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Genetic Diversity, Structure, and Differentiation of *Picea abies– Picea obovata–Picea koraiensis* Species Complex according to Data of Chloroplast DNA Microsatellite Analysis

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Abstract—The results of a comparative study of the genetic structure, parameters of intraspecific genetic diversity, and spatial differentiation of 35 populations of *Picea abies*–*P. obovata*–*P. koraiensis* species complex, located within the areas of distribution of "pure" species and in the contact zones of their ranges were presented. *P. abies* populations from Eastern Europe (Belarus, Poland); spruce populations from the European part of Russia, the Urals, and the West Siberian Plain, located in the zone of *P. abies* and *P. obovata* introgressive hybridization; *P. obovata* populations from various parts of the extensive range of this species in Siberia and Mongolia; and *P. koraiensis* populations from the Russian Far East and Northeast China were analyzed. The study of genetic variability and differentiation of populations was based on the analysis of variability of three microsatellite loci: Pt63718, Pt71936, and Pt26081, developed on the basis of *Pinus thunbergii* Parl chloroplast genome. The use of these DNA markers made it possible to obtain data indicating a fairly high degree of *P. koraiensis* genetic divergence from two other spruce species included in the complex, *P. abies* and *P. obovata*. Korean spruce growing in the Far East differs significantly from the populations of these species in the number and composition of haplotypes, the level of genetic diversity, and population structure, which gives us reason to consider it as a separate species.

INTRODUCTION

The spruce genus *Picea* A. Dietr. is one of the most important genera in the Pinaceae family in terms of economic importance and abundance of species. Currently, representatives of this genus are distributed circumpolarly across all continents of the Northern Hemisphere—from the polar regions almost to the subtropical zone. Species diversity of the genus *Picea* is represented by approximately 40–45 species, the majority of which are found in Eurasia and North America [1–4].

Six species of the genus *Picea* grow in the territory of Russia: *Picea abies* (L.) H. Karst., *P. obovata* Ledeb., *P. jezoensis* (Siebold & Zucc.) Carrière, *P. glehnii* (F. Schmidt) Mast., *P. koraiensis* Nakai, and *P. orientalis* (L.) Peterm. The largest and almost continuous range among them is occupied by Norway spruce *P. abies* and Siberian *P. obovata* [2], and the extensive zone of introgressive hybridization of these species covers almost the entire Russian Plain to the Urals and Western Siberia [1, 2, 5]. *P. abies* is distributed from Scandinavia to the Urals, as well as in the mountains of Western Europe (Alps, Carpathians, Balkans). In the Cis-Urals *P. abies* is gradually replaced by a similar species *P. obovata*. Siberian spruce grows in the northeastern part of European Russia, almost throughout the entire territory of Siberia, and in the south of the Far East to the coast of the Sea of Okhotsk and the lower Amur [6, 7]. Korean spruce *P. koraiensis* grows in the southern part of the Amur region and in Primorye. Morphological and ecological proximity, as well as the presence of transitional forms due to possible hybridization [2], do not allow us to establish a clear boundary between *P. obovata* and *P. koraiensis* [8–11].

Until now, the question of the species status of Korean spruce in the south of the Russian Far East remains open. Some researchers recognize its species

independence [2, 6−9, 12−15], others consider the spruce growing here as a variety of Siberian spruce [4, 11, 16, 17]. Study of polymorphism of morphological characters in spruce populations of the Far Eastern region [18], on the basis of which it was identified as Korean spruce, revealed a significant similarity of these populations with populations of Siberian spruce from the central regions of the species' range. In addition, it was found that the populations of *P. koraiensis* in the studied area do not exhibit the characteristic geographic confinement typical of independent species. Based on the obtained results, the authors suggested the absence of *P. koraiensis* in the Russian Far East.

Karyological analysis of *P. koraiensis* and *P. obovata* revealed the similarity of these species in the main morphological types of chromosomes. Significant differences between karyotypes were observed only in chromosome length, number, frequency of occurrence, and localization features of nucleolar regions [19]. According to the authors, satellite chromosomes found in *P. koraiensis* can be an additional criterion for distinguishing taxonomically controversial species *P. koraiensis* and *P. obovata*.

According to V.V. Potenko [20], *P. obovata* and *P. koraiensis,* growing in the studied region of the Far East, reveal reliably significant differences in genetic structure. On a dendrogram constructed using the pair-group method of cluster analysis (UPGMA) based on genetic distances calculated for 20 isoenzyme loci, the populations *P. obovata* and *P. koraiensis* are divided into two groups with a high level of bootstrap support. The degree of genetic differentiation of populations of these species is comparable to the level detectable by the isoenzyme method in *P. abies* and *P. obovata,* as well as in other closely related conifer species.

