Genetic diversity and structure of Siberian Stone Pine (*Pinus sibirica* Du Tour) populations

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Abstract

Siberian stone pine (*Pinus sibirica* Du Tour) is a key component of the Eurasian boreal forest ecosystems. However, due to the ongoing climatic changes and anthropogenic activities, the habitats of the species are constantly degrading and reducing. To these reasons, exploring the genetic resources of the species and determining the genetic diversity and structure of today's populations is essential. In this study, we assessed genetic diversity and differentiation in six Siberian stone pine populations from different forest zones in Middle Siberia. Based on seven microsatellite nuclear markers (nSSR), moderate level of genetic diversity (*He*=0.455) was detected. A population structure analysis divided the six Siberian stone pine populations into two groups. Southernmost populations were distinguished from the others. Analysis of molecular variance (AMOVA) showed that only 2 % of the genetic variation occurred among populations. Our findings suggest that extensive gene flow may prevent genetic differentiation among Siberian stone pine populations. Hence, further genetic diversity estimation with additional loci is needed for crucial insight into the gene pool of Siberian stone pine populations.

Keywords: *Siberian stone pine; nuclear microsatellite markers (nSSR); genetic diversity; genetic structure; Siberia.*

Introduction

Genetic diversity is a fundamental basis for the evolution of forest tree species and for their adaptation to changing environment. It helps to maintain ecosystem functions, stability and services. Since the industrial revolution, genetic diversity has been decreasing because of habitat degradation and population loss, unsustainable harvest, invasive species and increasing extreme climatic events (Hoban et al., 2020). Therefore, to efficiently conserve the genetic diversity of a species, the genetic diversity should be known (Graudal et al., 2020).

Siberian stone pine (*Pinus sibirica* Du Tour) is one of the main forest-forming coniferous species of the boreal ecosystems of Siberia. It is of great ecological, economic and social importance (Titov, 2007; Behk et al., 2009; Shah et al., 2019). The species` distribution range stretches from the western Urals through the West Siberian Plain to the Transbaikalia (the Baikal Lake region) and southern Sakha-Yakutia and from the Arctic Circle to northern Mongolia (Titov, 2007; Shuvaev and Ibe, 2021). Forests with a predominance of Siberian stone pine cover an area of about 40 million ha (Debkov, 2019). Siberian stone pine is a monoecious, wind-pollinated and zoochoric tree species (Gribkov, 2014). It is frost hardy, relatively hydrophilic, shade tolerant species and is often associated with Siberian spruce (*Picea obovata* Ledeb.) and Siberian fir (*Abies sibirica* Ledeb.) (Timoshok et al., 2014). Siberian stone pine is morphologically and ecologically heterogeneous and has a variety of

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morphological forms and ecotypes (Talantsev et al., 1978). Siberian stone pine forests are constantly exposed to numerous natural (climate change, pest outbreaks and diseases) and anthropogenic disturbances, which lead to changes in the species distribution range (Behk et al., 2009; Voronin et al., 2013; Gribkov, 2014; Kerchev et al., 2019). The group of anthropogenic factors is the most numerous and diverse. It includes logging, forest fires, land reclamation, environmental pollution, recreational, forestry and other types of human activity (Behk et al., 2009). All these factors may affect the gene pool of the species and lead to the decline in genetic diversity (Ellstrand et al., 1993; Ivetić et al., 2016). Understanding of the extent and patterns of genetic diversity in Siberian stone pine is essential for its conservation and exploitation, especially in the light of global climate change.

In the last decades, DNA markers such as microsatellites (SSRs) have been extensively employed in genetic studies such as population and conservation genetics, landscape genetics, paternity testing, and forest reproductive material traceability (Petit et al., 1998; Rajora and Mosseler, 2001; Oliveira et al., 2006). Nuclear SSR markers have several benefits over other markers, e.g. they are co-dominant, scattered all over the genome, robust, polymorphic and can be multiplexed. They yield important data for the estimation of gene flow patterns, genetic drift and inbreeding rates (Oliveira et al., 2006; Selkoe and Toonen 2006).

In this study, we used nuclear microsatellite markers to characterize the level of genetic diversity of Siberian stone pine populations. Specifically, we addressed the following questions: (1) What is the pattern of genetic diversity in Siberian stone pine populations? (2) Is there a genetic structure among the studied Siberian stone pine populations based on nuclear microsatellite genotypes?

