Plant Genetic Resources: Characterization and Utilization

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Population genetic variation of microsatellite markers developed for Siberian fir (*Abies sibirica* Ledeb.) and European silver fir (*Abies alba* Mill.) using whole genome sequencing data

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Abstract

The article presents the results of the search for microsatellite or simple sequence repeat (SSR) loci with tri-, tetra-, penta- and hexanucleotide tandem repeat motifs in the draft *de novo* assembly of the Siberian fir (*Abies sibirica* Ledeb.) genome and the development of convenient relatively highly and moderately polymorphic markers that can be easily genotyped even by simple gel electrophoresis. In total, 64 pairs of oligonucleotide polymerase chain reaction (PCR) primers for 32 detected microsatellite loci were designed and tested. Based on the testing results, 10 most promising polymorphic loci were selected and genotyped in eight natural populations of Siberian fir. Homologous microsatellite loci in the genome of European silver fir (*Abies alba* Mill.) were also identified by mapping Siberian fir contigs, containing SSR loci to the European silver fir genome assembly. A multiplex panel of 14 universal microsatellite loci was developed and genotyped in samples from four natural populations of *A. alba* and a small sample of eight Nordmann fir (*Abies nordmanniana* (Steven) Spach) trees.

Introduction

Over the past 15–20 years, genomic research has been rapidly developing due to the development of high-throughput DNA sequencing methods, called next generation sequencing technologies. This made it possible to sequence giant genomes, including conifer genomes, which are 4–9 times larger [~11–35 Gbp (Leitch *et al.*, 2019)] than the human genome [~3.06 Gbp (Nurk *et al.*, 2022)]. Thus, to date, more than 10 genomes of the conifer family Pinaceae have been fully sequenced, annotated and assembled (see for review Kuzmin *et al.*, 2019; Bondar *et al.*, 2022), including *Abies alba* (Mosca *et al.*, 2019) and partially *Abies sibirica* (Nystedt *et al.*, 2013).

Thanks to whole genome or partial genome sequencing of conifers, it became possible to develop easily genotyped species-specific microsatellite markers for various conifer species, for example, for such main species of the Eurasian boreal forests as *Pinus sibirica* Du Tour (Belokon *et al.*, 2016) and *Larix sibirica* Ledeb. (Oreshkova *et al.*, 2017, 2019). These markers facilitate population genetic studies, identification of the timber and plant material origin in the fight against illegal logging and control over the legal origin of plant material, effective reforestation, identification of clone material, etc. (Krutovsky *et al.*, 2019).

The main object of the study presented here was the Siberian fir (*A. sibirica* Ledeb.), one of the most important forest-forming species of the dark coniferous taiga with a large distribution zone, including the northeast of the European part of Russia, the Urals and most of Siberia. In general, representatives of the genus *Abies* (Mill) are of great interest to foresters, dendrologists, gardeners and landscapers. This genus is considered one of the largest in the Pinaceae

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family, including from 40 to 65 species according to various modern estimates (Semerikova *et al*., 2018).

However, despite its significance the genetic diversity of Siberian fir has been insufficiently studied. Most of earlier such studies were carried out using allozyme markers, which are less informative than simple sequence repeat (SSR) markers, and did not cover the entire range of the species (Goncharenko and Padutov, 1995; Larionova and Ekart, 2005; Semerikova and Semerikov, 2006; Larionova *et al*., 2007). Several studies were carried out also in Siberian fir on the dynamics of populations, population size and phylogeography using cytoplasmic DNA and amplified fragment length polymorphism (AFLP) markers (Semerikova and Semerikov, 2014, 2016; Semerikov *et al*., 2022). However, despite these studies, there is practically no information on the genetic diversity, structure and differentiation of Siberian fir populations in the Krasnoyarsk Territory and the Republic of Khakassia, as well as in many other large areas of its natural distribution. The genetic structure, subdivision and differentiation of populations of this species have not been studied under ecologically heterogeneous environmental conditions. This does not allow assessing the general state of the genetic resources of the species, their spatial distribution, which makes it difficult to develop programmes for the protection, rational use and reproduction of the genetic resources of the species.

