significantly differ between (5–7.2%) and within (4.2–7%) samples from different localities.

The distribution of substitutions was not equal along the nucleotide sequence of the *cox1* gene for *M. suifunensis* from the Primorsky Region. A conserved region within the sequence was detected in the range of 162–783 bp, which shortened upon addition of the nucleotide sequences from the Khabarovsk Region (the Anuj and Odyr Rivers) to the analysis (382–681 bp) (Fig. 3).

3.2. Intraspecific phylogeny

Different haplotype patterns were identified in a comparative analysis of samples of two representatives belonging to the superfamily Opisthorchioidea (*M. suifunensis* and *C. sinensis*) from the Russian southern Far East, which had the same size of 53 and 63 sequences, respectively. The MST for *C. sinensis* had complex star-like structure with a plurality of branches reaching the fourth order (Fig. 4B). A total of 41 haplotypes, including from one to six nucleotide sequences, were identified for Chinese liver fluke. The ancestral haplotype (haplotype in the centre of star-like MST) includes three nucleotide sequences from the most southern population (the Komarovka River). The MST for *M. suifunensis* combined only 13 haplotypes (Fig. 4A). Most of the samples ($n = 41$) were included in the main haplotype, while the remaining haplotypes consisted of only one sample: one haplotype was in a sample from the Komarovka River (MK736844), one haplotype was in a sample from the Anuj River (MK736869), two haplotypes were in a sample from the Odarka River (MK736857, MK736860), four haplotypes were found in the samples from the Arsenjevka (MK736848-MK736851) and Odyr Rivers (MN116484, MN116487, MN116488, MN116492). All sequences from the Komissarovka, Sorochevka and Ilistaya Rivers belonged to the main haplotype. Most of the haplotypes insignificantly differed from the main haplotype (1–2 nucleotide substitutions), while one sample from the Arsenjevka River (MK736850), one sample from the Anuj River (MK736869) and two samples from the Odyr River (MN116488, MN116492) differed from the main haplotype by four, five, three and four mutational steps, respectively.

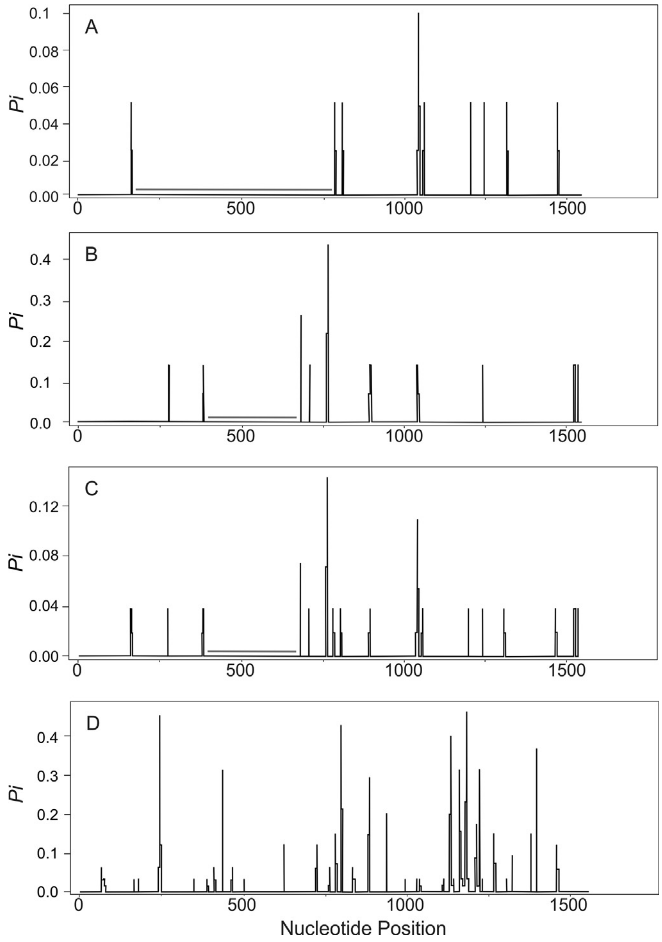

Fig. 3. Nucleotide distribution for the complete *cox1* gene sequences for populations of *Metagonimus suifunensis* from the Primorsky Region ($n = 39$, A), the Khabarovsk Region ($n = 14$, B), both regions ($n = 53$, C), and a population of *Clonorchis sinensis* ($n = 63$, D). *Pi* is nucleotide diversity; grey lines show the conservative region.

