ISSN 1022-7954, Russian Journal of Genetics, 2017, Vol. 53, No. 3, pp. 338–347. © Pleiades Publishing, Inc., 2017.
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PLANT GENETICS

Variability of Nuclear Microsatellite Loci in the Populations of Siberian Dwarf Pine (*Pinus pumila* (Pallas) Regel) from the Russian Part of the Range

N. V. Oreshkova[a], *, V. P. Vetrova[b], **, S. N. Goroshkevich[c], *, and E. A. Petrova[c]**

[a] *Sukachev Institute of Forest, Siberian Branch, Russian Academy of Sciences, Krasnoyarsk, 660036 Russia*
[b] *Kamchatka Branch of Pacific Geographical Institute, Far East Branch, Russian Academy of Sciences, Petropavlovsk-Kamchatsky, 683024 Russia*
[c] *Institute of Monitoring of Climatic and Ecological Systems, Siberian Branch, Russian Academy of Sciences, Tomsk, 634055 Russia*
**e-mail: oreshkova@ksc.krasn.ru*
***e-mail: v.vetrova@mail.ru*
****e-mail: gorosh@imces.ru*

Received April 4, 2016; in final form, April 21, 2016

Abstract—Variability of nuclear microsatellite loci was examined in Siberian dwarf pine. Six microsatellite loci (*RPS2*, *RPS6*, *RPS12*, *RPS124*, *RPS127*, *Pc18*) demonstrated different polymorphism levels in ten populations of Siberian dwarf pine. The average number of alleles per locus was 4.88, the average observed heterozygosity was 0.465, and the average expected heterozygosity was 0.510. About 13% of total genetic variability was explained by the genetic differences between the populations ($F_{ST} = 0.129$). Genetic distances between the examined populations of *Pinus pumila* inferred from the data on the SSR marker frequencies statistically significantly correlated with the geographical distances between the population samples. The level of genetic variability of the populations from Kamchatka Peninsula was lower than that demonstrated by continental and island populations. The genetic differentiation of the Kamchatka–Magadan and other populations of Siberian dwarf pine observed in our study can be explained in terms of their formation from different Pleistocene refugial centers.

Keywords: molecular genetic markers, polymorphism, population structure and differentiation, genetic distance

DOI: 10.1134/S1022795417020089

INTRODUCTION

Siberian dwarf pine (*Pinus pumila* (Pall.) Regel) is a species of five-needle pines, which belongs to the section *Quinquefolia* of the subgenus *Strobus* [1]. Unlike other members of the genus *Pinus* (with the exception of *P. mugo*), which are erect trees, *P. pumila* has a life form of a creeping pine. Siberian dwarf pine is native to the territory from the Lake Baikal in the west to the Pacific Ocean in the east. The northern border of its range reaches 70°30′ N, the eastern border runs along the Chukotka Peninsula, the southern border wedges out to 36° N in the high mountains of the Honshu Island, and the western border extends into southern Trans-Baikal and Pre-Baikal regions [2–5].

Owing to high ecological plasticity, *P. pumila* is widely distributed in various natural zones and altitude belts of North and East Asia. It is a typical element of the coldest northern part of the Pacific monsoon region [6, 7]. In the Northeast Asia, this species develops a formation of creeping alpine forest [7–9]. The formation of this type can be also found on cold seacoasts of the region [9]. *P. pumila* is a part of the second layer or understory in forest stands, while in open spaces, especially in the subalpine mountain belt, it forms dense impenetrable thickets [2, 5]. *P. pumila* is a zonal species only in the forest-tundra. In the taiga zone, as well as in mixed and deciduous forests, it is intrazonal species widely distributed on the forest boundaries, including bogs and shores, stone placers, and volcanic ash.

In Pre-Baikal region, Trans-Baikal region and the south of Yakutia, the range of Siberian dwarf pine overlaps with the range of Siberian pine (*Pinus sibirica* Du Tour), a related species of five-needle pines. Natural hybridization of the species in this region was described [2, 10], and genetic evidence of the hybrid origin of morphologically intermediate individuals was obtained [11]. In addition, the genetic structure,

the system of cross pollination, and the seed yield of the species and their hybrids in the hybridization zone were described [12–17]. Morphologically intermediate individuals were also found in the east (on the territory of Japan), in the overlapping zone of Siberian dwarf pine and another representative of five-needle pines, Japanese white pine (*Pinus parviflora*) [18]. The fact of hybridization was first proved by Watano et al. [19]. The polymorphism of chloroplast and mitochondrial genomes of these two species has been examined by Japanese researchers since the mid-1990s [19–22]. The contrasting patterns of introgression of two cytoplasmic genomes, i.e., the transfer of paternal cpDNA from *P. parviflora* to *P. pumila* and the transfer of maternal mtDNA in the reverse direction in the species in hybridization zone, was described [19–21].

