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PLANT GENETICS

Genetic Variation, Population Structure, and Differentiation in Scots Pine (*Pinus sylvestris* L.) from the Northeast of the Russian Plain as Inferred from the Molecular Genetic Analysis Data

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Abstract—The DNA polymorphism in *Pinus sylvestris* L. from Severodvinsk, Upper Vetluga, and Vetluga–Vyatka populations, which were isolated earlier based on specific features of the geographic variation of allometric cone indices, was examined by the ISSR method. It was demonstrated that the Severodvinsk population of *P. sylvestris* differed from the chorologically adjacent Upper Vetluga population with respect to all of the examined genetic indices, and the Upper Vetluga population differed from the Vetluga–Vyatka population. It was suggested that the main the reason for the lack of statistically significant differences between Upper Vetluga and Vetluga–Vyatka samples of *P. silvestris* with respect to genetic variation indices (P_{95}, H_E, n_a, n_e) may be their formation based on the gene pools of two glacial refugia. It was demonstrated that the proportion of the interpopulation component of total genetic diversity (G_{ST}), as calculated based on the ISSR marker polymorphism, reached a value of 0.488, which was an order of magnitude higher than the previous estimates obtained based on data from allozyme analysis. It was concluded that *P. sylvestris* cone allometric indices reflected the specificity of spatial population subdivision, like the genetic diversity and population genetic structure indices calculated based on ISSR-marker polymorphism. Population isolation and mapping based on two-step phenogenetic studies is suggested.

Keywords: genetic markers, intra- and interpopulational variability, *Pinus sylvestris* L.
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INTRODUCTION

The most important problem of our time is the conservation of forest biological diversity at different levels, including the ecosystem, species, and populations. Based on the hierarchical organizational structure of a whole variety of signs of life in the forest phytocoena, the decision-making strategy is seen at the population genetic level. From the viewpoint of microevolution, it involves the preservation of each elementary population (in the interpretation of N.V. Timofeev-Resovsky et al. [1]) and the natural genetic diversity within it. This is the main condition for the preservation of species diversity of forest ecosystem, including the woody plants that are the edificators of this ecosystem, which create a particular biological medium for other organisms.

The conservation of genetic diversity in woody plants is only possible with an analysis of the population-chorological structure of the species and the identification of each elementary population. A phene–gene geographic investigation of woody plants, which was carried out in 1980s through 1990s by phenotypic and allozyme methods, showed the presence of the phene and gene pool specificities in different parts of the species ranges, as well as wide clinal geographic variation of morphological characters upon relatively low genetic population subdivision [2–7]. For coniferous plant species, the values of the population subdivision index (F_{ST}, G_{ST}) usually varies within the range of 0.018–0.060 [3, 4, 8]. In other words, based on data from allozyme analysis, more than 94% of the total genetic diversity in this group of plants is found within the populations.

Research on intraspecific variation in woody plants that involved methods of molecular genetic analysis of DNA showed a considerably higher level of genetic population subdivision than at allozyme loci [9, 10], indicating the prospects for their use in population chorological studies.

Despite some progress, the problem of the population-chorological structure of the woody plants spe-