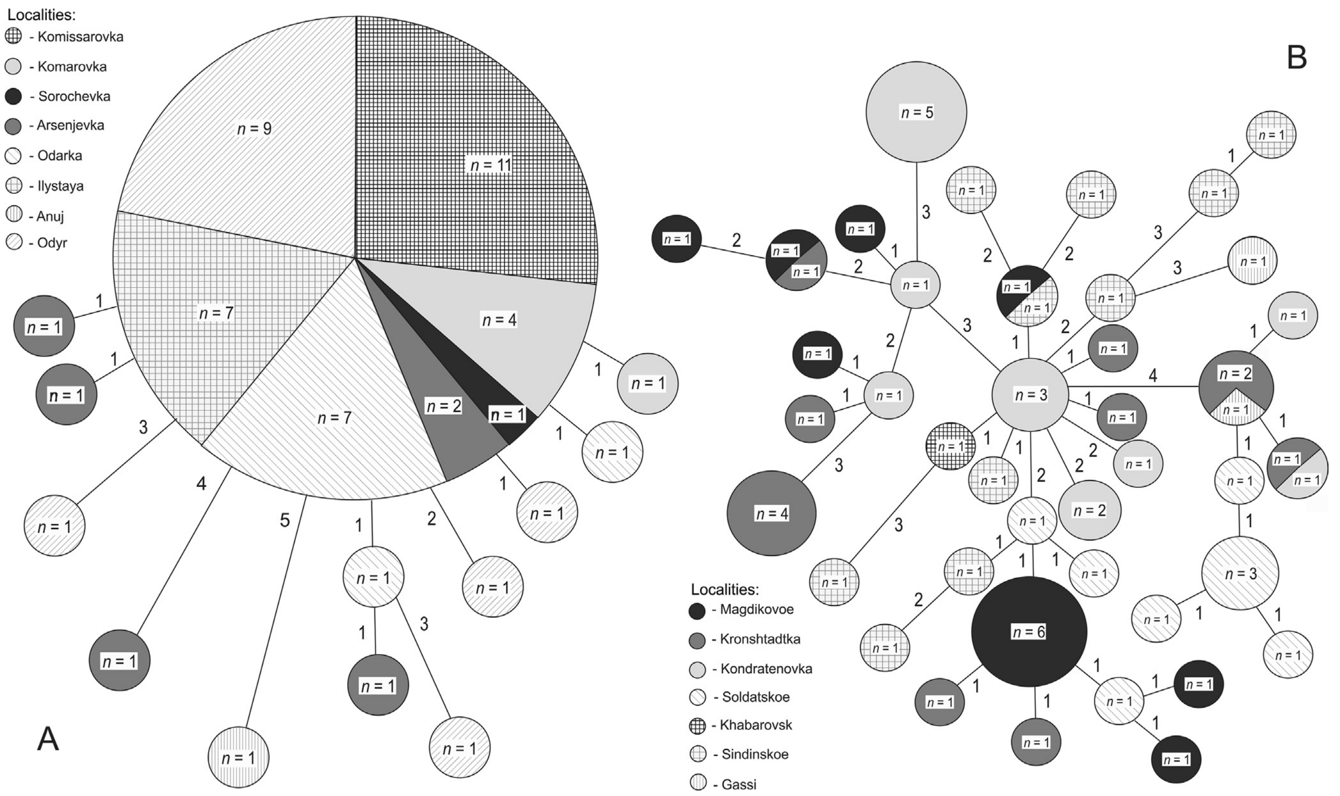

Fig. 4. Minimum spanning trees for the *cox1* haplotypes of *Metagonimus suifunensis* (A) and *Clonorchis sinesis* (B). *n* is number specimens in a haplotype; numbers of substitutions are shown on the branches.

3.3. Demographic analysis

For the total population of *M. suifunensis*, the peak of the observed pairwise differences was in the range of 0–1 on the mismatch distribution graph, and there is also a slight peak in the range of 4–5 pairwise differences with low frequency (=0.05) (Fig. 5). An *Hri* was calculated for all populations, except samples from the Sorochevka and Anuj rivers, which have one specimen for each locality (Table 4). The values varied from 0 to 0.20; however, they were statistically significant only for the Komarovka, Arsenjevka and Odyr populations (when $P < 0.05$). At the same time, *SSD* values were insignificant for populations from all localities at $P > 0.05$. The results of neutrality statistics were also not significant, despite negative values of Tajima's *D* and Fu's *Fs* for some populations (Table 4).

