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PLANT GENETICS

Analysis of Genetic Structure and Differentiation of the Bog and Dry Land Populations of *Pinus sibirica* Du Tour Based on Nuclear Microsatellite Loci

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Abstract—We evaluated the population structure of the bog and dry land populations of the Siberian pine *Pinus sibirica* (*P. sibrica*) in Western Siberia using nuclear genome markers. Six pairs of nuclear microsatellite loci were used for this analysis. We detected 30 allelic variants in 120 individuals of four populations of *P. sibirica*. We established that the studied populations differ by genetic structure. The most essential differences were identified between the Siberian pine population from oligotrophic bog and the group of populations from dry land within eutrophic bogs and near settlements *P. sibirica* forest ($F_{ST} = 0.019$; $D_N = 0.053$). We estimated that diversification of the West Siberian populations of *P. sibirica* exceeded 2.4% ($F_{ST} = 0.024$), based on an analysis of SSR markers.

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INTRODUCTION

The Siberian stone pine (*Pinus sibirica* Du Tour) is one of the main forest species in many areas of Western Siberia, where pine forests occupy 7667 ths ha, or about 24% of the area of coniferous forests [1]. The natural upland populations of the Siberian stone pine in Western Siberia is quite well researched with respect to forestry and agricultural selection, including plant populations in and near settlements and the cultivated population [2–5]. The West Siberian Wetland Plain is defined by highly mosaic forest conditions with respect to the Siberian stone pine [6]. In the southern taiga subzone in West Siberia, *P. sibirica* is represented in wetlands and wetland ecotopes, occupying a variety of trophic habitats [4, 7–9]. A substantial portion of stone pine grows in the marshes of eutrophic type of water-mineral nutrition [10], in contrast to oligotrophic swamps, where the Siberian stone pine is much rarer and occurs mainly in the form of clumps and solitary trees.

To date, only a few studies have examined the differentiation of the populations of the Siberian stone pine in wetlands and upland habitat conditions. It has been shown that the differences in ecotopic populations of *P. sibirica* growing in eutrophic wetlands and dry lands are manifested in a variety of characteristics, such as the formation and maintenance of male and female strobili, the characteristics of the pollination regimen, the morphological parameters of the generative structure, and seed yield and quality [6, 9–11]. It has been reported that the Siberian stone pine growing in the bog eutrophic conditions in Western Siberia differs by its karyotypic parameters from the chromosomal characteristics reported previously for this species [12]. The Siberian stone pine growing in oligotrophic bogs is poorly investigated. Several ecological forms of *P. sibirica* growing in the oligotrophic bogs of Western Siberia have been described, among which are the oppressed tree f. *turfosa* [13] and the dwarf shrub f. *pumila*, which is characterized by extreme morphological degradation and the lack of reproductive structures [14]. There also differences in the quality of seeds between the Siberian stone pine populations growing on the oligotrophic bogs and dry lands of Western Siberia [15].

Although there have been extensive studies on allozyme variations in the populations *P. sibirica* conducted for different regions of Western and Eastern Siberia (which were determined by their geographical location) [16, 17], the studies were attempted to identify the effects of environmental barriers in shaping the genetic structure and differentiation of the species in the wetland and upland growing sites and were conducted on a single pine population [9, 18]. These studies have demonstrated significant variation in the structural features and allelic composition of the isoenzymes of the Siberian stone pine populations in the coordinate system "hydration–richness of soil," as well as significant variation in the industrial values of the populations growing in the wetland and upland ecotopes in the southern taiga subzone of the West Siberia, which were integrated into a single swamp forest ecologic profile [9].

