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GENETIC POLYMORPHISM OF SIBERIAN LARCH (*Larix sibirica* Ledeb.) IN CONTRASTING ECOTOPES OF THE REPUBLIC OF KHAKASSIA

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Abstract. Using microsatellite markers (SSR), the genetic polymorphism of five coenopopulations of Siberian larch (*L. sibirica*) growing in the Republic of Khakassia was assessed. The highest values of the average number of alleles per locus were found in coenopopulations of *L. sibirica* from the steppe meadows of the valley of Lake Fyrkal (3.9 ± 0.458) and forested upland slopes in the valley of Lake Agaskyr (3.9 ± 0.348). The latter cenopopulation is also characterized by the maximum value of the effective number of alleles per locus (2.302 ± 0.283) and the highest rates of observed and expected heterozygosity (H_O = 0.487 ± 0.074, H_E = 0.492 ± 0.070). Analysis of the population structure indicates a 0.8% excess of heterozygous genotypes relative to the population (F_{IS} = −0.008 ± 0.031) and a 3.4% deficiency of heterozygous genotypes (F_{IT} = 0.034 ± 0.034) relative to the species. The differentiation of the studied cenopopulations of *L. sibirica* by SSR markers is 4.3% (F_{ST} = 0.043). The smallest genetic distance (0.036) was detected between ecotopically most similar (forest) coenopopulations of *L. sibirica*, and the largest genetic distance (0.077), on the contrary, was identified between ecotopically contrasting (swamp and steppe) coenopopulations.

Keywords: *genetic polymorphism, heterozygosity, conifers, microsatellite markers, larch, Larix*

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INTRODUCTION

Siberian larch (*Larix sibirica* Ledeb.) is one of the main forest-forming species of the boreal zone of Eurasia. In Russia, forests with larch (*Larix* Mill.) cover about 264 million hectares, or 38% of the total forested area. Within its distribution range, Siberian larch forms a wide spectrum of morphological variability, manifested in the presence of intraspecific forms and tree morphotypes adapted to various growing conditions [1]. When studying the genetic variability of *L. sibirica* populations in the north of Krasnoyarsk Territory, the Urals, Altai, and Tuva, no close relationship was found between the geographical position of the samples and the degree of their genetic subdivision, while maintaining a high level of intrapopulation genetic diversity [2, 3]. This identifies an important feature of *L. sibirica*: the main pool of genetic variability of the species is concentrated at the intrapopulation (cenopopulation) level, while the proportion of interpopulation (geographical) component is significantly smaller. Assessment of genetic diversity of several conifer species, including *L. sibirica*, at the cenopopulation level revealed the adaptive component of their intraspecific differentiation [4–7]. When selecting experimental objects for such studies, it is advisable to focus on those parts of the ranges where generally optimal growing conditions for the species are characterized

by significant soil-hydrological and phytocenotic heterogeneity, and the presence of orographic and phenological interpopulation barriers [8].

Such criteria are met by the southern Siberian part of the range *L. sibirica* within the Republic of Khakassia, where steppe, forest, meadow, and bog phytocenoses are concentrated in a relatively small area, complexly structured according to altitudinal mountain belts, exposures of low ridges, intermountain river valleys, and lacustrine basins with characteristic landscape mosaics and sharp environmental gradients [9]. Pure and mixed stands of *L. sibirica* of mountain taiga, forest-steppe, and floodplain-bog development series are widely represented here, differing in their morphotypic, age, and spatial structure, as well as the level and nature of anthropogenic transformation [10–12].

The aim of this work is to diagnose genetic diversity, structure, and intraspecific differentiation of Siberian larch cenopopulations growing in the Republic of Khakassia.

MATERIALS AND METHODS

The objects of the study were five cenopopulations of *L. sibirica* from various ecotopes of the Republic of Khakassia. Information about the location of collection sites, characteristics of phytocenoses, and tree morphology is presented in Table 1 and Fig. 1. Experimental materials (needle samples) were collected in July 2020. The total number of selected trees was 150, with 30 samples in each set.

For genetic analysis of Siberian larch samples, nuclear microsatellite markers were used, previously developed by researchers from the Laboratory of Forest Genomics of SFU for *L. sibirica,* as well as by Japanese researchers K. Isoda and A. Watanabe for Japanese larch (*Larix kaempferi* (Lamb.) Carrière) [13, 14].