4. Discussion

This study demonstrated that both *M. suifunensis* and *C. sinensis* populations are panmictic homogeneous populations, because any dissimilarity was not detected between different localities using two indices. For *M. suifunensis*, only a specimen from the northernmost locality (the Anuj River) significantly differed from other populations of the parasite, but this could be due to the lack of a sufficient number of samples for analysis. The values of all diversity parameters for *M. suifunensis* were lower than for *C. sinensis*. The obtained nucleotide diversity of *M. suifunensis* is six times less than that for *C. sinensis*. The haplotype diversity level was also 2.4 times lower for *M. suifunensis* than for *C. sinensis*. The low values of *M. suifunensis* variability are accompanied by the existence of a simple star-like structure of MST consisting of the peripheral haplotypes with low frequency and a small number of mutation steps. As a result, the graphic of mismatch distribution is sharply L-shaped, suggesting a recent population "bottleneck" for *M. suifunensis* with sudden expansion (Rogers and Harpending 1992), which was confirmed by values and a neutrality Tajima's

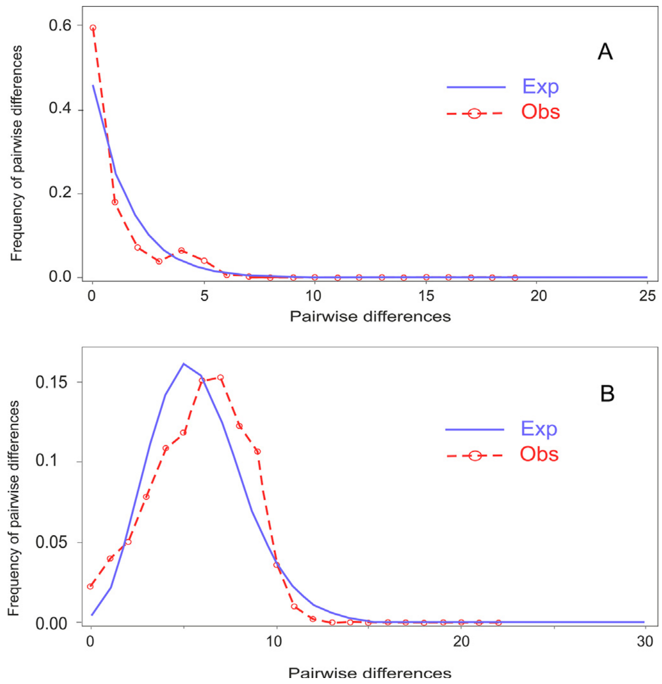

Fig. 5. Mismatch distribution of the complete *cox1* gene sequences of *Metagonimus suifunensis* (this study, A) and *Clonorchis sinensis* (this study, Chelomina et al., 2014 and specimen FJ381664 from GenBank, B). Exp and Obs are the expected and observed frequencies, respectively.

Table 4
Neutrality test statistics and demographic expansion parameters (with *P*-values) for *Metagonimus suifunensis* populations from six localities in Russia.

	Tajima's *D*		Fu's *Fs*		Goodness-of-fit tests			
	D	*P*	*Fs*	*P*	*SSD*	*P*	*HRI*	*P*
Komissarovka	0.00000	1.00000	n/a	1.00000	0.00000	0.00000	0.00000	0.00000
Komarovka	−0.81650	0.30000	−7.58197	0.00000	0.00722	0.76600	0.20000	0.95000
Arsenjevka	−1.40833	0.08000	−3.57660	0.01000	0.08922	0.17000	0.05778	0.98100
Odarka	−1.36240	0.12000	−18.17621	0.00000	0.00870	0.67300	0.16898	0.86900
Ilystaya	0.00000	1.00000	0.00000	1.00000	0.00000	0.00000	0.00000	0.00000
Odyr	−1.37840	0.11000	−19.11155	0.00000	0.36029	0.00100	0.13807	0.99800
Total	−2.46729	0.00000	n/a	0.00000	0.20859	0.00000	0.18818	0.99700

SSD, sum-of-squared deviations; *HRI*, Harpending's raggedness index.

D test (Tajima, 1989). This hypothesis could not be significantly rejected based on demographic expansion parameters (Harpending, 1994) For some populations, this result corresponds to significantly large negative values of Fu's *Fs* statistic and indicates the recent population growth from an ancestral population with a small effective population size (Fu, 1997; Excoffier and Schneider, 1999). Thereby, the data based on the *cox1* gene of mtDNA indicate a dramatic recent decline in the population size of this species in the Russian southern Far East. This decline led to a decrease in genetic diversity of *M. suifunensis*, unlike the population of *C. sinensis* in the same territory that was apparently not under the "bottleneck" effect, which was previously noted by Chelomina et al. (2014). It can be assumed that differences in the level of intraspecific genetic variability may be due to different rates of mutation accumulation in different species of trematodes. However, this seems unlikely for conservative protein-coding genes of representatives from one superfamily with the same area and common second and definitive hosts. At the same time, the influence of the marker length may be also excluded because the analysed region is rather extended. Moreover, for *M. suifunensis*, a lower level of variability was detected based on the nuclear marker, the ITS rDNA region. Only one non-parsimonious substitution was obtained in the analysis of 64 nucleotide sequences of this species (Shumenko et al., 2017), while for *C. sinensis*, this region has demonstrated different types of variability (Tatonova et al., 2017). The detected "bottleneck" effect may have two explanations, including the mass extinction of individuals or the founder effect and the relatively young age of the population.