Materials and methods

Plant material

Six native populations of Siberian stone pine were chosen within the natural distribution range of the species in Siberia (Tab. 1, Fig. 1). Three of them (KHO, URE and SAR) are situated in Southern-Siberian mountain zone, two (BER and KEM) grow in forest-steppe zone and CHU is distributed in taiga forest zone (Approval of the List of Areas of Forest Growth and Forest Areas of the Russian Federation, 2020). The distance between sampled individuals was 100 m to minimize the possibility of sampling closely related individuals. KHO and URE populations are Siberian stone pine dominant forests and the rest of the populations are located in mixed forests with other tree species like Siberian spruce (*Picea obovata* Ledeb.), Siberian fir (*Abies sibirica* Ledeb.), Scots pine (*Pinus sylvestris* L.), silver birch *(Betula pendula* Roth.). Initially, 300 individuals were sampled. Due to PCR failure, the number of analyzed trees was reduced to 222.

Molecular analysis

Genomic DNA was isolated from dried needles according to Dumolin et al. (1995). The quality and concentration of the extracted DNA were measured with Nanodrop spectrophotometry (Thermo Fisher Scientific), then diluted to a concentration of 10 ng/µl. Seven nuclear microsatellite markers were chosen for the genetic analysis: Ps_80612, Ps_364418, Ps_1375177, Ps_25981, Ps_31489, Ps_39709, Ps_1502048 (Belokon et al., 2016). The nSSR loci were amplified in two PCR multiplex reactions and using a universal tail-system (Blacket et al., 2012). The PCR mix contained 0.2 µM of each forward (specific primer plus tail) and universal labelled tail primer, 0.4 µM of each reverse primer, 1.75 mM of $MgCl_2$, 0,2 µM dNTPs, 0.6 units Taq Polymerase (DCSPol DNA Polymerase from DNA Cloning service), 1X PCR Buffer and 20 ng DNA template, for a total volume of 15 µl. PCR amplification was performed in a Labcycler gradient from SensoQuest with the following conditions: an initial denaturation of 3 min at 94°C, then 35 cycles of 30 s at 94°C, 30 s at 58°C, 1 min at 72°C and a final extension of 10 min at 72°C. Amplified fragments were analyzed on an ABI 3730 Genetic Analyzer (Thermo Fisher Scientific) with the GeneScan 500 LIZ (Thermo Fisher Scientific) size standard. Fragment scoring was performed with GeneMarker v3.0 (SoftGenetics, State College, USA).

Tab. 1
Geographical location of six Siberian stone pine populations in Siberia. Sample size (N); Southern-Siberian mountain zone (SSm); Forest-steppe zone (Fs); Taiga forest zone (T).

ID	Population	N	Forest zone	Associated tree species	Lat., Long.	Average elevation (m a.s.l.)
KHO	Khopto	37	Ssm	-	51.837, 95.423	1352
URE	Urener	37	Ssm	-	52.812, 95.615	1415
SAR	Sarala	37	Ssm	*Betula pendula*	54.707, 88.855	750
BER	Beret	37	Fs	*Picea obovata, Pinus sylvestris Betula pendula*	55.751, 93.154	396
KEM	Kemchug	37	Fs	*Picea obovata Betula pendula*	56.188, 91.566	287
CHU	Chunoyar	37	T	*Picea obovata, Abies sibirica, Pinus sylvestris*	57.569, 97.345	416