Table 1. Characteristics of the *P. sibirica* populations in the southern taiga subzone of the Tomsk region

Population code	Geographic coordinates	Growth conditions and forest type	Characteristics of the *P. sibirica*			
			number of samples	taxa parameters		
				heigh, m	diameter, m	age, years
K-1	56°54′ N, 83°04′ E	Iksa-Yara interfluve; dry clay land; spruce-fir Siberian pine forest in a moss and short grass land	30	32–38	36–58	227–384
K-2	56°33′ N, 84°04′ E	The first terrace above the river Ob’ floodplain; Village Melnikovo; loamy dry land; near settlement Siberian pine on sedge-forb land	30	29–36	31–52	186–262
K-3	56°20′ N, 84°34′ E	Bogged valley of the Zhukovka River; fallow deep (7.6 m) eutrophic swamp; mixed spruce-fir-larch Siberian pine forest on a forb-wort land	30	2–4	4–6	36–52
K-4	56°53′ N, 82°40′ E	Iksa-Bakchar interfluve; an oligotrophic bog to 2.7 m deep; a Scots pine-sphagnum-small shrub-cotton grass stand containing clumps of and single-standing Siberian pine trees.	30	4–7	11–16	92–211

Thus, the data available to date on a comparative analysis of the morphological and physiological characteristics of the reproductive sphere, cytogenetic parameters, and polymorphism of the protein-coding sequences of the isoenzymes in the wetland and upland pine Siberian stone populations are scant and can only reliably diagnose the ecotopic divergence of the pine populations, leaving open the questions of a possible genetic differentiation on the level of the direct carrier of genetic information, the DNA. In this study, we present qualitatively new data on the assessment of genetic variation, structure and population differentiation of the *Pinus sibirica* growing in wetland and upland ecotopes in the southern taiga subzone of West Siberia based on an analysis of DNA polymorphisms using nuclear microsatellite markers (Simple Sequence Repeat (SSR) markers). The advantages of microsatellite analysis are the large number of affected loci (independent markers) and alleles, high levels of polymorphism, and codominance effects, which make it relatively easy to identify the homozygous and heterozygous genotypes. These SSR marker qualities make them a highly informative and efficient tool to examine the genetic structure and differentiation of the studied populations.

MATERIALS AND METHODS

The objects of this study were four populations of the Siberian stone pine. A brief listing of the characteristics of these populations and their locations are presented in Table 1.

PCR analysis was performed on the base of a branch of the Russian Center of Forest Protection in Krasnoyarsk. Individual preparations of total DNA were isolated from 100–200 mg of dried pine needles collected from 30 trees in each of the four populations (total of 120 samples). DNA was isolated according to protocol for plant tissues using the cetyl trimethylammonium bromide (CTAB) method [19].

We used nuclear microsatellite loci (DNA fragments with repeated short nucleotide sequences) as genetic markers. The isolated DNA was used as a template for amplification in a Polymerase Chain Reaction (PCR) with six pairs of primers (Table 2). These primers were developed for the European stone pine (*Pinus cembra* L.), and used successfully in studies with *P. sibirica* [20]. The characteristics of the selected nuclear microsatellite loci are also shown in Table 2.

For PCR reactions, we used the DNA amplification kit GenePak PCR Core (Isogen Laboratory, Russia). Amplification of the selected microsatellite loci was conducted under the following conditions: DNA was melted 94°C for 15 min; then, 30 PCR cycles were carried out, denaturation for 30 s at 94°C, primer annealing for 30 s at 54°C, and elongation for 50 s at 72°C. Then, a final elongation interval was held at 72°C for 15 min.

Table 2. Characterization of nuclear microsatellite loci selected for analysis of genetic variation *P. sibirica*

Locus	Motif	Annealing temperature, °C	Product size, bp	N_A
Pc 1b	$(GT)_{19}$	54	174–212	9
Pc 7	$(CT)_6(AT)_4(GT)_{25}$	54	354–378	5
Pc 18	$(TG)_{12}$	54	152–158	4
Pc 22	$(CA)_7TA(CA)_{12}(TA)_2$	54	342–354	6
Pc 23	$(TG)_6CG(TG)_2$	54	205–235	4
Pc 35	$(GTT)_7$	54	152–162	2

N_A indicates the average number of alleles per locus.

Electrophoretic separation of the amplified DNA fragments was performed in a 6% polyacrylamide gel in a Tris-EDTA-borate electrode buffer in vertical electrophoresis chambers at 300 V for 2–2.5 h, followed by gel staining with EtBr solution to visualize the amplification products in UV light. As a standard DNA length markers, we used the product of the pBR322 plasmid digest with restriction enzymes *Hae*III and *Hpa*II.