The high importance of *P. pumila* for boreal, subarctic, and subalpine ecosystems provoked considerable interest of researchers, including geneticists, to this species. The first studies of the genetic polymorphism in the populations of *P. pumila* were performed in 1980s by analyzing the allozyme variability. Over the past period, the genetic control of isoenzymes in *P. pumila* was described [23, 24], and the genetic structure, subdivision, and differentiation of the populations from the Russian [16, 25–27] and Japanese [22, 28] parts of the range were described, as well as the system of cross pollination in the populations of the species [29]. All researchers pointed to the high level of intraspecific polymorphism of *P. pumila* compared to other species of five-needle pines [25, 26, 30, 31].

At present, the investigations and activities aimed at the gene pool conservation of forest-forming tree species actively use polymorphic DNA markers. Among these, the most effective are microsatellite loci, i.e., the DNA fragments, consisting of short repeated sequences (simple sequence repeat, SSR). These are highly polymorphic nuclear genome markers with codominant inheritance, which make it possible with high accuracy to obtain data on multilocus genotypes of individuals, to calculate the main indices of intraspecific variability, and to describe the population processes. This approach is widely used to study the population structure of tree species [32–34], including five-needle pines [35, 36]. Investigations of polymorphic nuclear microsatellite loci in the populations of Russian forest-forming tree species started rather recently [37–41]. The objective of this study is to test whether microsatellite markers developed for other species of five-needle pines can be used in *P. pumila* and to study their variability in the populations of *P. pumila* from the Russian part of the range.

MATERIALS AND METHODS

The experiments were performed using samples from ten populations of *P. pumila* from natural vegetation areas in the north of the Russian Far East, including the Kolyma and Koryak highlands and Kamchatka Peninsula, and samples of southern populations from Sakhalin Island and Kunashir Island, as well as one sample each from the Amur region and Cisbaikalia (Fig. 1).

Genomic DNA was extracted from 100–200 μg needles collected from 276 plants using the CTAB method [42]. The isolated DNA was used for polymerase chain reaction (PCR) with six pairs of primers (*RPS2*, *RPS6*, *RPS12*, *RPS124*, *RPS127*, *Pc18*) designed earlier for Weymouth pine (*Pinus strobus* L.) [43] and Swiss stone pine (*Pinus cembra* L.) [44]. The reactions were carried out using the GenePak PCR Core reagent kit (Isogene Lab., Ltd., Russia). The conditions of PCR amplification used in the present study were suggested and described by Echt et al. [43] and Salzer et al. [44]. The amplification products were separated by electrophoresis in 6% polyacrylamide gel. Assessment of the genetic diversity indices was carried out using the GenAlEx 6.41 software program [45]. The MICRO-CHECKER software program [46] was used to eliminate possible genotyping errors and to detect cryptic *null* alleles.

RESULTS

Variability of Microsatellite Loci in P. pumila

Analysis of six nuclear microsatellite loci in ten natural populations of *P. pumila* revealed 49 allelic variants. In our experiments, the *RPS2*, *RPS12*, *RPS124*, and *RPS127* loci were found to be highly polymorphic. In the studied populations, from 9 to 11 alleles of these loci were identified. The *RPS6* and *Pc18* loci, the heritability of which was determined by four allelic variants, were found to be less variable. It is worth noting that in Kamchatka populations, *RPS124* and *Pc18* behaved like monomorphic or weakly polymorphic loci with rare alleles.

An example of an electrophoregram of one of the highly polymorphic loci is given in Fig. 2.

The highest allelic diversity (35–36 alleles) was identified in three populations: from the Kolyma Highlands (MG), Amur region (TsM), and Cisbaikalia (S-M). Lower allelic diversity (19–30) was observed in all Kamchatka populations.