The primers were selected by adjusting and optimizing PCR amplification conditions. Based on the results of this testing, polymorphic loci demonstrating well-interpretable electropherograms were selected. The selected polymorphic microsatellite loci for further study of samples from *L. sibirica* populations are presented in Table 2.

Total DNA preparations were isolated using a modified method with cetyltrimethylammonium bromide (CTAB) from needle tissue samples dried with silica gel [15].

For PCR, ready reaction mixtures for DNA amplification "GenePak PCR Core" products from "Laboratory Isogen", LLC, containing inhibitor for "hot start" Taq-DNA-polymerase, deoxyribonucleoside triphosphates, and magnesium chloride were used. The amplification program included primary denaturation for 1 min at 94°C, then 9 "touchdown" cycles with 1°C decrease each cycle: 30 s at 94°C, 30 s at 63°C, 1 min at 72°C, followed by 24 cycles without "touchdown": 30 s at 94°C, 30 s at 53°C, 30 s at 72°C; final elongation was 10 min at 72°C. Amplification products were separated by electrophoresis in 6% polyacrylamide gel using Tris-EDTA-borate electrode buffer in chambers for vertical electrophoresis. Gel staining was performed in a solution of ethidium bromide. The DNA of *E. coli* plasmids pBR322, treated with the restriction enzyme Hpa II, was used as a standard-length marker.

Calculation of the main indicators for selection of five cenopopulations of Siberian pine was carried out using the GenAlEx 6.51b2 program [16]. Genotyping errors arising due to *null-alleles* were identified and corrected using the MICRO-CHECKER program [17]. For phylogenetic tree construction, adegenet and poppr packages in R were used, tree construction was performed by the unweighted pair group method with arithmetic mean (UPGMA). The phylogenetic tree was generated based on the standard genetic distance of M. Nei [18]. Tree visualization was performed using the online tool iTOL, version 6 [19].

RESULTS AND DISCUSSION

During the study of 10 nuclear microsatellite loci in five samples of Siberian larch from the Republic of Khakassia, 45 allelic variants were identified, 29 (about 64%) of which were common. In the cenopopulations we studied, the identified microsatellite loci partially differed in composition and frequency of occurrence of the detected alleles. The largest number of alleles was found in the following samples: swamp larch forest in the valley of Lake Agaskyr (AG-b) – 40 (of which 7 are rare, with a frequency of occurrence less than 5%); sparse larch woodland in the valley of Lake Fyrkal (FRK) – 39 (9 rare); steppe-like sparse larch woodland in the floodplain of the Karysh River (KR) – 38 (8 rare). The cenopopulation from the Tunguzhul River valley (TNZh) – the oldest and largest stand, on the contrary, has the smallest number of allelic variants – 33 (of which 3 are rare). The highest level of allelic diversity in the studied larch samples is found in the locus *bcLK232*, in which 8 alleles were identified. The locus *Ls_2672894* in the FRK cenopopulation was monomorphic.

Analysis of the identified genetic diversity *L. sibirica* in the studied stands of the Republic of Khakassia showed (Table 3) that the highest values of the average number of alleles per locus were found in samples AG-s (3.9 ± 0.348) – forested upland slopes in the valley of Lake Agaskyr and FRK (3.9 ± 0.458) – steppe meadows of the

Table 1. Bioecological characteristics of cenopopulations *L. sibirica* in Shirinsky and Ordzhonikidzevsky districts of the Republic of Khakassia