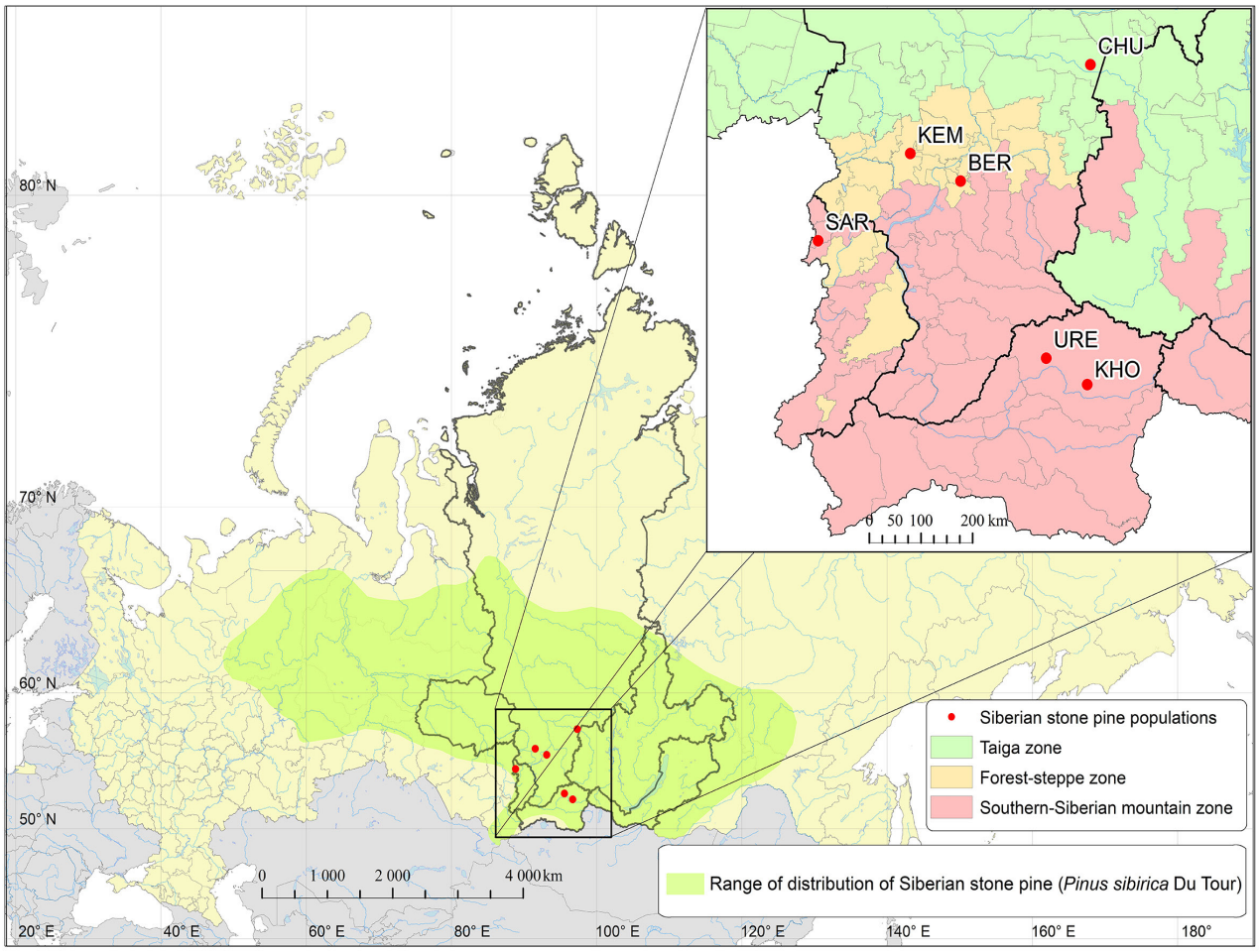

Fig. 1
Map of Russia showing the locations of the studied Siberian stone pine populations (acronyms are as in Table 1).

Genetic Data Analysis

Micro-Checker software (Van Oosterhout et al., 2006) was used to test all markers for null alleles and possible genotyping errors. Despite the high frequency of null alleles in several populations for Ps_80612, Ps_364418, Ps_39709 loci (0.153, 0.180 and 0.228 respectively), we decided not to omit these loci from the analysis.

GenAlEx v. 6.5 software (Peakall and Smouse, 2006) and POPGENE v. 1.32 (Yeh et al., 1999) were used to estimate the following genetic diversity parameters: number of alleles (*Na*) (Brown and Weir, 1983); number of effective alleles (*Ne*) (Brown and Weir, 1983); inbreeding coefficient of an individual relative to the subpopulation (*Fis*) (Hartl and Clark, 1997); inbreeding coefficient of an individual relative to the total population (*Fit*) (Hartl and Clark, 1997); genetic differentiation coefficient (*Fst*) (Hartl and Clark, 1997); observed heterozygosity (*Ho*) (Hartl and Clark, 1997); expected heterozygosity (*He*) (Hartl and Clark, 1997). The allelic richness (Ar) was computed in R (R Core Team, 2013) using the "hierfstat" package (Goudet, 2005).

Hierarchical analysis of molecular variance (AMOVA), implemented in GenAlEx v. 6.5 software, was used to determine the partitioning of the genetic variation among populations. The significance of differences was estimated using a permutation approach with 999 replications. The unweighted pair-group method with arithmetic mean (UPGMA) was used to perform cluster analysis on the Nei`s genetic distances data (Nei, 1972) and a Principal Component Analysis (PCA) was applied using the "FactoMineR" package in R (Lê et al., 2008) to show genetic relationships among populations.