We used program MICRO-CHECKER [21] to identify and eliminate possible errors in genotyping of the microsatellite loci. These errors in microsatellite studies arise predominantly due to the emergence of the hidden null alleles in homozygotes, which arise due to a mutation in the DNA at the site of primer hybridization, or due to incomplete annealing of the primers. Using this program, we evaluated the frequency of the potential null alleles at the studied loci and adjusted the calculated numbers of homozygous genotypes and the frequencies of the amplified alleles in the studied populations. The corrected genotypes for the four studied populations of the Siberian stone pine were used for calculations of the basic indicators of the intra- and inter-population genetic variations using the GenAlEx 6.41 program [22]. Correlations between geographic and genetic distances were assessed using the Mantel test [23].

RESULTS AND DISCUSSION

Analysis of six nuclear microsatellite loci in four natural populations of the Siberian stone pine from different areas of its natural distribution in the Tomsk region showed that all loci were polymorphic (Fig. 1). The most highly polymorphic of all four studied populations proved to be the *Pc 1b* locus, which was represented by nine allelic variants. The loci *Pc 7* and *Pc 22* also showed high population variability, with five and six identified alleles, respectively. The *Pc 35* locus was characterized by the lowest variability, manifested by only two alleles. Electrophoregrams presenting variations in all of the studied microsatellite loci are shown in Fig. 1.

Using the software MICRO-CHECKER, we uncovered several hidden null alleles at the *Pc 1b* locus in all four studied populations of the *P. sibirica* (K-1–K-4), and at the *Pc 7* and *Pc 22* loci in the oligotrophic bog population (K-4). In total, we found 30 allelic SSR variants in four *P. sibirica* populations. The frequencies of the hidden null alleles were calculated with the assumption that populations conform to the Hardy–Weinberg equilibrium [24]. The selected nuclear microsatellite loci differed in frequency and allele composition in the different populations of Siberian stone pine (Table 2). The greatest allelic diversity was detected in the population of the species from the oligotrophic bog (K-4) (Table 3).

To identify the level of genetic diversity in each of the four studied populations of Siberian stone pine, we calculated the basic indicators of genetic variability (Table 3). The obtained vales of the basic genetic variability parameters showed that the *P. sibirica* populations included in the study differ in the level of their genetic diversity. The highest levels for most genetic variability parameters ($N_A = 5.000$; $N_E = 3.122$; $H_E = 0.611$; $F = 0.107$), with the exception of observed heterozygosity ($H_O = 0.550$), were detected in the population of Siberian stone pine in oligotrophic bog (K-4). The highest level of observed heterozygosity ($H_O = 0.583$) was detected in the near-settlement population of *P. sibirica* (K-2). In the upland populations *P. sibirica* (K-1) and in the eutrophic swamp (K-3), the observed heterozygosity was 0.506 and 0.483, respectively. Overall, the obtained values for the main indicators of genetic polymorphism demonstrate a fairly high average level of genetic diversity in the Siberian stone pine populations in the upland and wetland ecotopes (Table 3) and are similar to the values for the Siberian pines reported by other authors [20, 25, 26].

We detected a deficiency in heterozygous genotypes in the marshes (K-3 and K-4) and upland (K-1) *P. sibirica* populations (Table 3). One explanation for heterozygote deficiency and elevated levels of inbreeding ($F = 0.107$) in the population of Siberian stone pine in the oligotrophic bog (K-4) may be the twofold (phenologically and geographically conditioned)