Cenopopulation sample code, geographical coordinates	Orographic and phytocenotic conditions, taxation characteristics of stands
TNZh 54°16' N, 89°38' E	Larch open woodland (13–15 trees/ha) with tall herb-meadow polydominant vegetation (over 140 species of herbaceous plants) on dark gray and sod-podzolic soils of gentle slopes in the Tunguzhuil River valley (right tributary of the Bely Iyus River). 600–640 m above sea level. Tree age 180–360 years, height 18-34 m, diameter 58–92 cm. Great diversity of large-sized tree morphotypes: low-stunted gnarled large-branched; with flattened-tent-like, widely spreading, dome-shaped and even double crown forms; highly tapering, multi-branched and thick-barked trunks, etc.
KR 54°24' N, 89°59' E	Larch sparse forest (6–17 trees/ha) with steppe-meadow vegetation on dry primitive soils of disintegrating blocky outliers, their rocky screes framing the floodplain of the Karysh River (Itkol Lake basin). 500–520 m above sea level. Tree age 90–140 years, height 5–8 m, diameter 13–16 cm. Solitary trees are represented by various forms: crooked-stemmed, small-branched, often with dry tops; in groups, straight-stemmed, strongly tapering, large-branched, stunted trees with tiered-asymmetric, or flattened, flag-shaped and other crown forms predominate.
AG-s 54°58' N 89°15' E	Sparse larch forest (40–60 trees/ha) on dry and fresh mineral soils along the southern slope in the valley of Lake Agaskyr — Pechishche River basin (left tributary of the Cherny Iyus River). 540–555 m above sea level. Tree age130–185 years, height 19–23 m, diameter 44–56 cm. Most of the large trees are characterized by low, spreading, branchy crowns of one-sided umbrella shape, possibly formed under the influence of systematic wind impact.
AG-b 54°58' N 89°16' E	Boggy larch forest with spruce on patchy-permafrost, medium-thick (up to 120 cm), excessively wet peat soils of the eutrophic series of water-mineral nutrition in the valley of Lake Agaskyr — Pechishche River basin (left tributary of the Cherny Iyus River). 540 m above sea level. The sample consists of thin undergrowth (2–2.5 thousand trees/ha, age 50–70 years, height 4–6 m, diameter 8–11 cm).
FRK 54°30' N 89°46' E	Park-type larch sparse forest (8–9 trees/ha) with low-growing herbs and shrubs on steppe meadows of the southern end of the Fyrkal Lake valley (right-bank floodplain of the Bely Iyus River). 540 m above sea level. Tree age 160–200 years, height 12–14 m, diameter 50–60 cm. Stunted, scattered solitary trees with strongly tapering trunks and large skeletal branches, forming flag-shaped, umbrella-shaped, cone-shaped, asymmetrically-domed, branched, shield-shaped and other crown forms.

Fyrkal Lake valley, and the effective number of alleles per locus – in the AG-s sample (2.302 ± 0.283). The highest indicators of observed and expected heterozygosity were also identified in the aforementioned forest sample AG-s (H_O = 0.487 ± 0.074, H_E = 0.492 ± 0.070). In three (AG-s, AG-b, FRK) of the five samples of *L. sibirica*, a deficit of heterozygous genotypes was detected. A high value of Wright's fixation index was found for the AG-b population on peaty soils of the swampy valley of Lake Agaskyr (0.054 ± 0.043).