Population structure was analyzed using STRUCTURE v.2.3.4 with a Bayesian clustering approach (Pritchard et al., 2000). Testing 20 independent runs with K from one to 10, each run had a burn-in period of 100 000 iterations and 500 000 Monte Carlo Markov iterations, assuming admixture model (with LocPrior) with correlated allele frequencies. The studied populations were separated into groups by the Structure Harvester program (Earl and VonHoldt, 2012) based on ΔK and mean L(K) values (Evanno et al., 2005). The average matrices of individual membership proportions for each population were estimated using CLUMPP v.1.1.2. (Jakobsson and Rosenberg, 2007). To further test the significance of differentiation between the detected population groups, a two-samples Wilcoxon test was carried out comparing the membership probabilities (individual Q-values) of Structure in R using "Stats" package

Tab. 2
Basic genetic statistics averaged across seven microsatellite loci for each Siberian stone pine population. Number of alleles (*Na*); number of effective alleles (*Ne*); allelic richness (A_R); observed heterozygosity (*Ho*); expected heterozygosity (*He*); inbreeding coefficient (*Fis*).

Population	*Na*	*Ne*	A_R	*Ho*	*He*	*Fis*
KHO	4.000	2.008	3.027	0.313	0.424	0.241
URE	4.000	2.121	3.102	0.305	0.445	0.367
SAR	3.714	2.120	3.198	0.375	0.490	0.255
BER	4.000	2.026	3.026	0.340	0.433	0.208
KEM	4.143	2.092	3.063	0.378	0.482	0.190
CHU	4.000	2.125	3.082	0.336	0.456	0.240
Overall Mean	3.976	2.082	3.083	0.341	0.455	0.250