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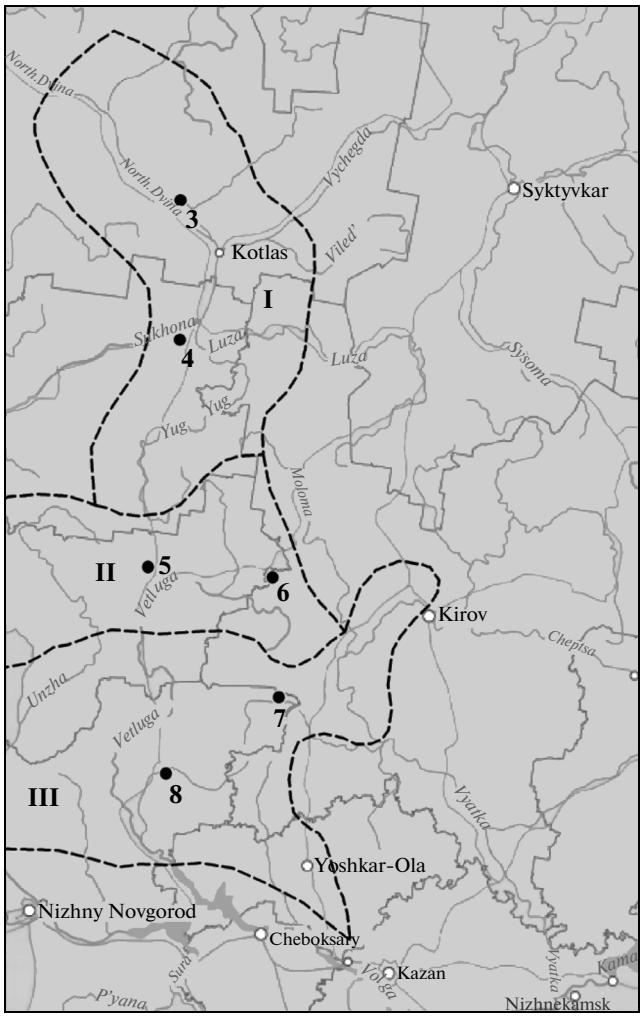

Fig. 1. Map showing the location of the populations and population samples of *P. sylvestris*. Populations: I, Northern Dvina; II, Upper Vetluga; III, Vetluga–Vyatka. The population boundaries are designated by dashed line; the samples are marked by numbers.

cies remained unsolved until nearly the end of the 20th century. The fundamental aspects of this structure, like volume, boundaries, factors, and features of elementary populations differentiation were especially poorly examined and controversial.

At the turn of 20th and 21st century, a system of methods to isolate the phenes and quantitative morphophenotypic marker traits of the tree genotypes, as well as for their range according to the levels of structural-biochorological organization of the species, was developed with the Scots pine (*Pinus sylvestris* L.), [11–14]. After that, a new (phenetic) phase of the study of population-chorological structure of Scots pine with highly informative markers of different levels of intraspecific organization began. The studies showed that, within certain geographical regions, the phene frequencies were characterized by homogeneity and specificity. This made it possible to identify and map the statistically significantly differing populations and groups [15–18] characterized by intrapopulation homogeneity and interpopulation heterogeneity of the phene pools.

However, the main criteria for spatial population isolation are the specific features of their gene pools [1, 19, 20]. This specificity can be explored by methods of molecular genetic analysis of DNA. Therefore, morphophenotypically isolated chorologically adjacent subdivisions of the species can be considered as populations only in the case of their relative internal homogeneity and the presence of marked differences between them in terms of genetic variation and genetic structure. The proportion of polymorphic loci (P_{95}) and heterozygosity (H_E) can serve as the measure of the population genetic variation [21].

Based on the above, the aim of the present study was to examine the genetic variation and genetic structure of the chorologically adjacent phenotypically identified and previously mapped [16] Scots pine populations and to evaluate the applicability of morphophenotypic markers in studying the population chorological structure of the species.

MATERIALS AND METHODS

Three chorologically adjacent populations of Scots pine, including that of Severodvinsk, Upper Vetluga, and Vetluga–Vyatka, that were isolated earlier based on specific features of the geographic variation of allometric cone indices [16] were examined. The range of the first population includes the basins of Yug River and Northern Dvina River. The range of the second population included the upper reaches of Vetluga River, and that of the third population includes the basin of the middle reaches of Vetluga River (Fig. 1).

Molecular genetic analysis was performed on two samples from each population (Fig. 1). The samples were selected to meet the criteria as follows: (1) samples that are equally geographically distant from each other (about 90 to100 km apart); (2) representativeness (sample size of 46 trees); (3) a distance between the trees from which the shoots are cut of at least 80 to 100 m; (4) uniformity of forest sampling sites (cowberry pine forests).

To carry out molecular genetic analysis, DNA was extracted from fresh vegetative buds of lateral shoots from 46 individual trees of each population sample by the Rogers method [22] with some modifications. The DNA concentration and quality was determined with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, United States), and the DNA concentration was aligned to 10 ng/μL. Analysis of the polymorphism was performed in 276 DNA specimens by polymerase chain reaction (PCR) with five primers. To examine the genetic variation in the samples of *P. sylvestris*, 1380 DNA specimens were amplified.