Genetic Diversity in the Populations of P. pumila

Analysis of the main genetic diversity parameters (Table 1) showed that the examined populations of *P. pumila* were considerably different in their levels. The highest, compared to other populations, level of genetic variability was revealed in the population from the Amur region (TsM). In this population, the average number of alleles per locus and the values of observed and expected heterozygosity under Hardy–Weinberg equilibrium constituted 6.000, 0.611, and 0.652, respectively. The populations from the Kolyma Highlands (MG) and Kunashir and Sakhalin islands,

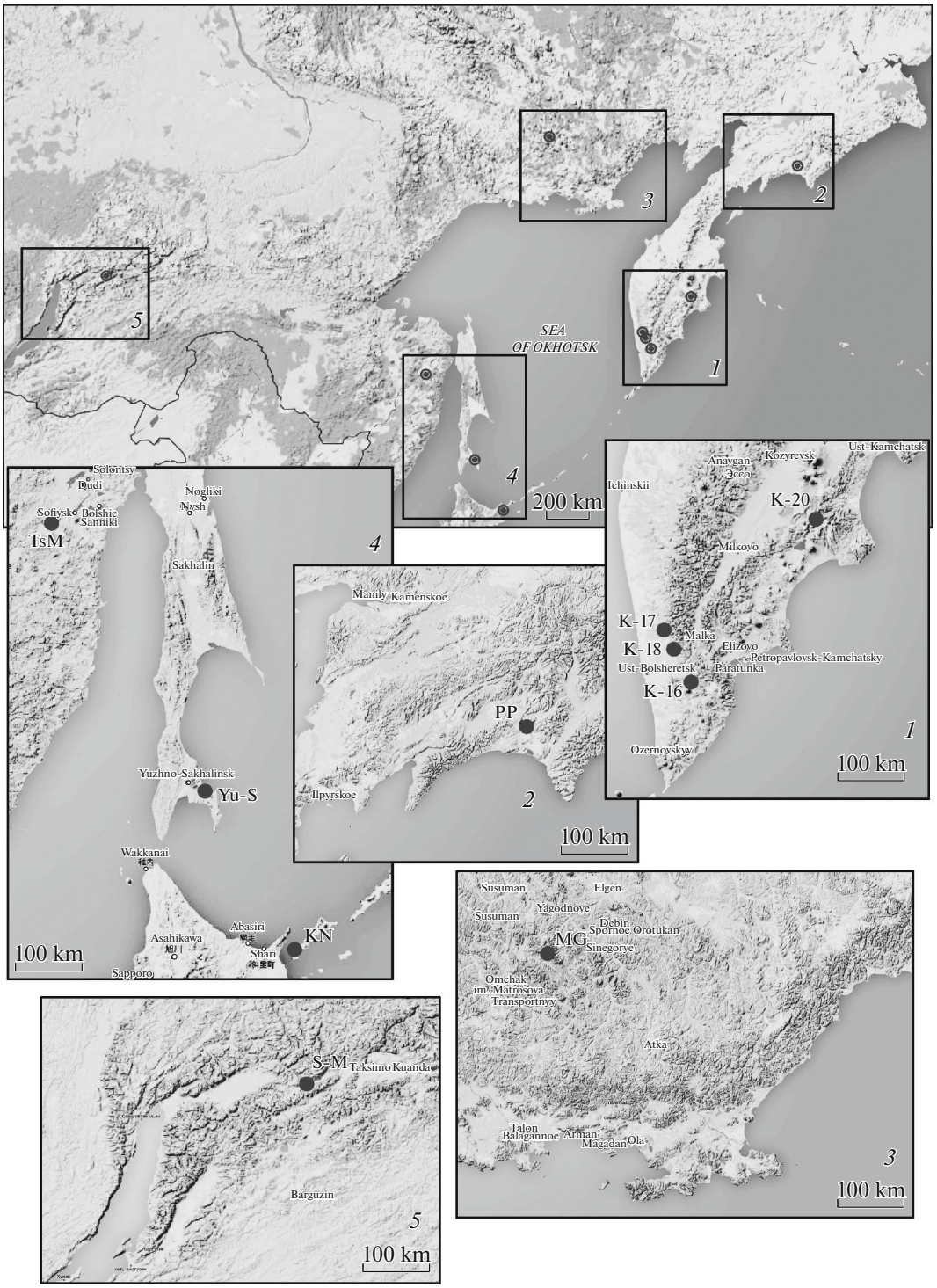

Fig. 1. Schematic map showing the distribution of the population samples of *P. pumila* in the examined regions. (*1*) Kamchatka krai; (*2*) Koryak Autonomous Okrug; (*3*) Magadan oblast; (*4*) Khabarovsk krai and Sakhalin oblast; (*5*) Republic of Buryatia. Populations: K-16, K-17, K-18, K-20, Kamchatka; PP, Koryak, near the settlement of Pakhachi; MG, Magadan; Yu-S, Yuzhno-Sakhalinsk; KN, from Kunashir Island; TsM, from Khabarovsk krai, near the settlement of Tsimmermanovka; S-M, from Republic of Buryatia, near the settlement of Severomuisk.

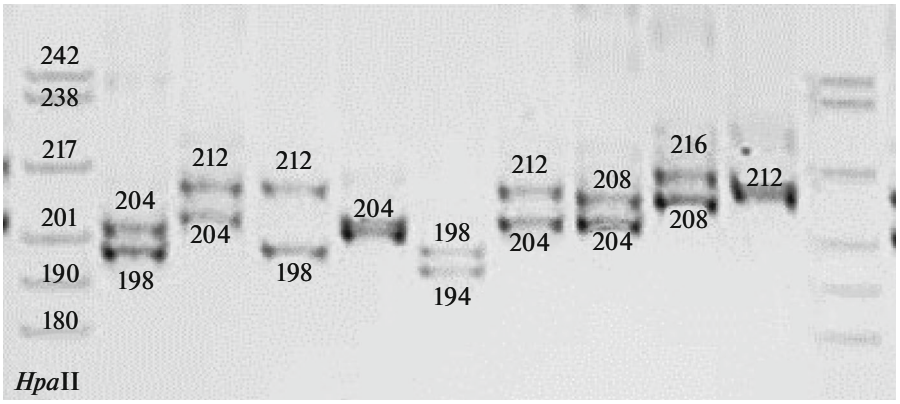

Fig. 2. Electrophoregram of nuclear *RPS127* microsatellite locus in *P. pumila*. Figures 194, 198, 204, 208, 212, and 216 on the electrophoregram are the allele designations according to the size of the amplified DNA fragment. *Hpa*II, standard size marker.

as well as the sample from Cisbaikalia (S-M), were characterized by medium values of the genetic variability parameters. The lowest level of intrapopulation genetic variability (N_A = 3.792; N_E = 2.027; H_O = 0.332; H_E = 0.375) was found in the populations of *P. pumila* from the Kamchatka Peninsula.