Under natural conditions, Korean spruce also grows in North Korea and the northeastern regions of China, where its species status is not in doubt among the scientific community [21, 22]. Using mtDNA and cpDNA markers, studies were carried out on five closely related spruce species of the complex *P. asperata* Mast.: *P. crassifolia* Kom.−*P. meyeri* Rehder et E.H. Wilson−*P. koraiensis*−*P. obovata* [23]. These species are morphologically similar but have separate ranges. It is known that in conifers, due to different pathways of transmission of genetic information (maternal or paternal), mitochondrial and chloroplast DNA are characterized by different rates of gene flow. Chloroplast DNA transmitted through pollen has higher rates of gene flow compared to mitochondrial DNA transmitted through seeds. In the course of a comparative study, it was found that the mtDNA markers used by the authors reveal mainly geographical variability, while cpDNA markers separate populations depending on their species. Four groups of populations of different species, differentiated from each other, have been identified. The most significant differences in cpDNA markers were observed between

P. obovata and other species of the spruce complex, including a group of two species, which includes *P. koraiensis*. The findings suggest that cpDNA markers with high gene flow rates are best suited for distinguishing spruce species.

Study of *P. koraiensis* genetic diversity and population structure based on analysis of single nucleotide polymorphism (SNP), carried out in Chinese populations of different geographical location and climatic conditions [24], revealed a fairly high level of intraspecific differentiation of populations of this species. The main reasons for the significant differences between the *P. koraiensis* populations studied in the region, according to the authors of this study, are their geographic isolation, climatic heterogeneity, and gene introgression. It was also found that among the factors promoting differentiation of *P. koraiensis* populations from the various habitats, the most important are temperature and precipitation.

Much more publications are devoted to the results of research on key species in Eurasia, *P. abies* and *P. obovata*. The variability of the morphological characters of these species in different parts of their ranges is covered in sufficient detail in the literature ([1, 5, 25, 26] etc.). Much attention is paid to discussing their taxonomic status.

To date, based on the analysis of the variability of genetic markers, data on the diversity, structure, and differentiation of *P. abies* ([27−43] etc.) and *P. obovata* ([28, 35−40, 43−52] etc.) populations in various parts of the ranges of these species have been obtained. Particular attention was paid to the study of genetic variability and population structure in the zone of introgressive hybridization [28, 35, 38, 40, 43], including the northwestern Russia [53−56].

Thus, when studying the allozyme variability of 26 loci in populations of the complex *P. abies*−*P. obovata* [28], a significant genetic similarity between the populations of these species was revealed. There were no fixed differences in allelic frequencies between them, nor any species-specific diagnostic loci. Based on this, the authors proposed to consider *P. abies* and *P. obovata* as two subspecies or geographic races of the same species.

In further studies, using other molecular markers ([36−38] etc.), data were obtained indicating that *P. abies* and *P. obovata* are two different species, significantly differentiated from each other.

The clearest separation of species was observed using mtDNA markers, in particular *nad1* (intron 2), inherited through the maternal line. According to the composition of haplotypes of this fragment in the complex *P. abies*−*P. obovata,* three groups of populations are distinguished: southern European, northern European, and Siberian [32, 37−39]. It has been established that mitochondrial haplotypes of *P. abies* in northern populations are grouped together with *P. obovata* haplotypes and differ from *P. abies* haplotypes in southern populations. Level of genetic differ-

entiation between European and Siberian populations by locus *nad1* according to data given in publications, was very high: $F_{ST} = 0.650$ [39] and $F_{ST} = 0.685$ [35]. A study of the spatial distribution of mtDNA and cpDNA haplotypes showed that the boundary separating the species is located along the Ob' River from the mouth in the north to the Ob' and Irtysh watershed in the south [39].

The distribution of chloroplast haplotypes across the species' range confirms introgressive hybridization between them. The fact of introgression of *P. obovata* to northern populations of *P. abies* has been established [32, 33, 35, 40], and the overall distribution of mitochondrial and nuclear variability indicates introgression of nuclear DNA of *P. abies* to southern populations of *P. obovata* [38].

As a result of processes of introgressive hybridization of *P. abies* and *P. obovata,* a large group of populations of hybrid origin has formed in the territory of the East European Plain [5, 26, 53, 54, 56, 57]. When analyzing the variability of nuclear and chloroplast SNPs in 55 populations of *P. abies* and *P. obovata* from Western Europe to the Yenisei River, it has been established that the hybrid zone extends from the Carpathians and Fennoscandia in the west to the Ob' River in the east [43]. The data on introgression of *P. obovata* into northern populations of *P. abies* and *P. abies* into southern populations of *P. obovata* obtained earlier by other researchers were confirmed. A joint demographic analysis was conducted, confirming multiple contacts of species during glacial cycles.

Recent phylogenetic studies of various species of the genus *Picea*, based on comparative transcriptomic analysis [58], also showed a close relationship between *P. abies* and *P. obovata*. These two species are included in a common clade with other spruce species in North Asia (*P. koraiensis*, *P. meyeri*, *P. asperata*, *P. crassifolia*, *P. retroflexa* Mast.) and the Japanese archipelago (*P. glehnii*, *P. koyamae* Shiras.), but are differentiated from them with formation of a separate cluster.