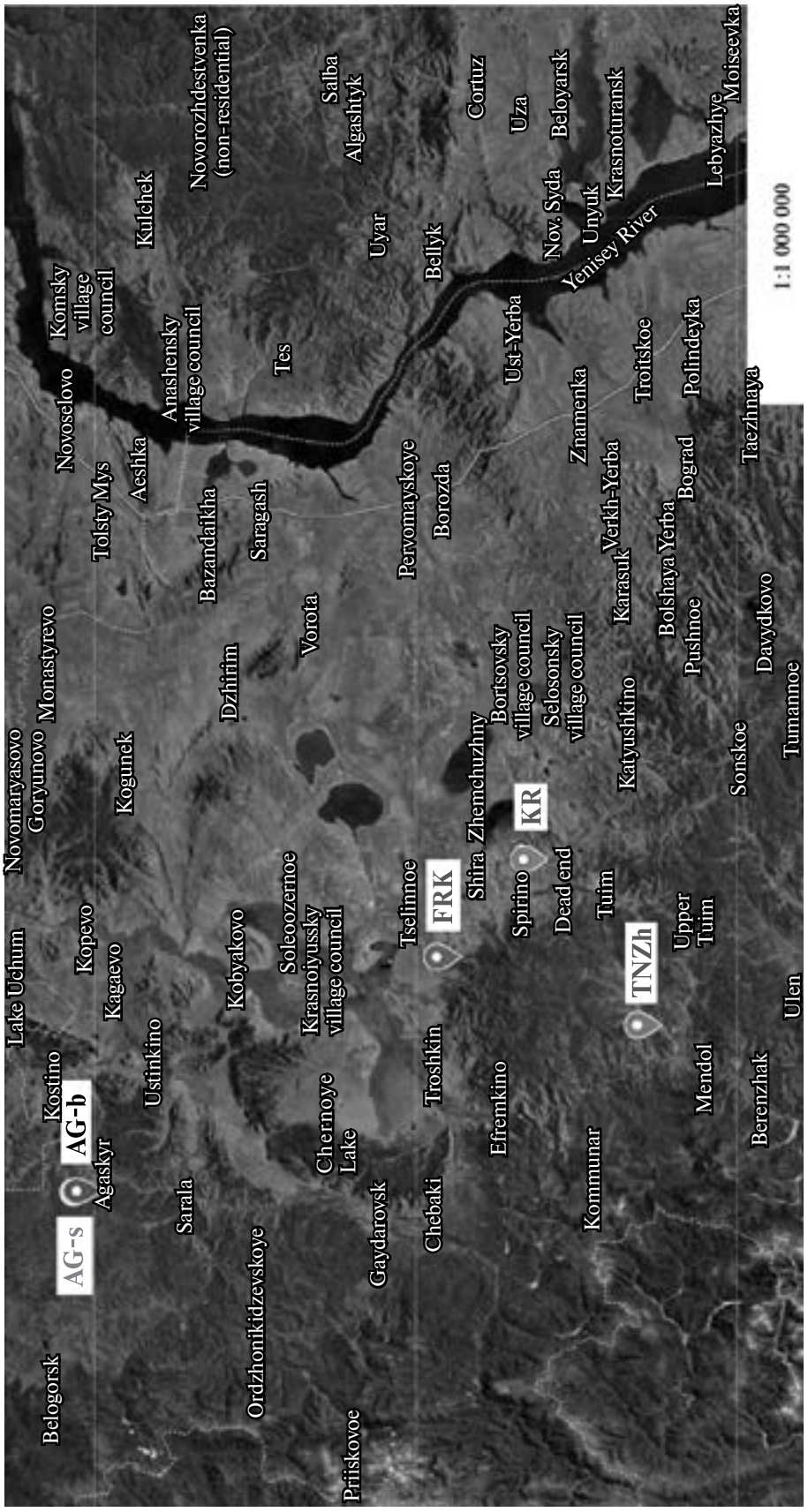

Fig. 1. Map of the studied cenopopulations of *L. sibirica* in Shirinsky and Ordzhonikidzevsky districts of the Republic of Khakassia (coding of cenopopulation samples corresponds to Table 1)

Table 2. Selected nuclear microsatellite loci for *L. sibirica*

Locus	Motif	Primer sequences	Fragment length	Source literature
bcLK232	$(AG)_{19}$	F: TGTTGCTGGGTTGTTGTTAGA R: GGGTAATAGTTCCAGTCTTTG	142–178	[14]
bcLK224	$(AG)_{17}$	F: GGAGAGGCCACTACTATTATTAC R: ATGCGTTCCTTCATTCCTCT	152–168	
bcLK066	$(TG)_{12}$	F: GCAACCGACAATGATTACATAG R: CCTAAAACTGAACTTTGCTCAAT	155–172	
Ls_954234	$(ATT)_{15}$	F: TGGCGTTTGGCTAAGTTGTAA R: GGTTGATTTATGTGTGTGTATGTGG	171–204	[13]
Ls_752897	$(AAG)_{15}$	F: GCAGATGTTGATACAGTGGAGG R: CAGCTTCATTTCGTGGCTAAT	216–264	
Ls_417667	$(AAT)_{16}$	F: CAGAGGATCTCATTCCTGTTGA R: CTCGAAGGCCAATTAGGATAAA	207–243	
Ls_2672894	$(TTTG)_{11}$	F: CAAAGGATGGAATGTGTCTCAA R: GTTGGTATGGTTTCCCAGAGTG	152–164	
Ls_2552367	$(CTAT)_{10}$	F: AAAGGTGCAATCACGTAAAGAC R: ATCGAAGCGGAAAATGTGTA	184–196	
Ls_1008427	$(ATAG)_{13}$	F: CACCCCTATCCCACAAATCTTA R: ATTTATCTTTGGCCCTCATGC	152–174	
Ls_305132	$(GTCGGA)_{7}$	F: GCAGAGCCGTTATTCGATCTAT R: CCCTCGTTTCCTCTTCTGACTA	210–240	