In general, the observed values of the main genetic polymorphism parameters point to a high level of genetic diversity in *P. pumila* (Table 1). Moreover, these values were close to those obtained for cedar pines [43, 44, 47–49].

In three Kamchatka populations (K-17, K-18, K-20), as well as in the population from the Kolyma Highlands (MG), the increased deficiency of heterozygous genotypes was observed (Table 1). In the population from Kunashir Island, as well as in South Kamchatka population sample from the Karymchina River valley (K-16), a small excess of heterozygotes was observed (Table 1). In the remaining populations (Yu-S, TsM, S-M), the heterozygote deficiency was about 0.067.

The Structure of Microsatellite Loci Variability in the Populations of P. pumila

On average, as follows from the analysis of the species population structure using Wright's fixation indi-

Table 1. Genetic variability indices in *P. pumila* inferred from the data of nuclear microsatellite analysis

Populations	N_A	N_E	H_O	H_E	F
K-16	3.500	2.205	0.378	0.379	–0.025
K-17	4.333	2.213	0.383	0.436	0.110
K-18	4.167	2.255	0.350	0.422	0.149
K-20	3.167	1.437	0.217	0.263	0.181
For Kamchatka populations, on average	3.792 ± 0.426	2.027 ± 0.223	0.332 ± 0.051	0.375 ± 0.056	0.105 ± 0.029
PP	5.000	3.092	0.444	0.488	0.055
MG	5.833	3.482	0.578	0.651	0.124
KN	5.333	3.356	0.583	0.595	–0.016
Yu-S	5.667	3.752	0.563	0.630	0.067
TsM	6.000	3.521	0.611	0.652	0.055
S-M	5.833	3.185	0.542	0.584	0.029
For non-Kamchatka populations, on average	*5.611 ± 0.370*	*3.398 ± 0.275*	*0.553 ± 0.035*	*0.600 ± 0.040*	*0.052 ± 0.020*
For all examined populations, on average	*4.883 ± 0.301*	*2.850 ± 0.206*	*0.465 ± 0.032*	*0.510 ± 0.036*	*0.071 ± 0.016*

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.