It should be noted, however, that despite the large number of works devoted to the study of *P. abies*, *P. obovata*, and *P. koraiensis*, by morphological characteristics and using various markers of nuclear and cytoplasmic genomes, many questions concerning the spatial distribution of genetic variability in the ranges of these species, the structure and differentiation of populations in the supposed zones of introgression of species, and phylogenetic relationships between species require additional, more detailed studies. Due to poor knowledge, the question of the species status of Korean spruce in the Russian Far East remains open. To determine the structure of spruce growing in this region and assess the degree of its genetic similarity with the structure of Siberian spruce populations, it is necessary to use DNA markers for the study of these two species, which are widely and successfully used to study the genetic diversity, structure, differentiation, and phylogeny of coniferous species, including number of species of the genus *Picea*.

The objectives of this work included conducting a comparative genetic study of the *P. abies*−*P. obovata*−*P. koraiensis* species complex using cpDNA markers. Transmitted via pollen cpDNA has higher rates of gene flow than mtDNA, distributed by seeds, and is best suited for identifying genetic differences between species. In our work we used chloroplast microsatellite loci (cpSSR) universal for the Pinaceae family [59], which were previously successfully used to study the genetic structure of spruce [29, 41, 49, 60] and other conifers [61−63]. The relatively high variability of cpDNA microsatellites, as well as the absence of recombination characteristic of cytoplasmic genomes and uniparental inheritance, which causes a twofold decrease in the effective population size, make cpSSR more sensitive to population reductions and isolation [63]. Currently, chloroplast microsatellites are one of the most informative markers for studying the population and phylogeographic structure of species.

MATERIALS AND METHODS

The study included four groups of populations of the *P. abies*−*P. obovata*−*P. koraiensis* species complex: populations of *P. abies* from Eastern Europe (Belarus, Poland); spruce populations from the European part of Russia, the Urals, and the West Siberian Plain, located in the zone of supposed introgressive hybridization of *P. abies* and *P. obovata*; populations of *P. obovata* from various parts of the species' extensive range in Siberia and in Mongolia; and also populations of *P. koraiensis*, naturally growing in the Primorski krai and Amur oblast of Russia, as well as in northeastern China. The study of the genetic structure, intra- and interspecific differentiation of populations was carried out based on an analysis of the variability of three microsatellite cpDNA loci: Pt63718, Pt26081, and Pt71936, developed from the chloroplast genome *Pinus thunbergii* Parl. [59]. Samples of needles and seed embryos were used as research material. A total of 35 spruce populations were analyzed. Their location, geographic coordinates, and variability parameters are shown in Fig. 1 and in Table 1.

Genomic DNA was isolated using the CTAB method [66] from needles dried in silica gel and from seed embryos. The DNA of each fragment was amplified in 10 µL of a mixture containing 1× PCR buffer (75 mM of Tris-HCl pH 8.8, 20 mM of $(NH_4)_2SO_4$, 0.1% of Tween 20), 2.5 mM of $MgCl_2$, 200 µM of each dNTP, 0.2 µM of forward and reverse primers, 0.5 units of Taq polymerase (manufactured by MBI Fermentas or Sibenzyme), and 10 ng of genomic DNA. The temperature profile corresponded to that given in [59]. Amplification products were analyzed using denaturing polyacrylamide gel electrophoresis followed by staining with silver nitrate. The size vari-
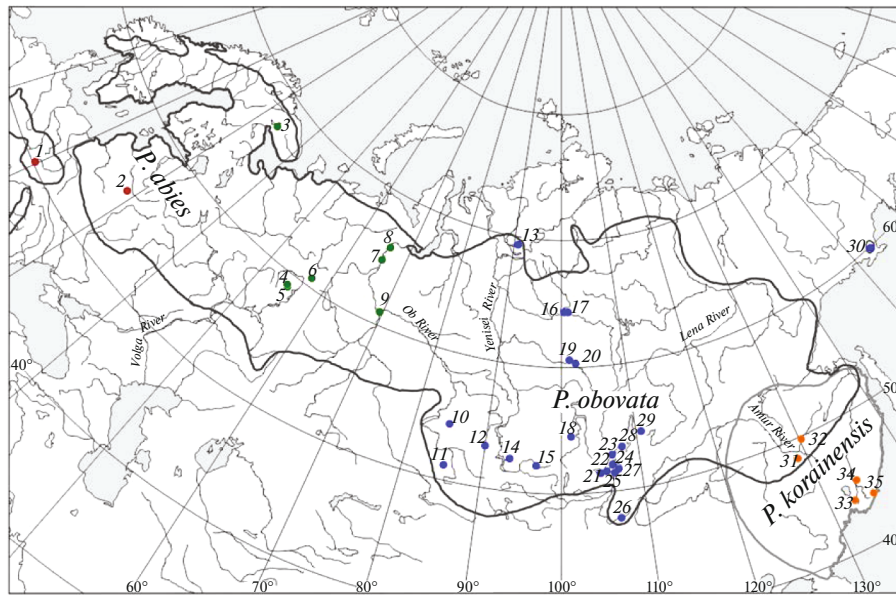
Fig. 1. Geographical location of the studied populations of the *P. abies−P. obovata−P. koraiensis* species complex. The black line indicates the range of Norway spruce [9] and Siberian spruce [6], and the gray line indicates the range of Korean spruce [64]. The numbers indicate the numbers of population samples from Table 1.

ant (the number of microsatellite repeats) was considered as an allele of a given locus, and the combination of alleles of three cpSSR loci in an individual was considered as a haplotype.