Molecular genetic analysis was carried out by ISSR (Inter Simple Sequence Repeats) analysis of DNA polymorphism [23].

Amplification was carried out in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, United States), according to standard ISSR-PCR protocol [24]. To test the quality of reagents, the reaction mixture was supplemented with 5 μL of deionized water instead of DNA, and this mixture was used as negative control (C–). The amplification products were separated by means of electrophoresis in 1.7% agarose gel in 1× TBE buffer, stained with ethidium bromide, and photographed in transmitted UV light with the Gel-Doc XR system (Bio-Rad, United States). The molecular sizes of the DNA fragments were determined molecular size marker (100 bp + 1.5 + 3 kb DNA Ladder, SibEnzim-M Ltd., Moscow). The fragment sizes were determined with the Quantity One software program in the Gel-Doc XR gel-documentation system (Bio-Rad, United States). In the examined populations of *P. sylvestris*, the polymorphism of 114 ISSR markers was analyzed.

For computer processing of the obtained data, they were presented in the form of a binary matrix in which the presence or absence in the profile of equally sized DNA fragments was treated, as a state of 1 or 0, respectively. In this case, only fragments reproducible in repeated experiments were taken into account, while the polymorphism intensity was not considered. Computer analysis of the data was performed with the POPGENE 1.31 software program [25] and with a specialized GenAlEx6 macro [26] for Microsoft Excel with the definition of the proportion of polymorphic loci (P_{95}) [27], the absolute number of alleles per locus (n_a), the effective number of alleles per locus (n_e) [28], and the expected heterozygosity (H_E) [29]. The following parameters were used to describe the genetic structure of a subdivided population: the expected proportion of heterozygote genotypes (H_T) in the general population as a measure of the total genetic diversity; the expected proportion of heterozygous genotypes (H_S) in a subpopulation as a measure of its intrapopulation diversity; the proportion of interpopulation genetic diversity in the overall diversity, or the population subdivision index (G_{ST}) [30].

The matrix of genetic differences [31] was calculated with the use of the binary character matrix. Based on it and with the unweighted pair group method with arithmetic mean (UPGMA) a dendrogram that reflected the degree of relatedness of the examined populations at the ISSR profiles was constructed as implemented in the Treecon 1.3b and POPGENE 1.31 software programs. The genetic distance between the samples (D) was determined by Nei's and Li's formulas [32]. The statistical significance of the differences between the mean index values of the chorologically adjacent populations was evaluated by univariate analysis of the variance [33].

RESULTS AND DISCUSSION

In three examined *P. sylvestris* populations, 114 ISSR markers were identified by PCR, of which 109 were polymorphic. Depending on the primer used, the number of amplicons varied from 13 to 20, and their sizes varied from 150 to 1650 bp. On average, in ISSR analysis, one primer initiated the synthesis of 16.5 amplicons. The number of polymorphic loci in the total plant sample varied from 20 to 25. Depending on the primer, the proportion of polymorphic loci (P_{95}) in the total sample varied 0.880 to 1.00, with an average value of 0.956.

The samples of each population are relatively uniform in the total number, as well as the number and percentage of polymorphic loci amplified both within individual primers and as a whole on the sample (Table 1). For instance, in the samples Ps_3 and Ps_4 from the Northern Dvina population, PCR with primer X-100 reveled 19 ISSR markers, including 16 polymorphic, the proportion of which constituted 0.842. Overall, 89 ISSR primers were identified in the Ps_3 sample with five primers; 72 of these which were polymorphic ($P_{95} = 0.818$). In the Ps_4 sample, 88 and 76 ISSR markers, respectively were identified ($P_{95} = 0.864$). PCR analysis with five primers showed that the Northern Dvina population had 17.3% more ISSR markers and 2.1 times more polymorphic loci than the Upper Vetluga population, while the number of ISSR markers and polymorphic loci in the Vetluga–Vyatka population was only 2.6 and 13% higher than in Upper Vetluga population.