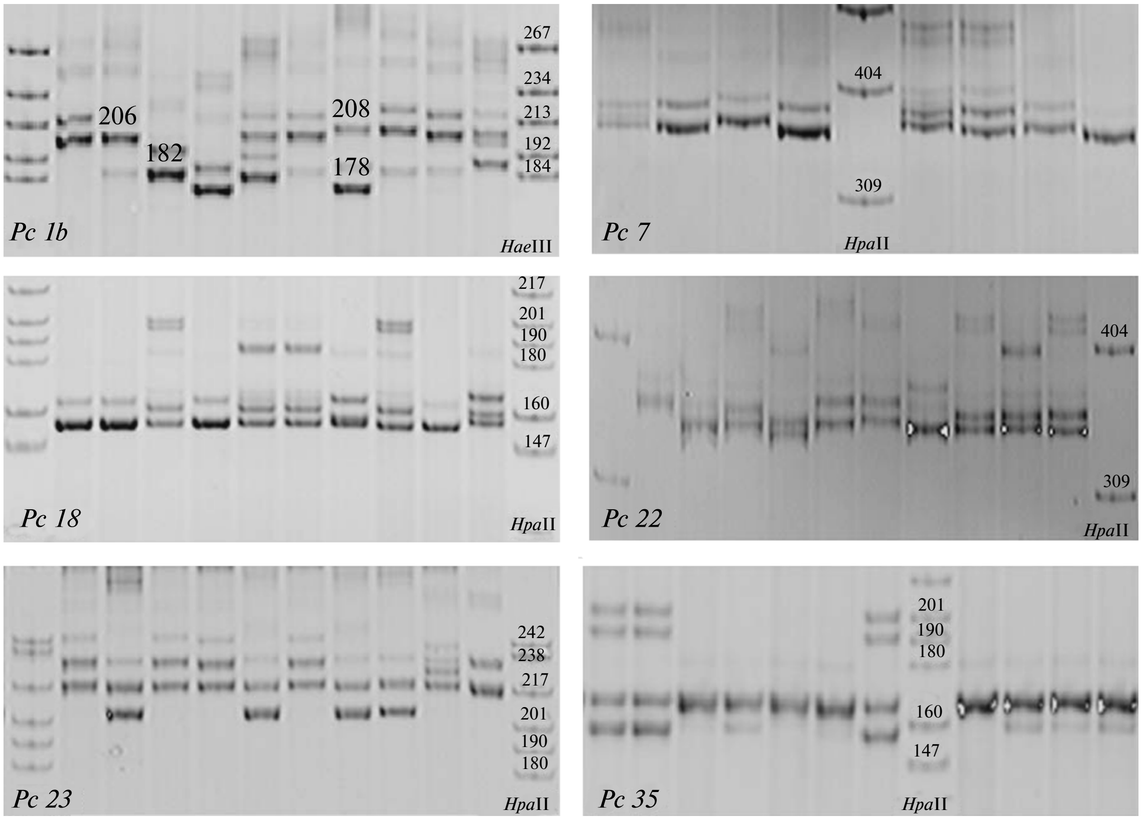

Fig. 1. Electrophoregrams of the nuclear microsatellite loci *Pc 1b, Pc 7, Pc 18, Pc 22, Pc 23*, and *Pc 35* of the Siberian stone pine. The values of 178, 182, 206, and 208 on the *Pc 1b* electrophoregram show allele annotations based on the length of the amplification product. *Hae*III and *Hpa*II indicate the standard DNA length marker sets obtained in a digest reaction of the pBR322 plasmid.

effect of its reproductive isolation from the trees species in dry lands. This hypothesis is based on the example of reproductive isolation between populations of another species of the *Pinus* genus, the Scots pine growing on oligotrophic bogs and dry lands, due to a mismatch in the phenophase of pollen release in these contrasting ecotopes [27, 28]. Thus, there is an increased likelihood of self-pollination in *Pinus* species in oligotrophic bogs that is due to phenological isolation, an overall decline in the amount of pollen, a reduction of the number of microstrobiles on each tree, and the number of microsporophylls in the microstrobili [29].

The deficit in heterozygotes, constituting $F = 0.057$ in the *P. sibirica* population in the upland (K-1) and $F = 0.045$ in the population of the species in the eutrophic swamp (K-3), can be also explained by a shielding effect produced by other species of coniferous trees, which impairs exchange of pollen between trees in these dense mixed tree stands. The peculiarity of the Siberian stone pine population in the eutrophic swamp is a lag in the pollen release relative to the adjacent upland stone pine forests and protogyny, resulting in a diminished pollen concentration during the period of high receptivity of female cones and a low pollination effect [6]. Furthermore, the trees in the eutrophic swamp sample (K-3) are young; their age is 40–50 years. It has been shown that a high frequency of homozygous genotypes in the young conifer populations is usually offset with age by a balancing selection in favor of heterozygotes [17].