Population genetic analysis was carried out on the basis of statistical parameters generally accepted in population genetics that describe the level of variability and differentiation. In each population, using GenAlex 6.502 [67], statistics of genetic variability were determined: the number of identified haplotypes (N_h), haplotype diversity (h), unbiased haplotype diversity (uh), an analysis of the hierarchical structure of AMOVA variability was carried out (within populations, between populations within species, between species) [68], fixation indices Φ_{PT} were calculated between populations. Fixation index Φ_{PT}, as well as the fixation index F_{ST}, most often used in population studies, reflects the degree genetic differentiation between populations; however, unlike the latter, it does not depend on the level of intrapopulation variability [69]. The statistical significance of fixation indices was assessed using 999 permutations. To identify the spatial distribution of genetic variability, Principal Coordinates Analysis (PCoA) was carried out on a matrix of paired interpopulation values Φ_{PT}, calculated from cpSSR-locus allele frequencies.

RESULTS AND DISCUSSION

In total, the analysis of 920 individuals from 35 spruce populations identified 20 alleles at three chloroplast loci. Figure 2 shows a diagram of the occur-

rence of alleles for each of the studied loci in four groups of populations of the studied species complex.

Of the seven alleles of the Pt63718 locus, only three (5, 6, and 7) are common. Allele 6 has the highest frequency of occurrence in all studied groups. Alleles 1, 3, and 4 are classified as rare. In the Pt26081 locus, of the eight identified alleles, the common and most prevailing is allele 6. Alleles 1, 4 were found only in populations of *P. obovata*, as well as in several populations from the contact zone of the ranges of this species with *P. abies*. Three rare alleles (3, 8, and 9) were found in stand-alone individuals in one or two populations of *P. obovata* and *P. abies × P. obovata*. In the Pt71936 locus, only allele 2 is common to the three studied spruce species. Allele 3 is quite widely represented in populations of *P. obovata* and *P. abies* but is missing from *P. koraiensis*. In the studied populations of this species, allele 1 predominates, the frequency of which varies from 0.900 to 1. In *P. obovata* allele 1 is also detected in some populations, but with a very low frequency (less than 10%).

The total number of alleles found at the three cpSSR loci varies significantly in the studied spruce populations. The lowest level of allelic diversity is observed in populations *P. koraiensis* (4−9 alleles). In the remaining analyzed populations, the total number of identified alleles ranges from 8 to 15.

The combination of alleles of the three cpSSR loci analyzed in an individual was considered a haplotype. In total, genotyping 920 spruce individuals, 57 haplotypes were identified in 35 populations. In individual populations, the number of identified haplotypes varied from 2 to 17 (Table 1). The distribution of haplo-

Table 1. Location and variability parameters of spruce populations included in the study