The Northern Dvina population was statistically significantly different (Table 2) from the Upper Vetluga population in the proportion of polymorphic loci (P_{95}), which were identified with individual primers, as well as the total set of primers. At the same time, the Upper Vetluga population was different from the Vetluga–Vyatka population—only with the use of the ISSR-1 primer ($p < 0.05$).

The samples from one population were rather homogenous in the expected heterozygosity (H_E) and effective number of alleles per locus (n_e) indices. These samples are especially homogenous in the absolute number of alleles per locus (n_a). In the Northern Dvina population, the value of this index varies within the range of 1.640–1.667; in the Vetluga–Vyatka population, it ranges from 1.333 to 1.342. In all samples from the Upper Vetluga population, the value of this index was equal to 1.307 (Table 3).

Based on the data from Table 3 and with univariate variance analysis [33], the statistical significance of the H_E, n_a, and n_e differences in the chorologically adjacent populations was assessed. The advantage of this method is that it makes it possible to perform this assessment by taking into account the variance of the sample values of the parameters on the intra- and interpopulation levels, rather than average population parameters, which increases the accuracy of the esti-

Table 1. Characteristics of ISSR markers in *P. sylvestris* population samples

Sample	The number of ISSR markers identified with the use of primers in the population samples											
	ISSR-1		CR-212		CR-215		M27		X10		Per sample	
	n1	n2	n1	n2	n1	n2	n1	n2	n1	n2	n1	n2
Northern Dvina population												
Ps_3	15	13 (0.867)	20	16 (0.800)	18	15 (0.833)	16	12 (0.750)	19	16 (0.842)	89	72 (0.818)
Ps_4	16	15 (0.937)	19	17 (0.895)	18	15 (0.833)	16	13 (0.812)	19	16 (0.842)	88	76 (0.864)
Upper Vetluga population												
Ps_5	13	6 (0.462)	16	8 (0.500)	15	7 (0.467)	13	6 (0.462)	16	8 (0.500)	73	35 (0.479)
Ps_6	15	7 (0.467)	17	7 (0.412)	17	8 (0.471)	13	5 (0.385)	15	7 (0.467)	76	34 (0.442)
Vetluga–Vyatka population												
Ps_7	15	8 (0.533)	13	6 (0.462)	14	7 (0.500)	16	7 (0.438)	18	10 (0.500)	77	38 (0.500)
Ps_8	15	8 (0.533)	16	8 (0.500)	15	8 (0.533)	15	6 (0.400)	17	10 (0.588)	78	40 (0.513)
Per total sample	20	20 (1.00)	25	22 (0.880)	22	22 (1.00)	22	20 (0.909)	25	25 (1.00)	114	109 (0.956)

n1, the number of identified ISSR markers; n2, the number of polymorphic ISSR markers; in the brackets is the proportion of polymorphic loci.

Table 2. Evaluation of the statistical significance of the differences between the proportions of polymorphic loci (P_{95}) in the chorologically adjacent populations of *P. sylvestris*

No. of comparison variant	Compared populations	The F_Φ criterion between the proportion of polymorphic loci identified with the help of amplifiation primers					
		ISSR-1	CR-212	CR-215	M27	X10	total
1	Northern Dvina and Upper Vetluga	250.9	36.6	33124.0	24.4	471.6	166.0
2	Upper Vetluga and Vetluga–Vyatka	72.2	0.3	8.2	0.01	1.7	5.5

$F_{0.05} = 18.1$, $F_{0.01} = 98.5$.

mate. The analysis showed that the Northern Dvina population was statistically significantly different from the Upper Vetluga population at all of the examined parameters, while the Upper Vetluga population was different from the Vetluga–Vyatka population with respect to the absolute number of alleles per locus at $p < 0.05$ (Table 4).

To characterize the population genetic structure, rare alleles with a frequency lower than 5% were used. There were two to three such alleles in samples from the Northern Dvina populations, one to three alleles in samples from the Upper Vetluga population, and one to two alleles in samples from the Vetluga–Vyatka population (Table 3). The proportion of these alleles in the populations on average was 2.9, 2.7, and 2.0%, respectively.