Meanwhile, abundant distribution in the genome, high reproducibility, codominance and relatively easy identification have made microsatellite markers popular in modern studies of conifer population genetics. Several microsatellite markers with mostly dinucleotide motifs were previously developed for some fir species (Hansen *et al*., 2005; Cremer *et al*., 2006; Cvrčková *et al*., 2015). However, they are suitable for genotyping only using capillary gel-electrophoresis, while microsatellite markers with longer motifs can be easily genotyped in any laboratories with simple slab gel electrophoresis. Therefore, our main objective was to generate universal SSR markers suitable for reliable genotyping in any laboratories with any gel electrophoresis equipment, such as for either simple vertical electrophoresis in polyacrylamide slab gel or much more challenging capillary gel electrophoresis requiring more expensive instruments.

Fir is a good bioindicator of environmental conditions in the areas of their growth (Świercz *et al*., 2022). Therefore, in addition to the main goal of this study to develop new highly informative microsatellite markers that can be easily genotyped in *A. sibirica* based on the data of whole genome sequencing of this species, we also searched for homologous microsatellite loci in the genome of a closely related species, European silver fir (*A. alba* Mill.), tested them in population samples and developed a multiplex genotyping panel of 14 microsatellite loci, which were successfully tested and genotyped in the samples from eight *A. sibirica* populations, four *A. alba* populations and a small sample of eight trees representing *Abies nordmanniana*.

Both *A. sibirica* and *A. alba* are important species of the boreal and temperate forests in Eurasia. These trees are up to 30 m tall and up to 0.5 m in diameter, living up to 200–250 years in natural conditions. Fir is very shade-tolerant, has low frost resistance and often suffers from late spring frosts. It grows best on well-drained soddy-podzolic loam soils with close underneath limestone occurrence. It avoids strongly podzolized and stagnant-moistened soils and does not grow on poor sandy soils. It can form both pure and mixed (fir-spruce) stands. In the mountains it rises to 2000 m above sea level, where it is present in a bushy form (Mauri *et al*., 2016; Dobrowolska *et al*., 2017; Zaitsev *et al*., 2018).

Materials and methods

Search for microsatellite loci and design of PCR primers

Nucleotide reads obtained as a result of whole genome sequencing of *A. sibirica* Ledeb. with low coverage were used to search for microsatellite loci and design their polymerase chain reaction (PCR) primers. The reads of *A. sibirica* were generated additionally in a project dedicated mainly to the sequencing and assembly of the genome of Norway spruce, *Picea abies* (L.) Karst. (Nystedt *et al*., 2013; https://www.ncbi.nlm.nih.gov/bioproject/PRJEB1894; the NCBI GenBank SRA accession number ERP002568). We assembled reads into contigs using CLC Assembly Cell v.4.4 (QIAGEN, Aarhus, Denmark). The obtained contigs were used to search for microsatellite loci using the GMATo v.1.2 software (Wang *et al*., 2013). To increase the reliability of genotyping of the prospective microsatellite markers, the search was limited to the search for contigs containing tandem repeats of only three-, four-, five- and six-nucleotide long motifs with thresholds for minimal number of repeats 15, 10, 6 and 7, respectively.

The PCR primers for amplification of the found microsatellite loci within the size range of 140–280 bp were designed using the WebSat online tools (Martins *et al*., 2009). For successful PCR amplification of microsatellite loci, their primers must be locus-specific, that is, the PCR annealing sites matching primer sequence pairs for a given marker should not occur anywhere else in the genome. Therefore, to design unique, single locus-specific primers, their sequences were mapped to the newly generated assembly of the *A. sibirica* genome using BLAST. Primer pairs that had annealing sites for more than one locus were excluded from further analysis. Single locus-specific PCR primers selected for further testing were synthesized by Evrogen (Moscow, Russia). In addition, Siberian fir contigs containing successful microsatellite markers were used to search for homologous microsatellite loci in the European silver fir (*A. alba* Mill.) genome (Mosca *et al*., 2019). Then, PCR primers designed for the found homologous loci were tested in four populations of *A. alba* and a small sample of *A. nordmanniana*.