No.	Populations	Location	Coordinates N/E	n	N_h	h	uh
		Picea abies					
1	Carpathians	Poland, Babia Gora (1364 m)	49°35′/19°33′	39	13	0.810	0.831
2	Domzheritsy	Belarus, Berezinsky Natural Reserve	54°44′/28°19′	22	11	0.851	0.892
	Average			30.5	12	0.831	0.862
		Contact zone of *Picea abies* and *Picea obovata* ranges					
3	Khibiny	Murmansk oblast	67°39′/33°38′	21	8	0.844	0.886
4	Yuryuzan	Chelyabinsk oblast, Yuryuzan River (487 m)	54°41′/58°48′	23	12	0.881	0.921
5	Iremel	Bashkortostan, Mount Iremel (1340 m)	54°33′/58°52′	25	10	0.662	0.690
6	Yekaterinburg	Sverdlovsk oblast	56°47′/60°48′	22	10	0.855	0.896
7	Kazym Mis	Khanty-Mansi Autonomous Okrug, Ob' River	64°40′/65°37′	19	11	0.859	0.906
8	Pitlyar	Yamalo-Nenets Autonomous Okrug, Ob' River	65°50′/65°55′	24	10	0.823	0.859
9	Khanty-Mansiysk	Khanty-Mansi Autonomous Okrug, Ob' River	61°02′/69°20′	20	10	0.820	0.863
	Average			22	10.1	0.821	0.860
		Picea obovata					
10	Elban	Novosibirsk oblast, Maslyaninskii district	54°18′/84°37′	29	10	0.842	0.872
11	Ilyinka	Altai, Shebalinsky district	51°22′/85°10′	30	7	0.758	0.784
12	Mayskii	Khakassia, Ust-Abakansky district	53°36′/90°00′	19	10	0.881	0.930
13	Putoran Plateau	Krasnoyarsk krai, Taimyr Dolgano-Nenets district	69°24′/90°42′	21	9	0.825	0.867
14	Ergaki	Krasnoyarsk krai, Ermakovsky district	52°47′/93°23′	29	11	0.842	0.872
15	Azas	Tuva, Todzhinsky district, Azas Natural Reserve	52°15′/96°20′	29	11	0.790	0.818
16	Kochechum	Krasnoyarsk krai, Evenki district	64°19′/100°14′	30	7	0.718	0.743
17	Tura	Krasnoyarsk krai, Evenki district	64°18′/100°25′	29	10	0.809	0.837
18	Tulyushka	Irkutsk oblast, Kuytunsky district	54°27′/101°12′	29	16	0.892	0.924
19	Tunguska Natural Reserve	Krasnoyarsk krai, Evenki district	60°37′/101°39′	29	10	0.759	0.786
20	Vanavara	Krasnoyarsk krai, Evenki district	60°21′/102°16′	30	14	0.904	0.936
21	Vydrino	Buryatia, Kabansky district	51°26′/104°43′	58	17	0.856	0.871
22	Khamar-Daban	Buryatia, Bushelai Tract	51°32′/105°52′	30	8	0.724	0.749
23	Kosaya Steppe	Irkutsk oblast, Ol'khon district	52°51′/106°04′	29	10	0.835	0.865
24	Kabansk	Buryatia, Kabansky district	51°59′/106°32′	25	11	0.851	0.887
25	Ubukun	Buryatia, Selenginsky district	51°28′/106°43′	30	12	0.784	0.811
26	Bogd-Uul	Mongolia, Bogd-Uul Natural Reserve	47°48′/106°51′	30	9	0.760	0.786
27	Krasnoyarovo	Buryatia, Ivolginsky district	51°51′/107°16′	29	12	0.837	0.867
28	Olkhon	Irkutsk oblast, Olkhon Island	53°15′/107°43′	27	9	0.842	0.875
29	Barguzinsky Natural Reserve	Buryatia, Barguzin River	54°35′/110°47′	29	9	0.787	0.815
30	Magadan	Magadan oblast, Magadan Natural Reserve	59°39′/153°04′	27	6	0.529	0.550
	Average			29.4	10.4	0.801	0.831
		Picea koraiensis					
31	Lesser Khingan	China, Heilongjiang Province	48°/129°	16	2	0.117	0.125
32	Bureya	Amur oblast, Bureya district, Talaya River	49°47′/130°14′	30	7	0.491	0.508
33	Anuchino	Primorsky krai, Anuchinsky district	43°59′/133°07′	13	3	0.272	0.295
34	Malinovo	Primorsky krai, Dalnerechensky district	45°29′/134°18′	14	2	0.133	0.143
35	Olga	Primorsky krai, Olginsky district	43°44′/135°17′	14	4	0.367	0.396
	Average			17.4	3.6	0.276	0.293
	Average of 35 populations (SE)			26.3 (1.4)	9.5 (0.6)	0.732 (0.035)	0.762 (0.036)

(n) Sample size, (N_h) number of haplotypes, (h) haplotype diversity, (uh) unbiased haplotype diversity [65].
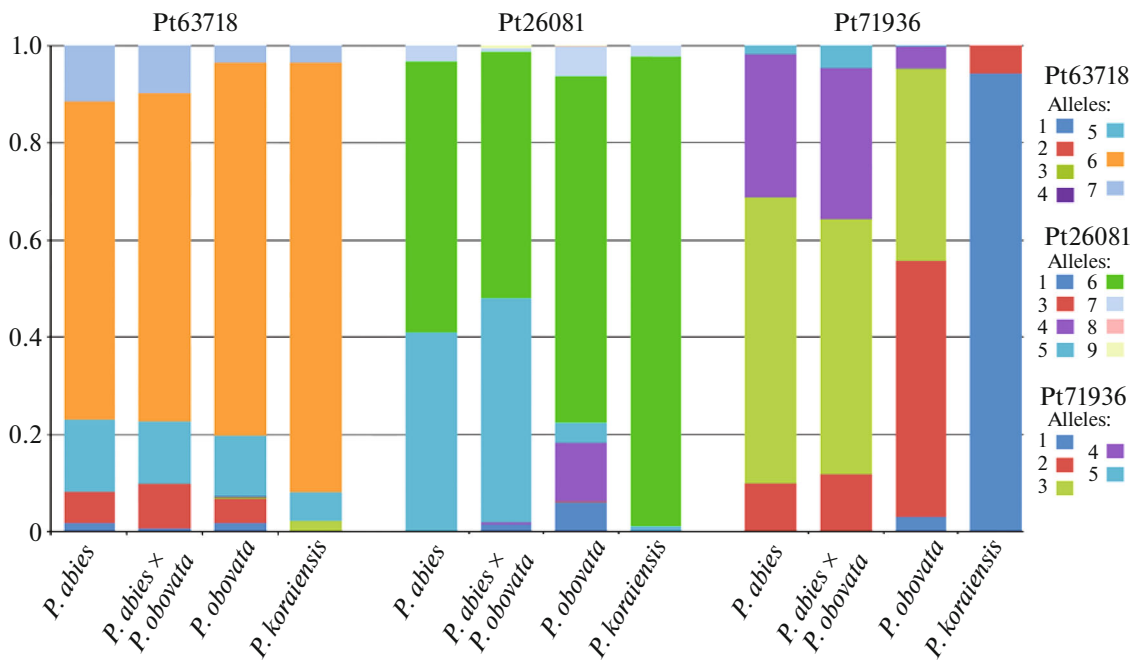
Fig. 2. Allele frequencies of three cpSSR loci in four groups of populations of the *P. abies*−*P. obovata*−*P. koraiensis* species complex.