The evaluation of the genetic structure and differentiation of chorologically adjacent morphophenotipically isolated populations was performed by their pairwise comparison in terms of total genetic diversity (H_T), average genetic diversity (H_S), and the subdivision coefficient (G_{ST}). The comparisons were performed in the samples as follows: (1) Ps_3 and Ps_4 of the Northern Dvina population and Ps_5 and Ps_6 of the Upper Vetluga population; (2) Ps_5 and Ps_6 of the Upper Vetluga population and Ps_7 and Ps_8 of the Vetluga—Vyatka population. In the first variant of the comparison, the total genetic diversity (H_T) con-

Table 3. Genetic diversity indices in the populations of *P. sylvestris*

Sample	H_E	n_a	n_e	R
Northern Dvina population				
Ps_3	0.224 (0.019)	1.640 (0.482)	1.380 (0.366)	4 (0.045)
Ps_4	0.212 (0.018)	1.667 (0.474)	1.354 (0.357)	2 (0.023)
Upper Vetluga population				
Ps_5	0.101 (0.016)	1.307 (0.463)	1.170 (0.307)	0
Ps_6	0.090 (0.015)	1.307 (0.463)	1.146 (0.281)	1 (0.013)
Vetluga–Vyatka population				
Ps_7	0.095 (0.015)	1.333 (0.474)	1.156 (0.290)	0
Ps_8	0.095 (0.014)	1.342 (0.477)	1.147 (0.257)	0
Per total sample	0.136 (0.016)	1.974 (0.161)	1.505 (0.330)	7

H_E, expected heterozygosity; n_a, absolute number of alleles per locus; n_e, effective number of alleles per locus; the standard deviation values are in brackets after the parameter values; R, the number of rare alleles, their proportion from the total number of the alleles is in brackets.

Table 4. Evaluation of statistical significance of the genetic diversity indices in the chorologically adjacent *P. sylvestris* populations

No. of comparison variant	Compared population	The F_Φ criterion of the indices		
		H_E	n_a	n_e
1	Northern Dvina and Upper Vetluga	224.0	657.9	139.6
2	Upper Vetluga and Vetluga–Vyatka	0	45.4	0.3

$F_{0.05} = 18.1$, $F_{0.01} = 98.5$.

stituted 0.307 on average; the average genetic diversity (H_S) was 0.157; and the subdivision coefficient (G_{ST}) was 0.488 (Table 5). In the second variant of the comparison, the values of these indices constituted 0.227, 0.115, and 0.484, respectively (Table 6). Thus, the samples from the Northern Dvina population and the adjacent Upper Vetluga population were different from the samples of the Upper Vetluga and Vetluga–Vyatka populations at a higher level of genetic diversity.

At the same time, both comparison variants were characterized by identical and very high values of the population subdivision index. These values were at least ten times higher than those obtained for the coniferous woody plants based on the allozyme analysis data.

The smallest pairwise genetic distances (D) were observed between samples within each population. The genetic distance was 0.072 between the Northern

Table 5. Parameters of genetic structure and differentiation of samples Ps_3 and Ps_4 from Northern Dvina and and Ps_5 and Ps_6 from Upper Vetluga populations of *P. sylvestris*

ISSR-primer	Nucleotide sequence (5' → 3')	H_T	H_S	G_{ST}
ISSR-1	$(AC)_8T$	0.329 (0.028)	0.145 (0.011)	0.558
CR-212	$(CT)_8TG$	0.300 (0.031)	0.167 (0.016)	0.444
CR-215	$(CA)_6GT$	0.317 (0.032)	0.168 (0.010)	0.470
M27	$(GA)_8C$	0.284 (0.026)	0.143 (0.08)	0.496
X10	$(AGC)_6C$	0.306 (0.031)	0.158 (0.016)	0.483
Average		0.307 (0.029)	0.157 (0.012)	0.488

H_T, expected proportion of heterozygous genotypes as a measure of total gene diversity in the full sample; H_S, expected proportion of heterozygous genotypes in a subpopulation, as a measure of its intrapopulation diversity, or the intrasample average gene diversity over all loci; G_{ST}, the proportion of the intersample genetic diversity from the total diversity, or the sample subdivision index; the standard deviation values are in brackets.