Plant material and DNA isolation

Total DNA was isolated from 100–200 mg of dried needles per individual tree sample collected from 240 Siberian fir trees in eight populations (30 trees per each population) in Central Siberia (Fig. 1) using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990).

About 50 mg of fresh needles per each sample were used for DNA isolation from 96 individual tree samples of *A. alba* representing four populations of anonymous origin (24 samples per each population, respectively) using the DNeasy®96 Plant Mini Kit and following the standard protocol (Qiagen, Hilden, Germany). The same kit was used to isolate DNA from germinated seeds of *A. nordmanniana*. The samples of *A. alba* were provided by Isogen GmbH (Göttingen, Germany; https://isogen.de) and the seed samples of *A. nordmanniana* by PlusBaum Samen GmbH (Nagold, Germany; https://plusbaum-samen.de).

PCR amplification and genotyping of microsatellite loci

GenePak PCR Core kits (IsoGene Laboratory Ltd, Moscow, Russia) containing hot-start *Taq*-DNA polymerase, deoxynucleoside triphosphates (dNTPs) and magnesium chloride were used for PCR amplification of selected nuclear microsatellite loci in

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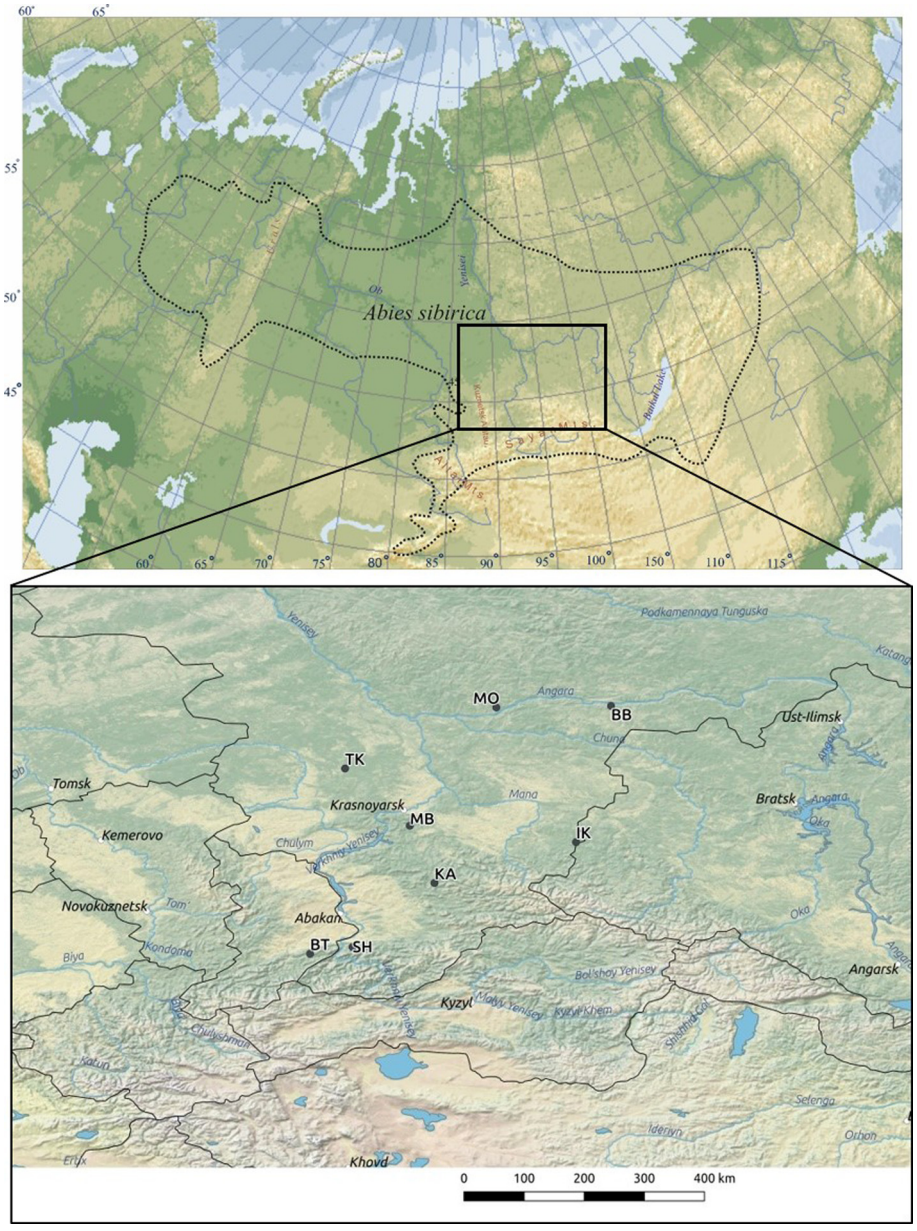