types is uneven: two haplotypes 6/6/3 (allele numbers in loci Pt63718/Pt26081/Pt71936) and 6/6/2 were found more often than others in the studied spruce populations. Haplotype 6/6/3 was absent only in populations of *P. koraiensis*, and haplotype 6/6/2, in the population *P. abies* (Domzheritsy) and in the populations of Khibiny, Yekaterinburg, Yuryuzan, Iremel, Khanty-Mansiysk from the contact zone *P. abies* and *P. obovata*. The highest frequency of occurrence of the 6/6/3 haplotype was observed in the population *P. obovata* from the Magadan Nature Reserve (66.7%); in the remaining studied populations *P. abies* and *P. obovata* its frequency varied from 4.5 to 56%, averaging 24.7%. Haplotype 6/6/2, which is maximally represented in the Siberian spruce population Khamar-Daban (43.3%), is found in other studied populations of spruce of this species with a lower frequency, averaging 26.4%. In *P. abies*, *P. koraiensis*, and in the contact zone of the ranges of *P. abies* and *P. obovata*, the haplotype 6/6/2 is also detected in a number of populations, but its frequency in these populations does not exceed 10%. The remaining haplotypes found in *P. obovata* occur much less frequently in the populations (2/6/2, 5/1/3, 5/6/2, 6/4/2, 6/7/2) or belong to the categories of rare and unique. In *P. abies* populations, in addition to the 6/6/3 haplotype, other haplotypes are also detected, the frequencies of which vary within the range of 2.6 to 27%. In the contact zone of the species' ranges, both rare and the most common (5/5/2, 6/5/3, 6/5/4, 6/6/2, 6/6/3, 6/6/4) haplotypes of *P. obovata* and *P. abies* are found.

Populations of *P. koraiensis* significantly differ in the composition of haplotypes from the populations of two other spruce species. In populations of this species, haplotype 6/6/1 predominates with a fairly high frequency (0.700−0.938). In addition, rare haplotypes 3/6/2, 5/6/1, 6/5/1, 6/6/2, 6/7/1, and 7/6/1 were found in some populations. It should be noted that the Bureya population of spruce, which we included in the *P. koraiensis* group, was previously considered by us to be a population of *P. obovata* [52]. However, as a result of preliminary analysis of the cpSSR-locus variation, it was found that this population significantly differs in the set of haplotypes from the populations of this species. Therefore, we considered it appropriate to include it in the group of *P. koraiensis* populations, with which it has more similarity in genetic structure. Even earlier, A.Ya. Orlov [70] noted that the Siberian spruce growing in the Bureya basin differs in its morphological and ecological features from the typical Siberian spruce and in a number of parameters is similar to the Korean spruce.

Data presented in Table 1 show that the haplotype number (N_h) and the unbiased diversity (uh) found in the studied populations of three spruce species vary over a fairly wide range: from 2 to 17 and from 0.125 to 0.936, respectively. The lowest parameters of variability are observed in populations of *P. koraiensis*. The average value of uh was only 0.293. In *P. abies* and *P. obovata* and in the contact zone of their ranges, the average level of haplotype diversity is almost 3 times higher than that of *P. koraiensis*: 0.862, 0.831, and 0.860 respectively. Among the studied populations

Table 2. Analysis of molecular variation (AMOVA) by cpDNA haplotypes in 35 populations of the *P. abies−P. obovata−P. koraiensis* species complex

Level of variability	d.f.	Sum of squared deviations	Variability component	Share of variability, %	p
Between groups	3	40.414	0.083	17.1	0.001
Between populations within groups	31	17.984	0.007	1.5	0.001
Within populations	885	349.551	0.395	81.5	0.001
Total	919	407.945	0.485	100	

from the contact zone of ranges of *P. abies* and *P. obovata*, the lowest unbiased haplotype diversity was observed in the high-mountain population of Iremel (0.690), the highest in the Yuryuzan population (0.921). In *P. obovata* minimum value of the parameter *uh* was found in the Magadan population (0.550), which is the extreme northeastern small-area isolated locality of Siberian spruce, separated at a considerable distance from the main range of the species [71]. The low cpSSR variability in this population is most likely the result of population decline and long-term isolation in the past ("bottleneck"), as confirmed by the high frequency (66.7%) of one of the six haplotypes found in the population. Maximum value of the parameter *uh* noted in the northern population of Vanavara from Evenkia (0.936). On average, for the 35 studied spruce populations, the parameter values N_h and *uh* are equal to 9.5 and 0.762, respectively.