Table 2. Values of F_{IS}, F_{IT}, and F_{ST}

Locus	N	χ^2	F_{IS}	F_{IT}	F_{ST}
RPS2	11	239.187 (55)***	0.079	0.229	0.163
RPS6	4	14.744 (6)*	0.005	0.037	0.032
RPS12	10	163.447 (45)***	0.108	0.166	0.064
RPS124	9	225.657 (36)***	0.079	0.319	0.260
RPS127	11	375.950 (55)***	0.131	0.296	0.191
Pc18	4	16.769 (6)*	0.078	0.135	0.061
Mean			0.080 ± 0.017	0.197 ± 0.043	0.129 ± 0.037

N, number of alleles; χ^2, test for heterogeneity at the statistical significance level of *** $P < 0.001$ and * $P < 0.05$; F_{IS}, the inbreeding coefficient of an individual relative to the populations; F_{IT}, the inbreeding coefficient of an individual relative to the species; F_{ST}, the inbreeding coefficient of the populations relative to the species as a whole; ±, standard error.

Table 3. D_N genetic distances between the examined populations of *P. pumila*

K-16	K-17	K-18	K-20	PP	MG	KN	Yu-S	TsM	Populations
0.071	–								K-17
0.099	0.097	–							K-18
0.072	0.062	0.160	–						K-20
0.083	0.055	0.111	0.124	–					PP
0.139	0.096	0.183	0.134	0.119	–				MG
0.333	0.284	0.327	0.422	0.253	0.194	–			KN
0.292	0.255	0.282	0.416	0.215	0.177	0.050	–		Yu-S
0.326	0.293	0.324	0.442	0.260	0.225	0.085	0.066	–	TsM
0.282	0.267	0.300	0.417	0.225	0.221	0.100	0.087	0.064	S-M

ces [50], each individual tree of *P. pumila* in the studied parts of the range showed 8% deficiency of heterozygotes ($F_{IS} = 0.080$) relative to the population and almost 20% ($F_{IT} = 0.197$) relative to the species as a whole (Table 2).

The F_{IS} and F_{IT} values for individual loci demonstrated in Table 2 showed that the highest deficiency of heterozygous genotypes was observed at the *RPS2*, *RPS124*, and *RPS127* loci. The inbreeding coefficient of the populations relative to the species, reflecting the degree of the population subdivision, was almost 13% ($F_{ST} = 0.129$). The 87.1% of total genetic diversity was found within the examined populations. Maximum population differentiation was observed at the abovementioned loci (*RPS2*, *RPS127*, *Pc18*). These loci were different in allele frequencies and allelic composition and could serve as diagnostic ones in genetic marking of the population samples from different geographic regions.

Genetic Differentiation of the Populations of P. pumila

Evaluation of the genetic differences between the studied populations of *P. pumila* using Nei genetic distances (D_N) [51] showed that the maximum differences in the genetic structure were observed between the populations from Kamchatka and the rest of the studied populations (Table 3). The genetic distance between the compared pairs of the studied populations ranged from 0.050 to 0.442, averaging 0.202. However, it should be noted that the population samples from Kamchatka were also considerably differentiated from one another ($F_{ST} = 0.052$; $D_N = 0.093$). The population from the Amur region (TsM) was less differentiated from the Sakhalin and Southern Kuril populations. The average genetic distance between these populations was 0.074. The closest with respect to the genetic structure were island populations from Kunashir and Sakhalin islands ($F_{ST} = 0.014$; $D_N = 0.050$).