Figure 1. Area of *Abies sibirica* and geographic location of the eight populations genotyped in the study using microsatellite markers. Krasnoyarsk Region: Boguchanskaya (BB, 58°21' N, 97°30' E, 419), Ordzhonikidzevskaya (MO, 58°19' N, 94°56' E, 293), Kytatskaya (TK, 56°57' N, 91°33' E, 232), Maganskaya (MB, 55°40' N, 93°00' E, 333), Kesovskaya (IK, 55°18' N, 96°43' E, 423), Artemovskaya (KA, 54°23' N, 93°33' E, 542), Shushenskoe (SH, 52°57' N, 91°43' E, 642); Republic of Khakassia: Tabatskaya (BT,52°48' N, 90° 46' E, 849).

the Axygen MaxyGene II Gradient Thermal Cycler (Axygen Scientific Inc., Union City, California, USA). A touchdown PCR program was used to reduce non-specific amplification, which included first DNA denaturation at 94°C for 1 min, then 9 cycles, including 30 s of denaturation at 94°C, annealing of primers for 30 s at 60°C, with a decrease by 1°C each cycle (up to 50°C), and 1 min of elongation at 72°C. The next 24 cycles included DNA denaturation at 94°C for 30 s, primer annealing at 50°C for 30 s and elongation at 72°C for 30 s. This was followed by an extension step at 72°C for 10 min, and the last stage was cooling at 4°C. Loci that showed stable interpretable PCR amplification spectra were selected for further use.

Amplification products were separated by 6% polyacrylamide gel electrophoresis using Tris-EDTA-borate electrode buffer in vertical electrophoresis chambers. The gel was stained in a solution of ethidium bromide followed by visualization in ultraviolet light. DNA fragments of the pBR322 *E. coli* plasmid digested by the *Hpa*II restriction enzyme were used as standard length markers.

PCR primers that had annealing sites also in homologous microsatellite loci of *A. alba* were also tested and genotyped in samples of 96 trees from four natural populations of *A. alba* and eight seedlings germinated from seeds of eight different *A. nordmanniana* trees using fluorescent labelled primers synthesized by Sigma Aldrich Chemie GmbH (Taufkirchen, Germany) and capillary gel electrophoresis with ABI PRISM 3130 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Visualization and processing of the results of fragment analysis (electropherograms) were carried out using the GeneMapper 4.0 program (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

Population genetics data analysis

Genetic variation parameters, such as average (N_a) and effective (N_e) number of alleles, observed (H_o) and expected (H_e) heterozygosity, fixation index (F_{IS}) and different parameters of genetic

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differentiation (Nei's standardized G_{ST} ($G'_{ST}N$), Hedrick's standardized G_{ST} ($G'_{ST}H$ and G''_{ST}), Jost's estimate of differentiation Dest) and assessment of the Hardy–Weinberg equilibrium (HWE) were calculated using GeneAlex v. 6.503 (Peakall and Smouse, 2006, 2012). Polymorphic information content (PIC) was calculated according to Botstein *et al.* (1980) using the MolMarker program (Jahnke *et al.*, 2022). The frequencies of null alleles were inferred and analysed using MICRO-CHECKER v. 2.2.3 (Van Oosterhout *et al.*, 2004) with data exported from GeneAlex.