In contrast, the near-settlement plantation of *P. sibirica* (K-2) showed an excess of heterozygote genotypes (Table 3), which is likely the result of a long-term positive selection conducted by the population. The near-settlement stone forests in the southern taiga subzone of the Western Siberia are a special type of a forest ecosystem. The high-yield pine seed gardens near settlements were formed with assistance by local residents, who cut down sterile and defective individual stone pines and removed the accompanying tree species present in the indigenous semidominat forest habitat [4]. In a study of trees in different selection conditions in a plantation of another *Pinus* species (the Scotch pine) conducted using ISSR markers, it was also determined that populations subject to positive planting are characterized by high levels of genetic variation and a considerable genetic distance from the normal natural populations and those under negative selection [30]. At the same time, analysis of the

Table 3. Indicators of genetic variability of Siberian stone pine, calculated based on nuclear microsatellite analysis

Population	Loci	N_A	N_E	H_O	H_E	F
K-1	*Pc 1b*	8.000	3.939	0.700	0.746	0.062
	Pc 7	4.000	1.773	0.400	0.436	0.083
	Pc 18	4.000	2.174	0.467	0.540	0.136
	Pc 22	5.000	2.903	0.633	0.656	0.034
	Pc 23	4.000	3.051	0.533	0.672	0.207
	Pc 35	2.000	1.342	0.300	0.255	–0.176
Population average		4.500 ± 0.806	2.530 ± 0.388	0.506 ± 0.061	0.551 ± 0.074	0.057 ± 0.053
K-2	*Pc 1b*	7.000	4.369	0.700	0.771	0.092
	Pc 7	4.000	1.921	0.500	0.479	–0.043
	Pc 18	4.000	1.474	0.367	0.322	–0.140
	Pc 22	5.000	3.147	0.733	0.682	–0.075
	Pc 23	4.000	2.773	0.833	0.639	–0.303
	Pc 35	2.000	1.514	0.367	0.339	–0.080
Population average		4.333 ± 0.667	2.533 ± 0.460	0.583 ± 0.082	0.539 ± 0.076	–0.091 ± 0.053
K-3	*Pc 1b*	8.000	5.556	0.767	0.820	0.065
	Pc 7	4.000	1.773	0.367	0.436	0.159
	Pc 18	4.000	2.048	0.433	0.512	0.153
	Pc 22	4.000	2.293	0.700	0.564	–0.241
	Pc 23	4.000	2.687	0.500	0.628	0.204
	Pc 35	2.000	1.142	0.133	0.124	–0.071
Population average		4.333 ± 0.803	2.583 ± 0.631	0.483 ± 0.094	0.514 ± 0.094	0.045 ± 0.070
K-4	*Pc 1b*	9.000	5.505	0.767	0.818	0.063
	Pc 7	5.000	2.100	0.467	0.524	0.109
	Pc 18	4.000	2.273	0.533	0.560	0.048
	Pc 22	6.000	4.265	0.733	0.766	0.042
	Pc 23	4.000	3.120	0.533	0.679	0.215
	Pc 35	2.000	1.471	0.267	0.320	0.167
Population average		5.000 ± 0.966	3.122 ± 0.619	0.550 ± 0.075	0.611 ± 0.074	0.107 ± 0.029
Average for all studied populations		4.542 ± 0.385	2.692 ± 0.255	0.531 ± 0.038	0.554 ± 0.038	0.029 ± 0.029

See Table 1 for population codes. N_A, mean 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.

isozyme and microsatellite loci revealed that individual tree heterozygosity in the Scotch pine plantations can vary significantly [31].

Analysis of the population structure of *P. sibirica* using the Wright's fixation indices [32] showed that an individual tree can have on average an almost 4% deficit of heterozygous genotypes ($F_{IS} = 0.037$) relative to the population and a 6% deficit in heterozygotes ($F_{IT} = 0.061$) in relation to the species as a whole (Table 4). The individual locus values, F_{IS} and F_{IT}, show that the most significant deficiency of heterozygotes are observed in the loci *Pc 1b, Pc 7, Pc 23, Pc 18.*

The inbreeding coefficient, calculated for the population in relation to the species as a whole (F_{ST}), reflects the degree of subdivision of the populations. The obtained vales ranged from 0.007 (*Pc 7*) to 0.035 (*Pc 22*), averaging 0.024. This indicates that only 2.4% of the genetic variation in the studied populations of