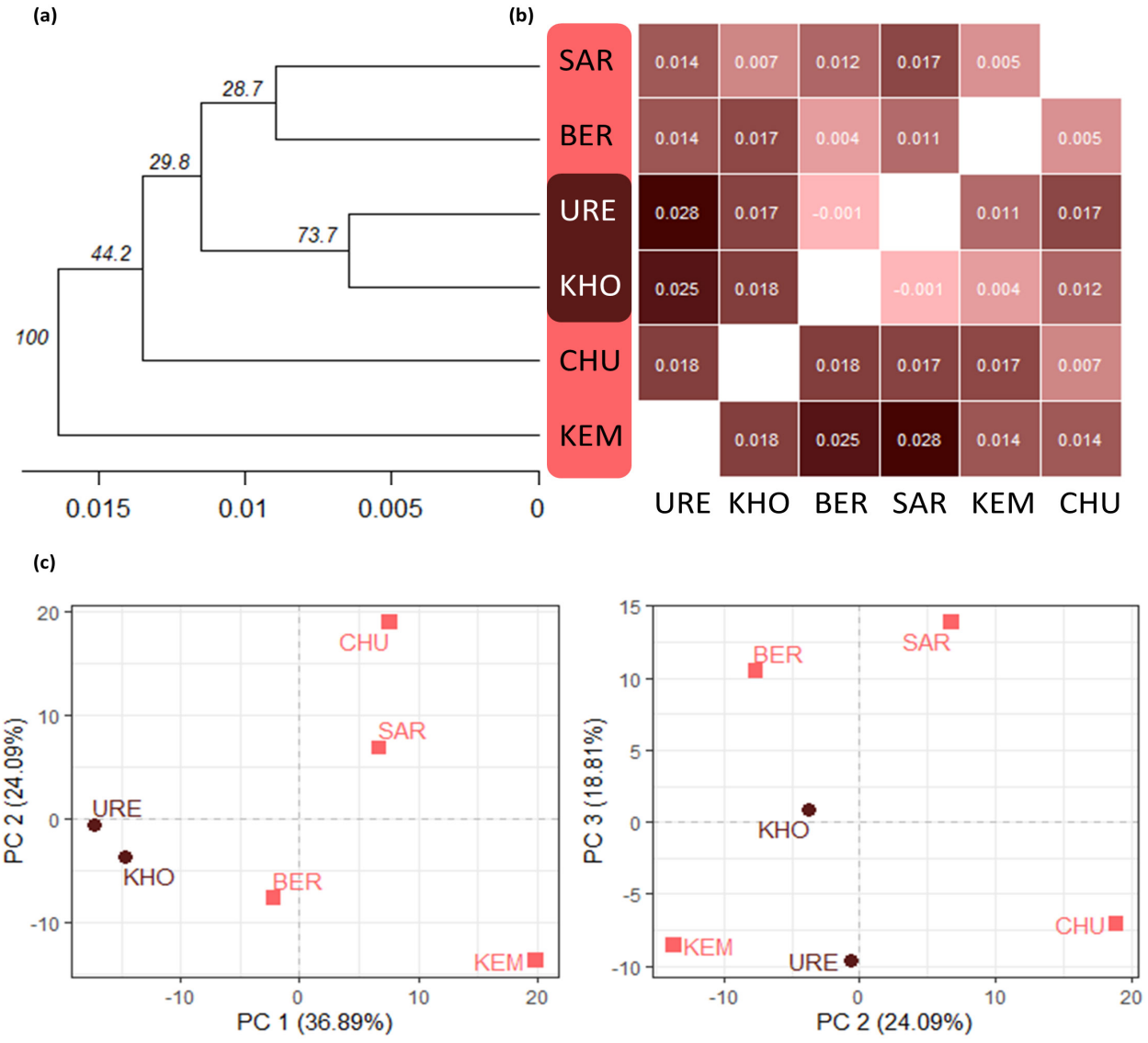

Fig. 2
UPGMA dendrogram based on Nei`s (1972) genetic distance between Siberian stone pine populations. Bootstrap values are presented at the branch intersections (a). Matrix of pairwise *F*st values among Siberian stone pine populations (b). Principal Component Analysis (PCA) (c). Colors used in the UPGMA-*F*st heatmap labels correspond to the result of Structure analysis at *K*=2.

Tab. 3
Analysis of molecular variance (AMOVA) at seven nuclear microsatellite loci. Degrees of freedom (df); sum of squares (SS); estimated variance of components (Est. Var.); percentage of total variance contributed by each component (%); probability (*P*).

Source	df	SS	Est. Var.	%	*P*
Among two groups: Group 1 (KHO, and URE) vs. Group 2 (SAR, BER, KEM and CHU)	1	6.444	0.022	1.0	
Among populations	5	37.261	0.091	2.2	0.001
Within populations	216	884.243	4.094	96.8	
Total	222	927.948	4.207	100	