Results

Abies sibirica

A total of 264,864 contigs containing tandem microsatellite repeats with three-, four-, five- and six-nucleotide motifs with a specified minimum number of repeats were found in the partial genome assembly of Siberian fir. We specifically focused on selecting loci with longer than dinucleotide motifs in order to improve scorability and comparability. Initially, 475 microsatellite loci with long enough neighbouring sequences on both ends to design PCR primers were selected, 115 of them were used to design PCR primers, and PCR primer pairs were finally synthesized by Evrogen Co. (Moscow, Russia) and further tested for 64 of them (see online Supplementary Table S1 for details and original contig sequences).

An initial test of the selected primers on four DNA samples from each population of Siberian fir showed that out of 64 primer pairs, 11 lacked an amplified product and 7 did not have the expected size of amplified product. The remaining 46 pairs were further tested on an enlarged sample of trees. According to the results of this test, 22 more loci had to be excluded, because 12 of them turned out to be monomorphic and 10 showed unstable amplification. As a result, 24 loci showed stable amplification under the selected conditions and were further used for genotyping entire population samples of Siberian fir trees (Table 1, online Supplementary Table S1).

Based on the results of genotyping of 24 selected loci in eight geographically distant populations of *A. sibirica*, another 14 loci had to be excluded due to poor or nonspecific amplification and a large number of null alleles. Using the MICRO-CHECKER program (Van Oosterhout *et al.*, 2004), hidden null alleles were identified for the following three loci: *As_43741* in the TK and SH samples, *As_563614* in the KA and MB samples and *As_420232* in the BB sample. Hidden null allele frequencies were calculated based on the assumption that populations are in HWE (Chakraborty, 1992). Some of the loci demonstrated significant deviation from HWE, but it was not systematic across all populations. Neither of the loci demonstrated deviation from HWE for all or the majority of populations (online Supplementary Table S2).

Thus, the 10 most reliable loci were selected (Table 1, online Supplementary Table S1), which were easily and unambiguously genotyped using a simple polyacrylamide gel in vertical electrophoresis chambers (online Supplementary Fig. S1).

Abies alba and Abies nordmanniana

Based on the results of mapping nucleotide sequences of 64 primer pairs selected for *A. sibirica* to the *A. alba* genome assembly, 36 *A. alba* homologous loci were selected and the corresponding primers were tested on four *A. alba* samples. Of these, 23 were stably amplified and well genotyped; 14 turned out to be polymorphic and 9 monomorphic. The polymorphic 14 markers were further tested on a larger set of eight samples using capillary electrophoresis on an ABI PRISM 3130 sequencer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). To do this, one of the primers in the pair was labelled with either 6-FAM or HEX fluorescent labels. Then, 14 markers were grouped into four PCR multiplexes and genotyped in 96 trees from four *A. alba* populations and eight seedlings representing eight different *A. nordmanniana* trees. Multiplex PCR primer groups were organized in such a way as to avoid overlapping ranges of allele sizes of different amplified loci labelled with the same fluorescent dye in the same PCR reaction. A maximum of four primer pairs per group were pooled together using two different fluorescent dyes (Table 2). Among 14 selected microsatellite loci tested on samples of Nordmann fir, one was not amplified at all, nine turned out to be polymorphic and four were monomorphic (Table 2).

Population genetic analysis

Polymorphism data for 10 microsatellite loci in eight populations of *A. sibirica* are presented in Table 3 (see also online Supplementary Table S2 for details).