Table 4. Calculated *F*-statistics parameters for the studied *P. sibrica* populations

Locus	F_{IS}	F_{IT}	F_{ST}
Pc 1b	0.070	0.097	0.029
Pc 7	0.076	0.083	0.007
Pc 18	0.069	0.097	0.030
Pc 22	–0.050	–0.013	0.035
Pc 23	0.084	0.101	0.019
Pc 35	–0.027	–0.001	0.026
Mean	0.037 ± 0.024	0.061 ± 0.022	0.024 ± 0.004

F_{IS}, individual inbreeding coefficient relative to the population. F_{IT}, individual inbreeding coefficient relative to the species; $F_{ST,}$ inbreeding coefficient for the populations relative to the whole species.

the Siberian stone pine is distributed among the populations. Thus, around 97.6% of the total genetic diversity is concentrated within the populations. The results of the χ^2-test indicate that a statistically significant heterogeneity of allelic frequencies in the studied populations was observed for three (*Pc 1b, Pc 18, Pc 22*) out of the six examined loci.

We further examined the degree of genetic diversification between the populations of *P. sibirica* by calculating the Nei's [33] genetic distances (D_N). This analysis (Table 5) showed that the upland population of the species (K-1) and the eutrophic swamp forest (K-3) are the most similar in their genetic structure ($F_{ST} = 0.012$; $D_N = 0.028$). Their difference from the near-settlement population (K-2) was slightly higher at $F_{ST} = 0.014$; $D_N = 0.031$. The maximal differences in the genetic structure were detected between the *P. sibirica* population in the oligotrophic bog (K-4) and the three others ($F_{ST} = 0.019$; $D_N = 0.053$). The established level of differentiation of the Siberian stone pine populations included in this study is clearly illustrated by the location of the populations in the plane of the two major components of the Nei's matrix (Fig. 2). We performed the Mantel test ($r = 0.274$, $P = 0.31$) to show that there is no correlation between the values of genetic distance (D_N) and geographic distance.

Thus, the results of our study of DNA polymorphisms in the wetland and upland populations of Siberian stone pine revealed a considerable genetic diversity of the nuclear microsatellite loci and a high degree of genetic differentiation. We propose that the significant genetic heterogeneity of the studied populations of *P. sibirica* is associated with the contrasting differences in their water-mineral nutrition regiments, which induce a change in the vectors of natural selection in the oligotrophic, eutrophic swamps and dry lands, and influence the long-term positive selection in the near-settlement plantations. Previous studies of isozyme polymorphisms in the *Pinus* species identified a relationship between the population allelic structure and trophic and humidity conditions of the soil and determined a high genetic subdivision between the wetland and upland populations of *P. sibirica* [9], as well as the *P. sylvestris* growing in these ecotopes [27, 34–36]. Further studies of DNA polymorphisms in a large number of more remote and ecotopically distinct populations of Siberian stone pine should allow a more detailed evaluation of their level of genetic variability, determine the degree of differentiation, and examine the level of interpopulation genetic drift in the species.

Table 5. Genetic distances D_N between the studied populations of *P. sibirica*

K-1	K-2	K-3	K-4	
***				K-1
0.030	***			K-2
0.028	0.034	***		K-3
0.052	0.042	0.065	***	K-4

See Table 1 for population codes.

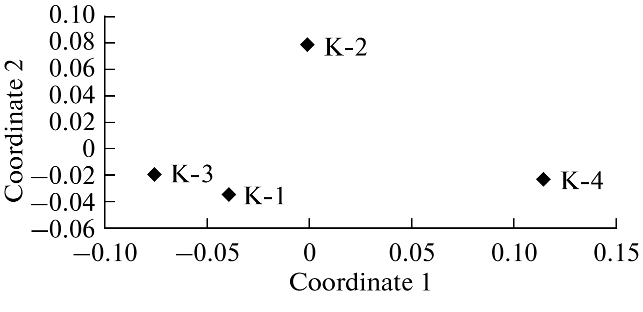

Fig. 2. Genetic distances for the studied *P. sibirica* populations. Projection on the plane of the two major components of the Nei's genetic distances matrix. See Table 1 for population cod numbers.

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