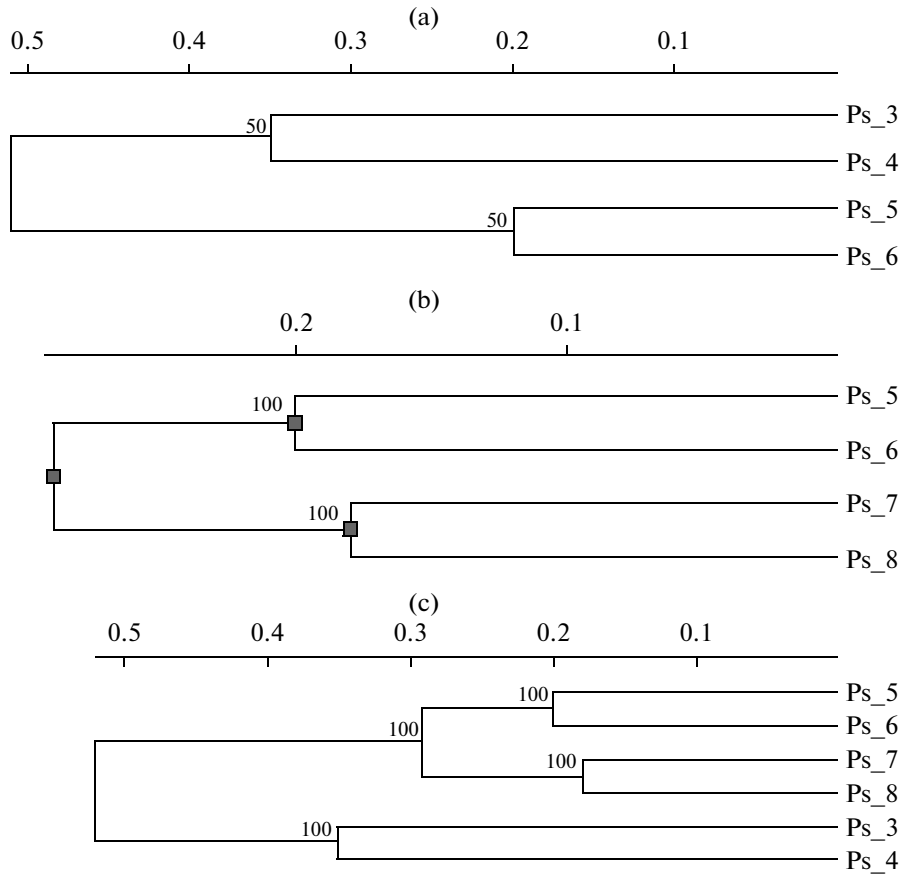

Fig. 2. UPGMA dendrogram constructed based on the ISSR profile for samples of *P. sylvestris* from the populations as follows: (a) Northern Dvina and Upper Vetluga; (b) Upper Vetluga and Vetluga–Vyatka; (c) Northern Dvina, Upper Vetluga, and Vetluga–Vyatka. Scale on the top, genetic distances according to Nei and Li [32]. Figures on the dendrogram designate the bootstrap support values (in %).

Dvina population samples Ps_3 and Ps_4 and 0.089 between samples Ps_5 and Ps_6 of the Upper Vetluga population, while the genetic distance between the populations was five times higher (Table 7). The genetic distance between samples Ps_5 and Ps_6 of the Upper Vetluga population, and that between samples Ps_7 and Ps_8 of the chorologically adjacent Upper Vetluga population were equal to 0.113 and 0.073, respectively, while the distance between the populations was 2.6 times higher (Table 8).