The studied microsatellite markers for Siberian fir turned out to be moderately polymorphic with the number of alleles (N_a) varying from 2 (*As_99437, As_2687764, As_3458593*) to 4.9 (*As_563614*) per locus on average for eight populations (Table 3). The effective number of alleles (N_e) varied from 1.6 to 3.7 at the *As_3666591* and *As_563614* loci, respectively. Observed (H_o) and expected (H_e) heterozygosity varied from 0.31 to 0.71 and from 0.36 to 0.73 for *As_3666591* and *As_563614*, respectively. The PIC was high and varied from 0.33 to 0.73 for the same pair of markers *As_3666591* and *As_563614*, respectively. The lowest (0.01) and highest (0.12) F_{ST} values were calculated for the *As_99437* and *As_3666591* loci, respectively. $G'_{ST}H$ varied from 0.01 to 0.25 for the *As_3458593 and As_420232* loci, respectively. The highest $G'_{ST}N$, G''_{ST} and Dest values were 0.12, 0.25 and 0.17, respectively (Table 3). F_{IS} values ranged from −0.41 to 0.13, but none of them was statistically significant (Table 3). However, it should be noted that the analysis for hidden null alleles using MICRO-CHECKER revealed null alleles at three loci (*As_43741, As_420232, As_563614*) in five populations, which were taken into account while calculating the parameters of genetic variation.

Some of the loci demonstrated significant deviation from HWE, but it was not systematic across all populations. Neither of the loci demonstrated deviation from HWE for all or the majority of populations (online Supplementary Table S3).

The results of population clustering are presented in Fig. 2. According to the results of the Mantel test, no significant correlation was found between genetic and geographic distances.

Polymorphism data for 14 microsatellite loci in four populations of *A. alba* and 13 microsatellite loci in a sample of eight trees of *A. nordmanniana* are presented in Table 4 (see also online Supplementary Tables S3 and S4 for details).

Most of the microsatellite markers were highly polymorphic with number of alleles (N_a) ranging from 1.8 (*As_420232*) to 10 (*As_1898225*) alleles per locus in four populations of *A. alba* on average (Table 4). The loci *As_1998225* and *As_2280886* showed the highest genetic variation in all populations (online

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Table 1. Characteristics of 24 successfully amplified polymorphic microsatellite loci selected for *Abies sibirica* based on genome sequencing data