AMOVA analysis showed that 17.1% of total genetic diversity ($\Phi_{RT} = 0.171$, $p = 0.001$), identified in a study of four population groups of the *P. abies−P. obovata−P. koraiensis* species complex, distributed between population groups, 1.5% ($\Phi_{PR} = 0.017$, $p = 0.001$), between populations within groups, and the remaining 81.5% of variability is realized within populations ($\Phi_{PT} = 0.185$, $p = 0.001$) (Table 2).

A fairly high proportion of intergroup variability, many times greater than the proportion of variability among populations within groups, indicates significant genetic differentiation of spruce trees included in the species complex under study. To visualize genetic differences between the studied species based on a matrix of paired index values Φ_{PT}, calculated by allele frequencies of the three analyzed loci, PCoA was performed (Fig. 3).

In Fig. 3a the results of ordination of 35 spruce populations on the plane of two principal coordinates
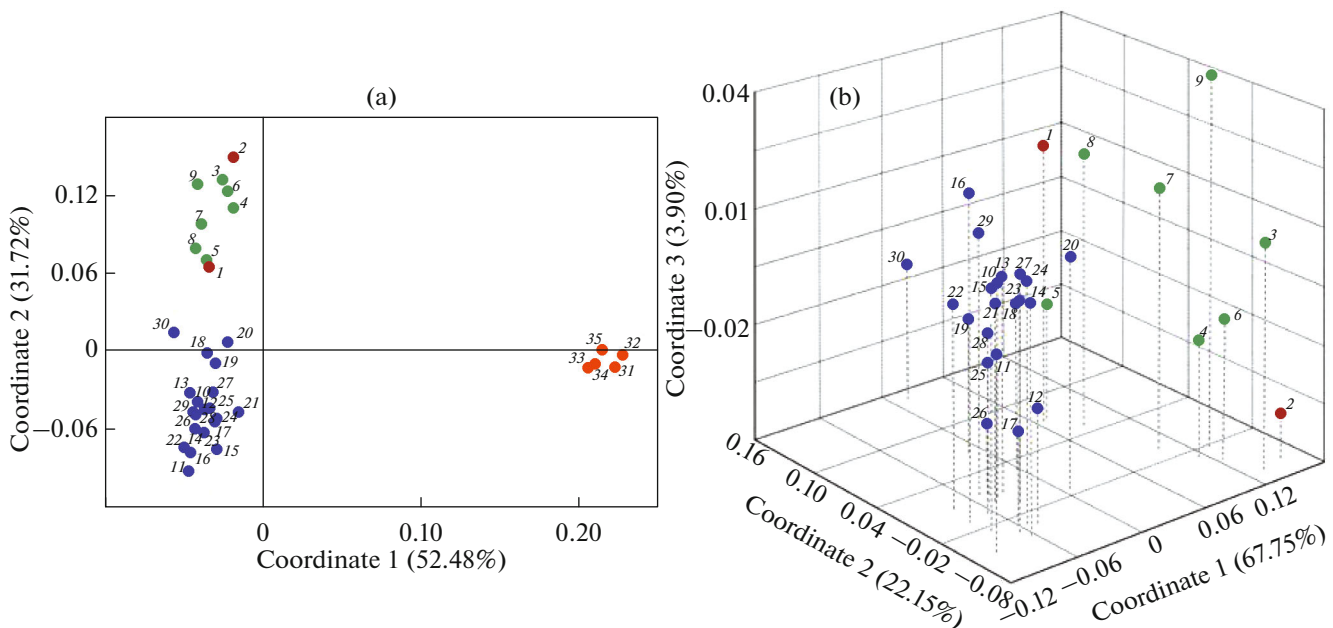
Fig. 3. Ordination of the studied spruce populations on the plane of principal coordinates according to the matrix of pairwise Φ_{PT} values, calculated from allele frequencies of three cpSSR loci: (a) ordination of 35 populations of the *P. abies−P. obovata−P. koraiensis* species complex; (b) ordination of populations of *P. obovata*, *P. abies* and populations from the contact zone of the ranges of these species. The numbering of populations corresponds to Table 1. Populations of *P. obovata* are indicated in blue; *P. abies*, in red; populations from the contact zone of *P. obovata* and *P. abies* ranges, in green; *P. koraiensis*, in orange.

are presented. The studied populations form several spatially separated groups. One of the groups, the most numerous, is represented by populations of *P. obovata*. Only population of *P. obovata* from Magadan oblast is located at some distance from the group of populations of this species, which indicates a significant difference in the haplotype composition of the Magadan population from the rest of the Siberian spruce populations included in the analysis. The peculiarity of this population is that it is dominated by the 6/6/3 haplotype with a fairly high frequency of occurrence (66.7%), which is found in other populations of this species with a lower frequency, averaging 21.7%. Previously, when studying the genetic diversity and differentiation of Siberian spruce populations by nuclear microsatellite loci, it was also shown that the Magadan population is maximally differentiated from the rest of the studied Siberian spruce populations and is characterized by the lowest level of genetic diversity [48].