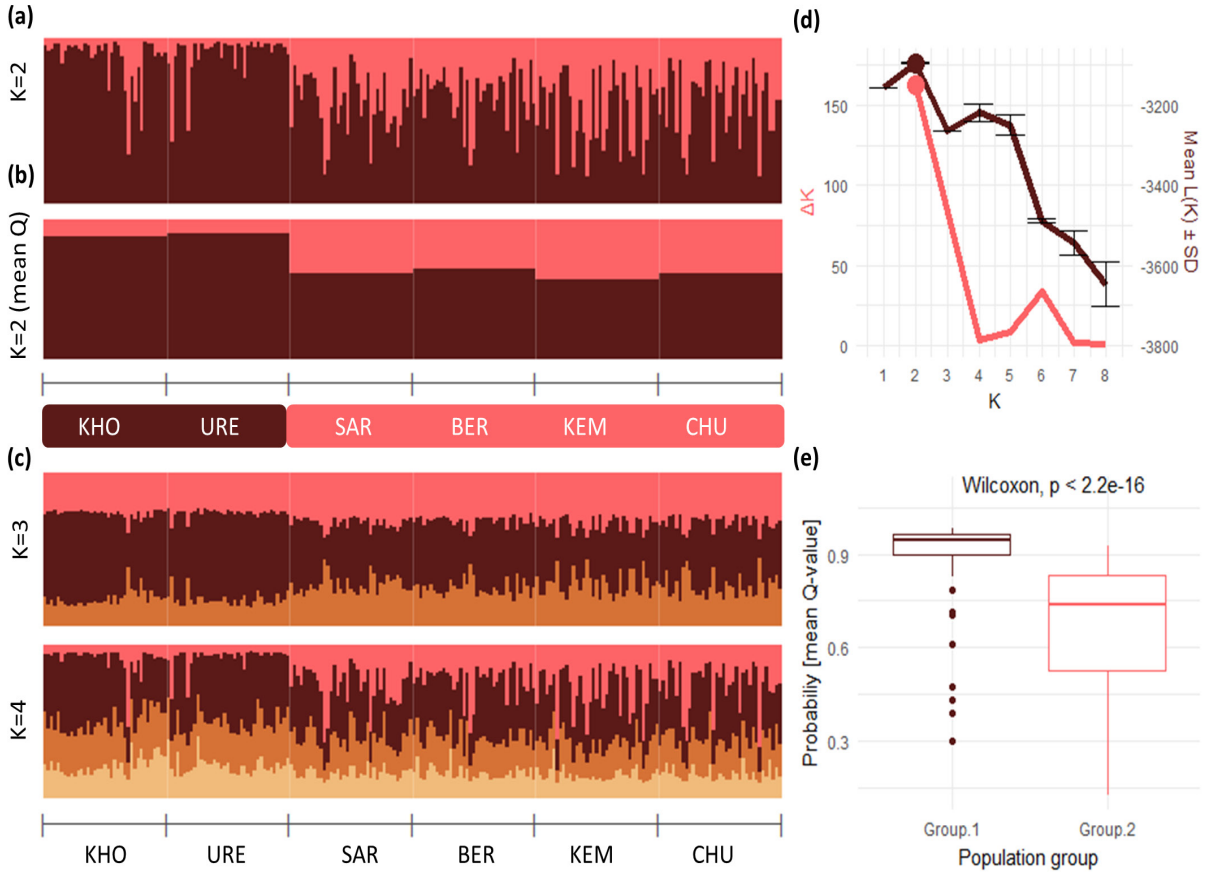

Fig. 3
Results of population genetic structure analysis of six Siberian stone pine populations (acronyms are as in Table 1). (a) Estimated population structure (*K*=2). (b) Genetic structural plot with group mean (*K*=2). (c) Estimated population structure (*K*=3 and *K*=4). (d) Estimation of the best subpopulation numbers based on Δ*K* and mean L(*K*) (±SD) values. (e) Wilcoxon test comparing membership probabilities of the two genetic groups.