The established level of differentiation of the studied populations of *P. pumila* is clearly demonstrated by the positions of the populations on the plane of two coordinates (Fig. 3). According to the Mantel test, genetic distances between the studied populations of *P. pumila* based on SSR marker frequencies considerably correlated with geographical distances between the population samples ($r = 0.428$, $P = 0.01$). These

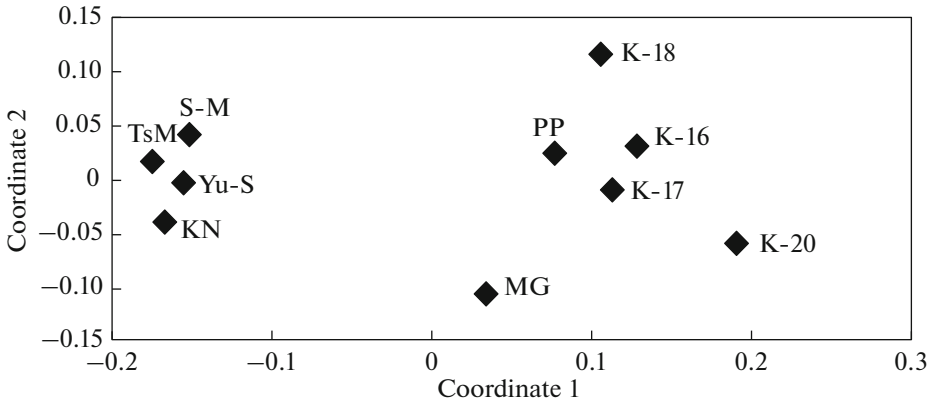

Fig. 3. The projection of the studied populations of *P. pumila* on the plane of two coordinates based on the PCA analysis of Nei genetic distance matrix.

findings point to genetic uniqueness of the examined populations of *P. pumila*.

DISCUSSION

In this study, the analysis of genetic variability of *P. pumila* was for the first time carried out using microsatellite marker loci of nuclear DNA designed for other pine species of subsection *Strobus* (hereinafter, model species). The question arises of how different is the variability of these loci in the species for which they were designed and *P. pumila*.

Table 4 presents the data on variability of the examined loci in *P. strobus* [43], *P. cembra* [44], and *P. pumila* (present study). It should be noted that such discussed genetic diversity index as heterozygosity obviously depends on the number of examined individuals and samples. For example, the design of the *RPS* markers was based on the data obtained for only 16 unrelated individuals of *P. strobus* growing in Wisconsin and Minnesota [43]; the design of the Pc markers was based on the data for 40 individuals growing in two populations of *P. cembra* [44].

Comparative analysis of the variability of investigated microsatellite loci showed that the number of alleles of these loci in *P. pumila* was almost two times higher than in model species (Table 4). The most variable in model species was the *RPS12* locus (11 alleles), the variability of which was higher than the average. This locus was also considerably variable in the populations of *P. pumila* (10 alleles). However, the maximum number of alleles was found at the *RPS2* and *RPS127* loci. Note that, in the analysis of mass samples of *P. strobus* (more than 200 individuals in each study), the number of detected allelic variants was ten at the *RPS2* locus, nine at *RPS6*, twenty-one at *RPS12*, and three at *RPS127* [52, 53]. Testing of primers for the *RPS2* and *RPS12* loci was carried out using the DNA specimens from five species of Mexican five-needle pines. It was found that the number of allelic variants at *RPS2* and *RPS12* loci in *Pinus strobiformis* (the number of analyzed samples $N = 5$) constituted

Table 4. Variability of the examined microsatellite loci in model species and in *P. pumila*

Locus	Species for which the marker was designed			*Pinus pumila*		
	N	size, bp	H_O	*N*	size, bp	H_O
*RPS2**	4	149–171	0.312	11	146–180	0.630
*RPS6**	4	159–164	0.500	4	164–170	0.374
*RPS12**	11	163–209	0.812	10	148–172	0.663
*RPS124**	4	147–153	0.500	9	148–178	0.357
*RPS127**	2	194–196	0.375	11	192–222	0.598
*Pc18***	3	152–156	0.300	4	145–151	0.168

N, number of alleles; bp, base pairs; H_O, observed heterozygosity. * [43]. ** [44].

four and five; in *Pinus flexilis* ($N = 2$), two and one; in *Pinus ayacahuite* var. *veitchii* ($N = 6$), seven and three; in *Pinus ayacahuite* ($N = 15$), seven and nine; and in *Pinus chiapensis* ($N = 11$), four and eight variants, respectively [54].

Variability at the *Rs18* locus was investigated in four West Siberian populations of another species of cedar pines, Siberian pine (*Pinus sibirica* Du Tour). Four allelic variants of this locus with the sizes ranging from 152 to 158 bp were detected. The average heterozygosity of the trees at this locus was 0.450 [39].