The second group, located at some distance from the *P. obovata* population group, includes populations of *P. abies* and spruce populations from the contact zone of ranges *P. abies* and *P. obovata* (Fig. 3a). This group is divided into two subgroups that are poorly differentiated from each other (Fig. 3b), one of which is represented by the Lower Ob' populations of spruce (Kazym Mys, Pitlyar, Khanty-Mansiysk) and the high-mountain population *P. abies* from the Carpathians, and the other unites two Ural populations (Yuryuzan, Yekaterinburg), the Khibiny population from the Murmansk region, and the *P. abies* population Domzheritsy. The Iremel population from high-mountain ranges in the Urals is spatially separated from the other studied spruce populations from the contact zone of species ranges and has a lower level of haplotype diversity compared to them. In recent work examining population structure and shared demographic history of *P. abies* and *P. obovata* based on an analysis of the variability of nuclear and chloroplast SNPs [43], spruce individuals from Mount Iremel were also included. It was shown that this high-mountain population is characterized by low nucleotide diversity, and its individuals group separately from nearby low-mountain populations, forming a separate cluster.

Populations of *P. koraiensis* form a separate group on the plane of two main coordinates, the spatial location of which indicates a fairly high degree of divergence from groups of Norway and Siberian spruce populations (Fig. 3a).

The results obtained based on the analysis of the variability of three cpDNA microsatellite loci indicate significant differences between the studied groups of populations of the species complex *P. abies−P. obovata−P. koraiensis* by the composition and number of identified haplotypes, the level of genetic diversity, and population structure. The most significant differentiation in these parameters is observed between populations from the contact zone of ranges *P. abies* and *P. obovata,* populations of *P. abies* and *P. obovata* on the one side, and populations of *P. koraiensis*, on the other side (Φ_{RT} = 0.369; Φ_{RT} = 0.403, and Φ_{RT} = 0.336, respectively). Degree of differentiation between populations of two closely related species of *P. abies* and *P. obovata* turned out to be an order of magnitude lower (Φ_{RT} = 0.043). Recent phylogenetic studies of various species of the genus *Picea*, based on comparative transcriptomic analysis [58], also showed that *P. abies* and *P. obovata* are genetically much closer to each other than to *P. koraiensis*.

Our study found that the highest level of genetic diversity among the four population groups included in the analysis is observed in Norway spruce populations and populations located in the contact zone of *P. abies* and *P. obovata* ranges (Table 1). The identification in this zone of haplotypes characteristic of Siberian spruce and haplotypes found only in Norway spruce once again confirms the existence of a wide zone of introgressive hybridization between these species. When comparing the species included in the complex, a noticeable decrease in the level of genetic diversity in the eastern direction was also discovered. In *P. abies* the average population value of the *uh* index is 0.862; in *P. obovata*, 0.831; and in *P. koraiensis*, 0.293. A lower level of genetic diversity in Siberian spruce compared to Norway spruce was also found in a comparative analysis of populations of *P. abies* and *P. obovata* via a maternally inherited mitochondrial fragment *nad1* (intron 2) and paternally inherited chloroplast region *trn*T−*trn*L [35−37, 39].

Data obtained during the study on significant genetic divergence of spruce growing in the Russian Far East from two other species of the *P. abies−P. obovata−P. koraiensis* complex, including from Siberian spruce, confirm its status as a separate species *P. koraiensis.* It should be noted, however, that spruce from Amur oblast, which we previously studied for the mitochondrial locus *mh44* [52], reveals similarities with other populations of Siberian spruce, while in terms of the haplotype composition of the chloroplast microsatellites we studied, this population is closer to the studied populations of Korean spruce. It is dominated by the same haplotype as in other populations of this species. The discrepancy between the data obtained from the analysis of cytoplasmic genome markers that differ in the type of inheritance confirms the presence of a zone of introgressive hybridization between Siberian spruce and Korean spruce, the existence of which was first suggested by E.G. Bobrov [2]. In this regard, additional research is needed involving a larger number of population samples of both Korean spruce and Siberian spruce, especially from a previously poorly studied vast region east of Lake Baikal. More detailed studies of the populations of these species in the Russian Far East will provide data on the boundaries of the ranges of "pure" species of *P. obo-*

vata and *P. koraiensis*, as well as zones of their interspecific hybridization.

Thus, the results obtained from a comparative study of the *P. abies−P. obovata−P. koraiensis* species complex indicate efficiency of using microsatellite loci cpDNA with high gene flow not only to determine the level of genetic diversity of species, to identify the degree of their differentiation, but also to establish the species status of controversial taxa.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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