Consider factors that could affect the mass extinction of the parasite. First, it can be associated with a sharp decrease in the number of one of the hosts. Any changes in the genetic structure of a parasite population are usually associated with historical processes in the host population. According to the Fahrenholz rule, the evolution of the two components of this system (a parasite and its host) corresponds to each other (Rannala, 1992). In life cycles of *M. suifunensis* and *C. sinensis*, the second and definitive hosts are cyprinid fish and fish-eating mammals including humans, respectively; that is, reducing the number of these animals could have an impact on both parasite species immediately. At the same time, the first intermediate hosts of these species are different snails. For *C. sinensis*, they are snails of the family Bithyniidae, which inhabit stagnant water bodies (Posokhov, 2004; Tang et al., 2016), while for *M. suifunensis*, they are snails of the family Semisulcospiridae living in rivers (Besprozvannykh and Ermolenko, 2005; Shumenko et al., 2017). Due to the differences in ecology of the first intermediate hosts of these parasites, it can be supposed that there is a connection between the possible decrease in the number of the semisulcospirid snails and a significant reduction in the population of their parasite, *M. suifunensis*. However, in the recent past the climatic conditions were stable on the explored territory, since the Late Pleistocene glacial did not occur in the Primorsky Region (Korotky et al., 1996), which correlates with the high level of genetic variability found for *C. sinensis*. Therefore, non-climatic causes may have influenced the number of snails and, indirectly, the level of *M. suifunensis* variability. Nevertheless, since the representatives of bithyniids and semisulcospirids occupy different ecological niches, the number of both groups of snails could to varying degrees depend on external factors including climatic conditions. In any case, to gather support for the hypothesis that a recent decrease in the number of snails influenced the variability of the parasites, it is necessary to estimate the variability level of both flukes, *M. suifunesis* and *C. sinensis*, and their first intermediate hosts.

Another possible cause of low variability in the *M. suifunensis* population compared with *C. sinensis* is the loss of some individuals due to localization of *M. suifunensis* metacercariae in second intermediate hosts. The *C. sinensis* metacercariae are located in the muscle tissue of the second intermediate hosts and easily survive winter freezing temperatures in the Russian Far East. At the same time, *Metagonimus* metacercariae are localised on fish scales and, as studies have shown (Besprozvannykh and Ermolenko, 2005), do not survive the winter period. That is, each year there is a complete renewal of the metacercariae. In accordance with this, the probability of accumulation of various haplotypes of the Chinese liver fluke is more likely, while the *M. suifunensis* haplotypes should be limited in each locality as a result of the natural loss of metacercariae and annual re-infection of the snails. Considering the indicated features of *Metagonimus* circulation, as well as the habitats of the first intermediate hosts of trematodes are to some extent isolated, each locality harbouring *M. suifunensis* should accumulate its own set of haplotypes. This is most likely to occur even if there is some exchange between the micropopulations of *M. suifunensis* due to more mobile definitive hosts, mammals and fish-eating birds. For example, different haplotype patterns were previously found for *C. sinensis* in different parts of the parasite area (Chelomina et al., 2014; Solodovnik et al., 2018). However, the fish used in the life cycle of *M. suifunensis* were caught in different years and in different localities, but a limited number of haplotypes were present in the whole area. This means that the different locations of *C. sinensis* and *M. suifunensis* in fish tissues do not affect the variability of parasites in the Russian Far East.

Contrarily, the decreased genetic variability may be also due to the "founder" effect. For example, a small number of the parasite could be transferred from the previously formed centre of species distribution. A constant concentration of genetic diversity is usually maintained only in the centre of the species distribution, whereas a reduction of allele variants is possible at the periphery (Lessica and Allendorf, 1995; Bunnell et al., 2004). If the recent introduction of the parasite had a direction from south to north, low variability would be present in the whole area of *M. suifunensis* in the Russian Far East. That is, most likely, in the Khabarovsk Region (the Anuj River), we would expect to identify a sequence that is identical or only slightly different from the major haplotype identified in the Primorsky Region. However, the specimen found from this locality differs from the major haplotype by five mutational steps. Another population from Khabarovsk Region (the Odyr River) has the intermediate geographical position between localities in the Primorsky Region and the northernmost locality in the Anuj River. This population is characterised by a higher level of variability, despite most of the samples being included in the major haplotype. Apparently, the *M. suifunensis* distribution centre is the Khabarovsk Region and peripheral populations are located in the Primorsky Region.