Table 3. Indicators of genetic variation calculated for five samples of *L. sibirica* based on SSR analysis results

Cenopopulation samples	N_A	N_E	H_O	H_E	F
TNZh	3.3 ± 0.260	2.03 ± 0.224	0.487 ± 0.060	0.454 ± 0.058	–0.083 ± 0.053
KR	3.8 ± 0.416	2.04 ± 0.172	0.467 ± 0.056	0.469 ± 0.056	–0.008 ± 0.055
AG-s	3.9 ± 0.348	2.30 ± 0.283	0.487 ± 0.074	0.492 ± 0.070	0.012 ± 0.053
AG-b	3.6 ± 0.340	1.87 ± 0.174	0.390 ± 0.057	0.419 ± 0.059	0.054 ± 0.043
FRK	3.9 ± 0.458	2.21 ± 0.248	0.473 ± 0.073	0.483 ± 0.068	0.030 ± 0.047
On average for all studied cenopopulations	3.7 ± 0.162	2.09 ± 0.098	0.461 ± 0.028	0.463 ± 0.027	0.0004 ± 0.023

Note. N_A – average number of alleles per locus, N_E – effective number of alleles per locus, H_O – observed heterozygosity, H_E – expected heterozygosity, F – fixation index, ± – standard error

Analysis of data on genetic polymorphism of the studied samples showed that, in general, the values of observed and expected heterozygosity (H_O = 0.461; H_E = 0.463) are comparable with similar indicators reported for *L. sibirica* from other habitats [3, 20–22]. However, the identified reduced allelic diversity (N_A = 3.7; N_E = 2.09) may be related to small sizes and orographic isolation between cenopopulations of *L. sibirica* that are territorially close but growing in contrasting ecotopes.

It is also worth noting that the increased values of the main indicators of genetic variability obtained by other authors are also associated with different sets of microsatellite loci used in studies. For analysis of population-genetic variability of Siberian larch, previously, mainly highly variable dinucleotide microsatellite loci were used [3, 20–22]. In our work, we also used dinucleotide loci, but the main proportion was represented by tri-, tetra-, and hexanucleotide loci (7 out of 10 loci).

In three (AG-s, AG-b, FRK) out of five samples of *L. sibirica*, a deficit of heterozygous genotypes was revealed, with the largest observed in the population from the bog larch forest in the valley of Lake Agaskyr (F = 0.054). This indicates probable inbreeding in the population, formed at the time of sampling mainly by young trees – offspring of a small number of trees that survived after intensive logging carried out in the larch forests of Khakassia in the mid-20th century. The population from the Karysh River valley is closest to the equilibrium state (F = −0.008) – it is the least anthropogenically disturbed larch stand in this study. A deficit of heterozygous genotypes was previously also identified in studies of genetic diversity of natural populations of Siberian larch from the Urals [3, 22].

Analysis of the population structure and the degree of genetic subdivision of Siberian larch cenopopulations (Table 4) [23] showed that in the studied samples there is a 0.8% excess of heterozygous genotypes relative to the population (F_{IS} = –0.008 ± 0.031) and a 3.4% deficit of heterozygous genotypes (F_{IT} = 0.034 ± 0.034) relative to the species. Per-locus values show that the most significant deficit of heterozygotes is observed in loci *Ls_1008427* and *Ls_954234*. The coefficient of population inbreeding relative to the species as a whole (F_{ST}), reflecting the degree of population subdivision, varies

Table 4. Values of Wright's F-statistics for *L. sibirica*

Locus	*N*	χ^2	F_{IS}	F_{IT}	F_{ST}
bcLK232	8	63.751 ($1.32 \cdot 10^{-4}$)***	0.020	0.109	0.091
bcLK224	4	49.813 ($5.125 \cdot 10^{-9}$)***	0.053	0.117	0.068
bcLK066	4	58.437 ($9.344 \cdot 10^{-11}$)***	–0.093	–0.048	0.041
Ls_954234	5	157.324 ($1.155 \cdot 10^{-28}$)***	0.092	0.118	0.028
Ls_752897	5	50.711 ($1.974 \cdot 10^{-7}$)***	–0.043	0.000	0.041
Ls_417667	3	94.971 ($1.873 \cdot 10^{-20}$)***	–0.191	–0.170	0.018
Ls_2672894	2	0.086 (0.770)ns	–0.037	–0.024	0.013
Ls_2552367	5	150.415 ($3.063 \cdot 10^{-8}$)***	–0.005	0.039	0.044
Ls_1008427	5	56.307 ($1.798 \cdot 10^{-8}$)***	0.156	0.201	0.053
Ls_305132	4	63.819 ($7.514 \cdot 10^{-12}$)***	–0.027	0.002	0.029
Mean			–0.008 ±0.031	0.034 ±0.034	0.043 ±0.007