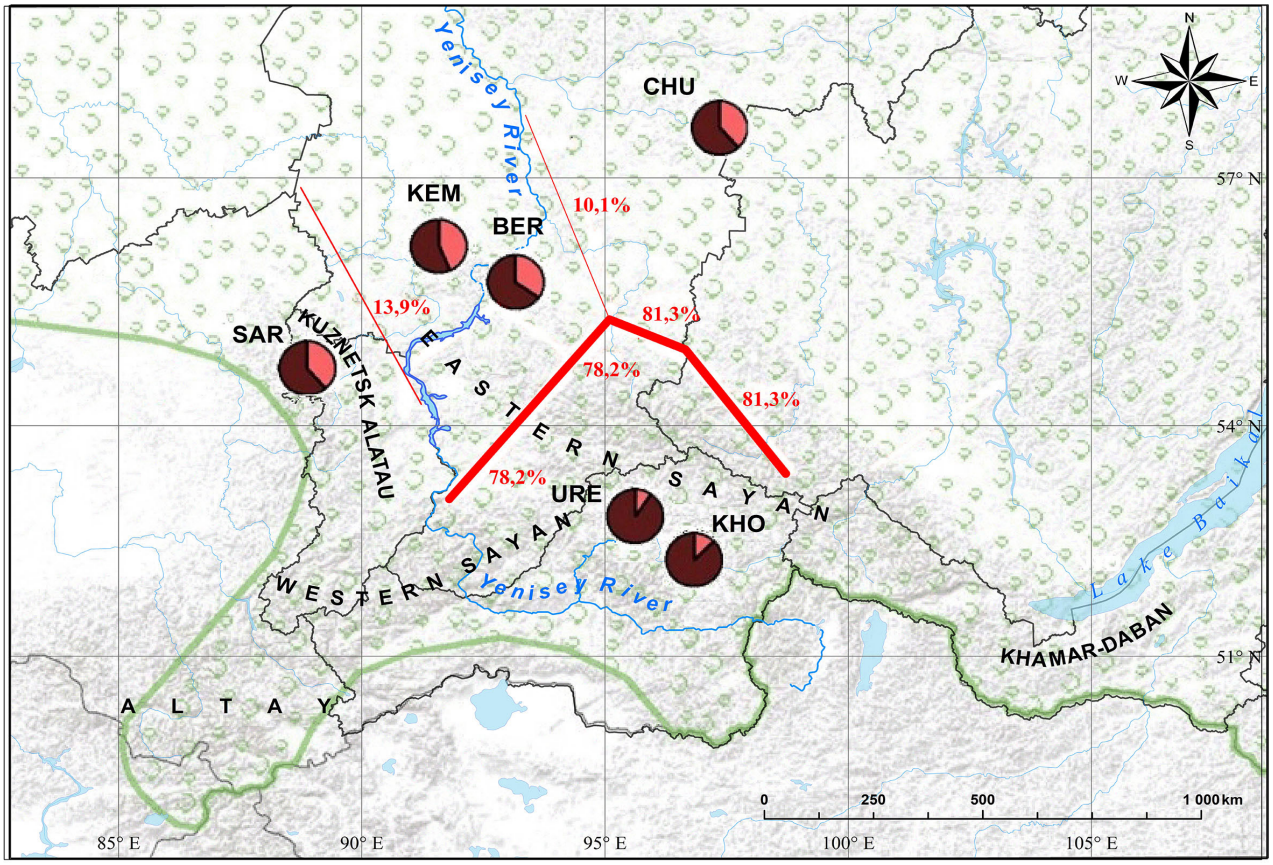

Fig. 4
Identification of genetic barriers among six Siberian stone pine populations (acronyms are as in Table 1), revealed by Barrier analysis (the genetic barriers are shown in red bold lines with bootstrap value).

v4.2.0. Throughout the analysis, 95 % confidence intervals (CI) were applied. Also, an AMOVA analysis was conducted comparing variance between groups, as detailed above.

Potential barriers to gene flow among the studied populations were identified using Monmonier's maximum-difference algorithm (Monmonier, 2010) implemented in BARRIER software v.2.2 (Manni et al., 2004). We generated 1000 *D* distance matrices (Nei's standard genetic distance corrected for sample size) in MSA software (Dieringer and Schlötterer, 2003) by bootstrapping over the seven nSSR loci. The matrices were subsequently used to estimate possible populations boundaries.

To detect the presence of isolation by distance (IBD), the correlation between geographical distances and genetic distances between population pairs was tested with Mantel test (Mantel, 1967). The test was performed with the "adegenet" R package with 1000 bootstrap replicates (Jombart, 2008).

Results

Among populations, the mean number of alleles per locus (*Na*) was 3.976 (3.714-4.143) and the mean effective number of alleles (*Ne*) was 2.082 (2.008-2.125) (Tab. 2). The BER population had the lowest values for allelic richness (A_R = 3.026) and SAR population had the highest value (A_R = 3.198). The mean expected heterozygosity (*He*) varied between 0.424 (KHO) and 0.490 (SAR). There was an excess of homozygotes across all populations (mean *F*is = 0.250).

Analysis of molecular variance (AMOVA) showed that most of the variability was accumulated within populations (96.8 %) and only a small part of it was accounted for by the interpopulation (2.2 %) and intergroup (1.0 %) variability components (Tab. 3). The significant among groups differentiation might indicate a possible existence in the past of separate Siberian stone pine refugia in Middle Siberia.

UPGMA clustering based on all loci showed that two groups are clearly differentiated, with a bootstrap value of 100 %, one containing KEM population from forest-steppe zone, and the other one consisting of the remaining populations (Fig. 2a). UPGMA clustering based on loci containing no null alleles also indicated two clusters, one containing CHU population and the other one comprised of the remaining populations (see Fig. S1 in Supplementary material for the results of UPGMA clustering for loci containing no null alleles). Principal Component Analysis (PCA) showed two population clusters according to the first and second main components (60,98 %)

(Fig. 2c). The KHO and URE populations formed one group, while all remaining populations formed the other group. The second and third principal components (42,90 %) showed no obvious clustering. No obvious clustering was also detected based on loci containing no null alleles (Fig. S1c)

STRUCTURE analysis was used to determine the optimal numbers of genetic groups in the studied Siberian stone pine populations. The highest value of Δ*K* and mean L(K) statistics was obtained when *K*=2 (Fig. 3d). Two Siberian stone pine populations located in Southern-Siberian mountain forest zone (Group 1: KHO and URE) showed the highest membership value in one of the two genetic clusters (in brown color in Fig. 3a, b). Similar groups were also identified when *K*=3 and *K*=4 (Fig. 3c). Also, significant differentiation was observed with the Wilcoxon test comparing membership probabilities of the two genetic groups ($p < 2.2e\text{-}16$) (Figure 3e), as well as with the AMOVA analysis ($F\text{st} = 0.01$, $p = 0.001$). In contrast, the other four populations were highly admixed (Group 2). The estimation of the contribution of genotypes in each population showed that the URE and KHO populations contained a higher proportion of genotypes originated from the south of Middle Siberia, compared to other samples from the core distribution area. The SAR, KEM, BER and CHU populations had a smaller share of genotypes from the south of Middle Siberia. The possible explanation for this might be the uneven ancestral migration from two different refugia.