The patterns of the genetic distance (D) variation between samples within populations and between chrorologically adjacent populations compared in pairs are supported by dendrograms (Fig. 2). On the first dendrogram (Fig. 2a), the samples formed two clusters. The first cluster included samples Ps_3 and

Table 6. Genetic structure and differentiation parameters of the Upper Vetluga samples Ps_5, Ps_6 and Vetluga–Vyatka samples Ps_7, Ps_8

ISSR-primer	Nucleotide sequence (5' → 3')	H_T	H_S	G_{ST}
ISSR-1	$(AC)_8T$	0.196 (0.045)	0.080 (0.008)	0.592
CR-212	$(CT)_8TG$	0.277 (0.032)	0.121 (0.012)	0.563
CR-215	$(CA)_6GT$	0.220 (0.033)	0.132 (0.017)	0.401
M27	$(GA)_8C$	0.187 (0.032)	0.121 (0.014)	0.354
X10	$(AGC)_6C$	0.253 (0.040)	0.124 (0.014)	0.512
Average		0.227 (0.037)	0.115 (0.013)	0.484

The parameter designations are as in Table 5.

Table 7. Pairwise genetic distance matrix between samples of the Northern Dvina (Ps_3, Ps_4) and Upper Vetluga (Ps_5, Ps_6) populations

Samples	Ps_3	Ps_4	Ps_5	Ps_6
Ps_3	0			
Ps_4	0.072	0		
Ps_5	0.385	0.376	0	
Ps_6	0.419	0.406	0.089	0

Table 8. Pairwise genetic distance matrix between samples of the Upper Vetluga (Ps_5, Ps_6) and Vetluga–Vyatka (Ps_7, Ps_8) populations

Samples	Ps_5	Ps_6	Ps_7	Ps_8
Ps_5	0			
Ps_6	0.113	0		
Ps_7	0.244	0.214	0	
Ps_8	0.264	0.242	0.073	0

Ps_4 of the Northern Dvina population, and the second cluster included samples Ps_5 and Ps_6 of the Upper Vetluga population. On the second dendrogram (Fig. 2b), the samples grouped in a similar manner. The first cluster included samples Ps_5 and Ps_6 of the Upper Vetluga population, and the second cluster included samples Ps_7 and Ps_8 of the Vetluga–Vyatka population. On the combined dendrogram (Fig. 2c), the samples formed two clusters with high bootstrap support. The first cluster consisted of the samples Ps_5, Ps_6, Ps_7, and Ps_8, which were divided into two groups (Ps_5, Ps_6 (1) and Ps_7, Ps_8 (2)). The second cluster included samples Ps_3 and Ps_4.

Thus, the molecular genetic investigation based on ISSR analysis of DNA polymorphism showed that Northern Dvina population of Scots pine was different from the chorologically adjacent Upper Vetluga population with respect to all examined parameters. At the same time, the Upper Vetluga population differed from the Vetluga–Vyatka population with respect to the genetic structure indices, including total genetic diversity (H_T), average genetic diversity (H_S), and the subdivision coefficient (G_{ST}).

The main reason for the absence of statistically significant differences between the Upper Vetluga and Vetluga–Vyatka populations in terms of the genetic variation indices may be their formation based on the gene pools of two glacial refugia, the spread of ancestral forms out of the refugia, and the subsequent landscape and geographical differentiation and phenogenetic microevolutionary divergence under the influence of elementary factors of evolution [16].

It can be suggested that the microevolution process resulted in gene pool alignment in chrologically adjacent populations in terms of genetic variation indices rather than differentiation. If so, with respect to these indices, the Vetluga–Vyatka population should be statistically significantly different from the neighboring Volga–Vetluga population, since they were formed based on the gene pool of one glacial refugium [15, 16]. This suggestion is verified.

An important result of this study is that a very high population subdivision was revealed based on analysis of the ISSR marker poplymorphism ($G_{ST} = 0.488$).

Based on these data, it can be concluded that the allometric cone indices, like the genetic diversity and population genetic structure indices (which are calculated based on the ISSR marker polymorphism), reflect the specificity of the spatial population subdivision. This makes it possible to isolate populations of *P. sylvestris* through the two-step phenogenetic studies by morphophenotipic and genetic methods. At the first step, geographic variation of the cone allometric indices is examined, and the population boundaries are mapped. At the second step, the variation of genetic markers in the isolated populations is examined.

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