Note. *N* – number of alleles; χ – heterogeneity test; significance level: ns – not significant; * – P < 0.05; ** – P < 0.01, *** – P < 0.001; F_{IS} – inbreeding coefficient of an individual relative to the population; F_{IT} – inbreeding coefficient of an individual relative to the species; F_{ST} – coefficient of interpopulation differentiation; ± – standard error

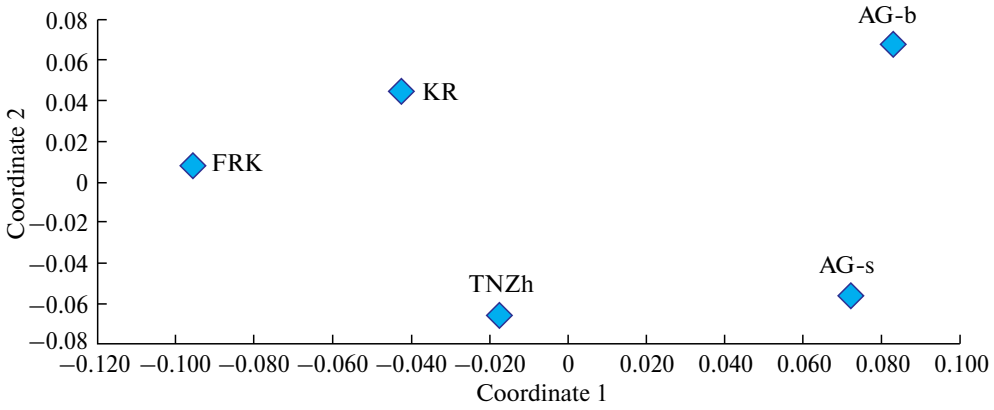

Fig. 2. Projection of the studied cenopopulation samples of *L. sibirica* on the plane of two coordinates according to PCA analysis of the genetic distance matrix (coding of cenopopulation samples corresponds to Table 1)

from 0.013 (*Ls_2672894*) to 0.091 (*bcLK232*), averaging 0.043 ± 0.007. This indicates that only about 4.3% of the identified genetic variation in the studied samples of Siberian larch is distributed among populations. About 95.7% of all genetic diversity is concentrated within populations. The greatest contribution to the differentiation of the studied populations is made by loci *bcLK232* and *bcLK224* (Table 4).

Analysis of the heterogeneity of allele frequencies using the χ^2 criterion showed that in one (*Ls_2672894*) of the ten studied loci, the observed differences are not statistically significant. In other loci, the differences in allele frequencies are highly significant ($P < 0.001$) (Table 4).

The results of the genetic variation distribution test (AMOVA) taking into account hierarchical levels showed that the greatest genetic diversity was found within individuals (94% out of 100%). Genetic diversity between individuals, constituting 2% and 4%, accounts for the interpopulation component.

Assessment of genetic differentiation between the studied populations of Siberian larch was performed using the standard genetic distance (D_N) of Nei [18] based on the frequencies of alleles of 10 microsatellite loci. The smallest genetic distance (0.036) was found between ecotopically maximally similar (forest) cenopopulations TNZh and AG-s (Table 5). The greatest genetic distance (0.077), on the contrary, was diagnosed between ecotopically contrasting cenopopulations – the bog AG-b and the steppe-like FRK.

The revealed level of genetic differentiation in five samples of Siberian larch clearly shows the location of cenopopulations on the plane of two coordinates (Fig. 2).

The results of the Mantel test ($R = 0.479$, $P = 0.100$) indicate the absence of correlation between the matrices of genetic and geographical distance in the studied region [24].