A genetic barrier analysis detected one barrier against gene flow with strong bootstrap supports (78-81 %) (Fig. 4). The barrier delimited two southernmost populations (URE and KHO) located in the Southern-Siberian mountain forest zone. All putative barriers between the other populations were weak, indicating non-significant separation.

We further analyzed the correlation between genetic and geographical distances for the six Siberian stone pine populations using the Mantel test. The results showed that there was no significant correlation between genetic distance and geographic distance ($R = 0.102$, $p = 0.12$).

Discussion

The genetic diversity and differentiation of Siberian stone pine populations from different forest zones in Siberia were assessed based on polymorphism of seven nuclear SSR markers. Since Siberian Stone pine is of great ecological and economic importance in Siberia, it is essential to have knowledge about its current genetic pattern.

The study showed that the level of genetic diversity in Siberian stone pine populations from Middle Siberia was moderate (*He*=0.455). Slightly higher value was observed by Shuvaev and Ibe (2021) for the ten Siberian stone pine populations in the West Siberian Plain (West Siberia) (*He*=0.482) and lower genetic diversity (*He*=0.401) was obtained by Oreshkova et al. (2020) for the seven Siberian stone pine populations from the Kuznetsk Alatau Mts. (south of Western Siberia). The KHO population, which is located in the Eastern Sayan Mts., showed the lowest levels of genetic diversity (*He*=0.424). The highest genetic diversity (*He*=0.490) was detected in SAR population situated on the territory of the Kuznetsk Alatau mountain range.

The values of the inbreeding coefficient (*F*is) observed in our study were positive in all populations, thus indicating the heterozygote deficiency. The highest *F*is value (*F*is=0.367) was detected in one of the southernmost populations (URE). Homozygote excess is a common feature in conifer species and might be the result of the selection against heterozygotes, assortative mating, or the presence of null alleles (Hamrick et al., 1992; Şofletea et al. 2020).

The AMOVA results showed that only 2 % of the total genetic variation occurred among populations. Low differentiation among Siberian stone pine populations revealed in this study is in line with previous reports on Siberian stone pine using allozyme and nuclear simple sequence repeat (nSSR) markers (Petrova et al., 2014; Shuvaev and Ibe, 2021).

According to the STRUCTURE analysis, the two southernmost populations (KHO and URE) located in the Southern-Siberian mountain forest zone are genetically very similar and show slightly different coancestry values when two putative genetic groups are considered. Further, a genetic discontinuity between the two southern populations (KHO and URE) and the rest ones is supported by BARRIER analysis.

Our results, based on the analysis of nuclear microsatellite markers, indicate that a moderate level of genetic diversity exists in Siberian stone pine populations and that, despite large geographic distances there is limited genetic differentiation among populations. The current pattern of genetic diversity in Siberian stone pine populations might be the result of historical processes such as long-term alterations in geographic, climatic and ecological conditions (Shuvaev and Ibe, 2021). The lack of polymorphism at the seven nuclear SSR loci used in this study, which are prone to null alleles, is indeed not surprising for a conifer species with high rate of genome duplication. Further studies including NGS-based SSR genotyping or SNP arrays would be desirable to disentangle patterns of genetic diversity and structure in this species.

Conclusions

In the present study, seven nSSR markers were used to estimate the genetic diversity within and among six natural populations of Siberian stone pine in Siberia. All populations showed moderate values of genetic diversity, while one of the southernmost populations was less diverse. Accordingly, this population should be at the focus of a long-term study aimed at monitoring of population dynamics to prevent loss of genetic resources. Additionally, the two southernmost populations (KHO and URE) seem to be genetically distinguished from the other four. Whether this slight genetic difference correlates with the forest type zones is still needed to be addressed with more loci and populations. Nevertheless, the results showed a low level of genetic differentiation among Siberian stone pine populations which could be explained by efficient long distance gene flow. The presented findings can be used in

long-term monitoring of the state of Siberian stone pine genetic resources in Siberia.

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Supplementary Materials

Fig. S1 - Results of UPGMA clustering and PCA for loci containing no null alleles.

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