This hypothesis about the advance of *M. suifunensis* from north to south, from the lower Amur located in the Khabarovsk Region to the Primorsky Region on the Russian Far East, is also supported by the fact that *Metagonimus* from Korea (KC330755) is identical to partial *cox1* nucleotide sequences of *M. yokogawai* from Japan, but differs from the representatives from Russia (*M. suifunensis*) at the species level (Tatonova et al., 2017), that is, one species is distributed in Korea and Japan, but another one is located in Russia. Consider the possible processes of divergence of these species and the formation of modern areas. This could be due to geological processes occurring in the history of designated territories. According to genetic data obtained in this study, *M. yokogawai* and *M. suifunensis* diverged in the Late Miocene (approximately 6 million years ago). During this period, there was a decrease in the level of the Sea of Japan, as well as the rise of certain sections of the Earth's crust, which led to the integration of Sakhalin and Hokkaido with the mainland in the north (Matyushkov et al., 2014). At the same time, the paleo-Amur River was formed, the delta of which moved closer to the northern part of Sakhalin. Apparently, there was an exchange of malacofauna (and their parasites) between Japan (Hokkaido), Sakhalin and the Russian Far East. Later, as a result of the Sea of Japan transgression in the Pliocene, a significant part of Sakhalin was inundated (Bogatov et al., 2006), and the exchange between the faunas became impossible. A land bridge connecting the territory of Hokkaido, Sakhalin and the continent appeared again, and level the Sea of Japan was approximately 100–130 m lower than the present level (Korotky et al., 1996). In the south, only a narrow strait (approximately 10 km wide and 12 m deep) divided Kyushu Island (Japan) and the Korean Peninsula. The waters of the semi-enclosed Sea of Japan, bounded by land from the ocean, were desalinated when it was almost completely separated from the ocean and large rivers (Amur, Yellow and Yangtze) flowed into it (Lee et al., 2008). Such conditions ensured the free exchange of fauna elements, including associated parasites, in circulation with freshwater inhabitants. However, the continental *Metagonimus* population seems to have been isolated in the Russian Far East, since in addition to the drop in sea level, the Late Pleistocene was characterised by the presence of a glacial period (Korotky et al., 1996; Lee et al., 2008). In that regard, the first intermediate hosts of *Metagonimus* could not spread in the more northern and cold parts of paleo-Amur. We made this conclusion based on the fact that modern semisulcospirids also do not spread in the Amur basin above the 52nd parallel north due to low temperatures (Posokhov, 2004). The final rupture of the land links of the territories of Japan and the continental Russian Far Eastern led to the restriction of the genetic exchange between the *Metagonimus* populations of these regions. This eventually led to the formation of *M. suifunensis*, which is morphologically similar to *M. yokogawai*, but genetically different from it due to their independent evolutions. At the same time, the exchange of faunas between the southern Japanese Islands and the Korean Peninsula was easily feasible during the regression in the Late Pleistocene. The later breakdown of land links between Japan and Korea ensured the preservation of the general morphogenetic features of *M. yokogawai* in these territories. As for the further fate of the *Metagonimus* on the Russian Far East, with a high degree of probability, the expansion of the parasite was carried out in the region through the Amur River basin into the southern parts of the region, the basins of the Ussuri River, Khanka Lake and the Razdolnaya River, which was mentioned above.

Unfortunately, according to molecular data, the expansion time of *Metagonimus* in the Russian Far East is currently impossible to calculate, since almost all indicators derived from the analysis of molecular data are statistically unreliable as a result of a small number of informative sites. It is also unclear at this time exactly where the continental frontier between *M. suifunensis* and *M. yokogawai* occurs. The molecular data for other regions are practically absent. For example, for *M. yokogawai* from China with an unknown sample locality, there are only ITS2 sequences and these data are unpublished. It is also necessary to clarify the species identities, their phylogenetic relationships and variability levels of the first intermediate hosts of the parasites, because these data could significantly expand the possibilities for solving the problems of the fauna formation of both *Metagonimus* and other trematode species.

Therefore, our results provide the first step towards understanding evolutionary processes for Metagonimus spp., but to obtain answers to all issues mentioned above, additional data are needed on parasites and their hosts in different parts of eastern and southeastern Asia.

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