The phylogenetic tree for five samples of Siberian larch from different ecotopes of the Republic of Khakassia, constructed by the unweighted pair group method with arithmetic mean (UPGMA), generated based on the standard genetic distance of M. Nei [18] with an

Table 5. Nei's pairwise genetic distances for cenopopulation samples of *L. sibirica*

Cenopopulation samples	TNZh	KR	AG-s	AG-b	FRK
TNZh	0.000				
KR	0.038				
AG-s	0.036	0.060			
AG-b	0.061	0.052	0.041		
FRK	0.046	0.042	0.072	0.077	0.000

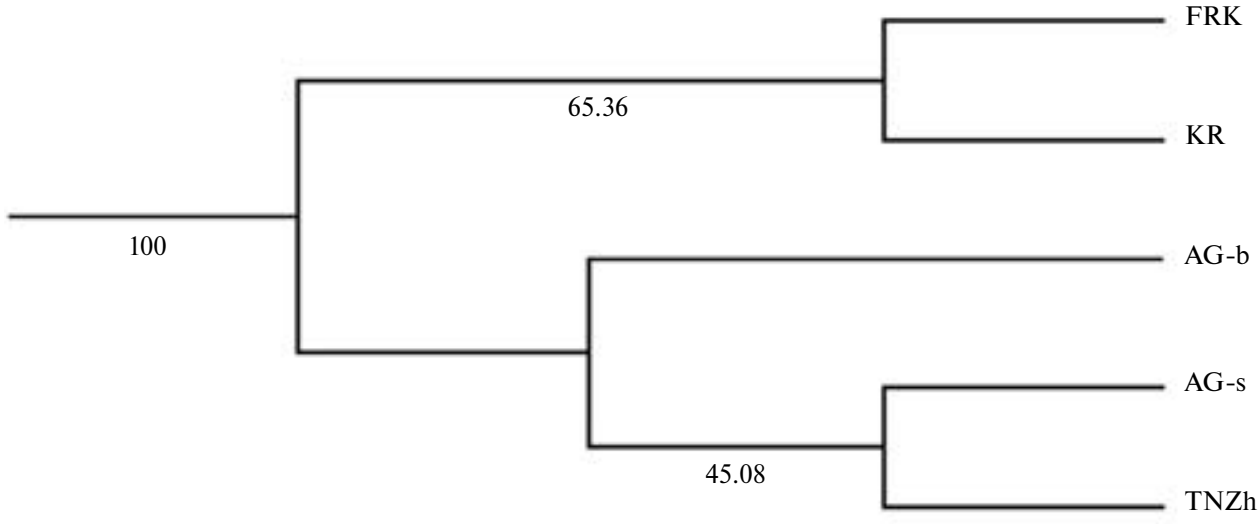

Fig. 3. Phylogenetic tree of five cenopopulations of *L. sibirica* (coding of cenopopulation samples corresponds to Table 1)

assessment of bootstrap support for branching nodes, illustrates the grouping of cenopopulations (Fig. 3).

Two groups with bootstrap support of 100% are clearly distinguished on the dendrogram. The first group, with a bootstrap value of 65.36, includes samples from the Karysh River valley (KR) and from the Fyrkal Lake valley (FRK) (F_{ST} = 0.019, D_N = 0.042). These cenopopulations, landscape isolates at the extreme limit of woody vegetation distribution in the Shira steppe, are maximally similar to each other in ecological conditions and taxation characteristics relative to other samples. The second cluster united forest samples of larch from the eastern spurs of the Kuznetsky Alatau: the valleys of the Tunzhugul River and Lake Agaskyr (F_{ST} = 0.024, D_N = 0.046). The cenopopulations forming these samples (swamp and upland larch forests) are ecotopically polyvariant in terms of water-mineral nutrition conditions, soil characteristics, and phytocenotic structure. It is very likely that the combination of these factors determines the extremely low bootstrap support values for this cluster, and, consequently, the questionable reliability of its topology.

In general, based on this phylogenetic tree with low bootstrap values within the obtained clusters — "steppe" and "forest" — it can be said that larch cenopopulations within each of these two contrasting ecotopes of the Republic of Khakassia are genetically heterogeneous and weakly differentiated. Probable reasons for this include intrapopulation heterogeneity of growing conditions and weak reproductive isolation of territorially adjacent Siberian larch cenopopulations within a given intermountain valley.

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STATEMENT OF COMPLIANCE WITH ETHICS REQUIREMENTS

This article does not contain any studies involving humans or animals.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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