In general, the level of variability of the studied loci in populations of *P. pumila* can be characterized as high. Comparative analysis of the population genetic variability parameters in the *P. pumila* related species, *P. cembra* and *P. sibirica*, showed that, according to the available data, these species were characterized by similar level of variability, while their gene pool structures were essentially different.

The values of another genetic diversity parameter, the effective number of alleles, in the populations of the mentioned species was 2.78 and 2.69, and the expected heterozygosity was 0.564 and 0.554, respectively [35, 39], indicating that these values were close to those obtained by us for *P. pumila* (Table 1). At the same time, genetic subdivision of the populations of *P. pumila* ($F_{ST} = 0.129$) (Table 2) was several times higher than in *P. cembra* and *P. sibirica* (0.024 and 0.055) [35, 39]. This difference is primarily caused by genetic differentiation and the peculiar features of Kamchatka populations of *P. pumila*.

It is known that *P. pumila* appeared in the northeast of Asia, in particular, on the Kamchatka Peninsula, with cooling of the climate in the Pliocene 3–2.5 million years ago [55]. The data on its origin are contradictory. Very characteristic fossils (needles and seeds) of *P. pumila* or its ancestor close to it in morphology were found in Pliocene deposits not only in Alaska but also in the Canadian Arctic archipelago [56]. Phylogenetic trees of five-needle pines constructed on the basis of the mitochondrial DNA data clearly divide modern species into Eurasian and American clusters, unambiguously assigning *P. pumila* to the American one [57]. The chloroplast phylogenies, on the contrary, place this species in the Eurasian cluster [1]. Comparison of the available data suggests that *P. pumila* originated in northeast Asia by hybridization of American and Eurasian ancestors [57, 58].

Genetic differentiation of the Magadan–Kamchatka populations and the rest of the *P. pumila* populations in our study may be explained in terms of their formation from different Pleistocene refugial centers. In the Pleistocene history of the northeast of Asia, there were several (at least three) periods of glaciation that were not mantle, but exclusively mountain–valley. Therefore, periglacial (subarctic and subalpine) flora, including *P. pumila*, was perfectly preserved in local refugia [55]. Apparently, this explains the specific features of the Kamchatka and Magadan populations observed in the present study. Being separated by glaciers, these populations were genetically depleted owing to a small effective population size and differentiated from one another owing to isolation and genetic drift. According to P.V. Krestov, in Northeast Asia, the Pleistocene refugia, supporting the development of forest vegetation, were located on the islands and on the coast of the Sea of Okhotsk and the Sea of Japan, with the Sikhote-Alin refugium as the largest [59]. Coastal areas of Northeast Asia could have been the centers of the *P. pumila* norward and eastward expansions. By the Early Holocene, *P. pumila*, as well as other subalpine and subarctic shrubs (*Rhododendron aureum*, *Duschekia fruticosa*) that survived the Pleistocene aridization in the Pacific regions, reached Beringia [60].

The very weak genetic subdivision of highly differentiated geographically southern populations established in the present study, from Stanovoe Plateau to the South Kuril Islands, indicates that all these regions were populated by *P. pumila* from one place, which was located, apparently, somewhere within the relatively narrow strip between the coast of the Sea of Okhotsk and the Central Yakut Plain. The population of the Southern Kurils and Sakhalin Island by *P. pumila*, probably, followed the same pattern. This is consistent with the Pleistocene paleogeography, because in cold periods the Southern Kurils, Hokkaido, and Sakhalin were a single land mass connected to the mainland [61].

We suggest that rapid dispersal of *P. pumila* during Pleistocene can be explained by a combination of climate cooling favorable for it and uniqueness of its adaptation to these climatic conditions. The life form of a creeping pine made it possible for this species to occupy an ecological niche in which it had almost no competitors. Most likely, the latter circumstance led to the high level of genetic diversity in southern populations.

ACKNOWLEDGMENTS

We thank N.V. Sinelnikova (Institute of the Biological Problems of the North, Far Eastern Branch, Russian Academy of Sciences, Magadan) for providing the population sample of Siberian dwarf pine from Magadan oblast.

This study was supported by the Russian Foundation for Basic Research (grant no. 15-04-03924).

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Translated by N. Maleeva