		Nucleotide sequence of PCR primers (5'-3')		
Locus*	Motif	Forward	Reverse	Allele length, bp
As_43741#	$(TCA)_{15}$	CTCAAAGGTAGTGTTTCTCAGTGGTAT	CAATGATGACAATGATGTTGGC	201, 207, *219*, null
As_99437#	$(ATG)_{17}$	GGGAGATTGATTATGTGGATCA	CATGCAAGGGGACTAATATCTTC	205, *229*
As_279869	$(TTA)_{15}$	ACGAAGGTGAAGGGTTCTATCA	TTAAGGGGAATTGTTGTGGGTA	*253*
As_301948	$(ATC)_{16}$	CAATGATGATGGACTAGATGGA	AAGTCTGATGGGTTATGCTTGT	*214*
As_878430	$(AAT)_{15}$	TTACCCTCGAATCTAAAGGTGG	CTATCCCACAAACAAAGGGGT	*260*
As_1177607	$(TAA)_{17}$	GTGATCTATGCCTCTCCAATGA	ACTGGTCACAATGGTTTGAATG	*188*
As_1563074	$(ATT)_{18}$	GTGAGGAGGAGATTTGTAAGTGC	CAGATGAGTACGAACCACTACCC	*142*
As_420232#	$(TAAA)_{10}$	CCTTCCACCTTCCATGATATTC	TCCAAGAAAAGGCACAAGAAGT	168, 176, *182*, 188, 192, null
As_563614#	$(ATAG)_{12}$	AAGTTGTTTGATCCCTCTCGAC	CATGTAATGCTCCAATCAGGAA	148, *156*, 160, 164, 168, 180, 192, 222
As_721750	$(TATG)_{10}$	CCCTTACCTCCCTAACACTTGA	GGCAAGATAGATGATTGACACC	*147*
As_2280886	$(TGTA)_{10}$	CACTAAAAGAGGAGCCATGTCC	GACACCTGCAATCACCAAGAC	*201*
As_2687764#	$(TATG)_{10}$	GATGAGAGATGCTTGTGAATCC	GGAGGTTATCATGGAAGTGGAC	218, *222*, 226
As_2996373	$(ATAC)_{11}$	TCGCTTCCACACTGACCAC	TTACACACAAAAGGGCTCACAC	*272*
As_3602348	$(CATA)_{10}$	GTCGCAGAATGTTGAATTGCT	CATGGATGGAGAGGACAAGTTT	*278*
As_347605	$(GAAAA)_{6}$	AAGGTTCTTAAATTCCAACCCC	GACTTGAGGATGAGGTTCTTGC	*253*
As_1133359	$(GATTT)_{6}$	GGATGCAGGGTTTCATTTATTC	CCAGAAGATTCGACATGGAAA	*238*
As_1356647#	$(GATAA)_{7}$	TAGACTTTGAGGTGATGGGCTT	TTGCTCCGTGATCTTAGGTTTT	238, 243, 248, *250*
As_2288295#	$(AGATA)_{6}$	TGTTTTCCAGATTTGTGAGTGC	AGTCCCGTTGACCTACTTTGTG	167, *172*, 177
As_3458593#	$(TTATC)_{7}$	CTCCCACATCAATCCCATTTAC	TGGTTTCCTGACGATGACTCTA	*225*, 230
As_404145#	$(CCAGCT)_{8}$	TAACGACCTGCTGCTATTTTCA	GGGCACCATTCTCAATTTCTAC	173, 179, 185, *187*, 191
As_1898225	$(TCTTCA)_{9}$	ATGCTGCCCATTGAGAATTAGT	ACCGCTCTTCCCTTTACTTTCT	*270*
As_1970306	$(GCAAGA)_{7}$	TTTGGACTGAGATTTGGGC	CGATTATGAAAGAATGGGTAGC	*248*
As_2631734	$(CACTCC)_{7}$	CCCATCCTATCTCCTACACTTCA	GGGTTGATATGTGAAAAGGGAA	*169*
As_3666591#	$(TATTCC)_{7}$	CGTTCTTTCACTTTTGTTGGC	TCCTTTGCCATTGTTCATGT	238, *256*, 262

*Number in the name of the loci refers the contig number in the *A. sibirica* genome assembly. #Ten most reliable polymorphic microsatellite loci finally selected for genotyping eight *A. sibirica* populations. Allele length in the contig is italicized.

Supplementary Table S3). The effective number of alleles (N_e) varied from 1.3 to 6.6 in the *As_420232* and *As_1898225* loci, respectively. The observed (H_o) and expected (H_e) heterozygosities varied from 0.21 to 0.84 and from 0.20 to 0.85 for loci *As_420232* and *As_1898225*. The PIC was high and varied from 0.20 to 0.85 for the same pair of markers *As_420232* and *As_1898225*, respectively. The highest (0.09) and lowest (0.01) F_{ST} values were calculated for *As_2631734* and *As_1970306*, respectively. The $G'_{ST}H$ varied from −0.01 to 0.22 for the *As_1970306* and *As_2280886* loci, respectively. The highest $G'_{ST}N$, G''_{ST} and *D*est were 0.10, 0.23 and 0.19, respectively (Table 4). In total, differentiation was not high ranging from 0.03 to 0.08 depending on the parameter, but significant for each parameter (Table 4).

The F_{IS} values varied from −0.37 to 0.69, and significant deficiency of heterozygotes was observed for four loci in single populations, for *As_563614* and *As_1089795* in more than two populations (online Supplementary Table S5). It is likely caused mainly by a high frequency of hidden null alleles in these a few loci found by MICRO-CHECKER (online Supplementary Table S6).

Discussion

Abies sibirica

In general, the values of the main parameters of genetic polymorphism ($N_a = 2.9$, $N_e = 2.2$, $H_o = 0.55$, $H_e = 0.51$) of *A. sibirica* indicate a relatively lower level of diversity compared to studies of *A. alba* based on other microsatellite markers (Cremer *et al.*, 2006; Cvrčková *et al.*, 2015), but similar to the parameters for *A. alba* obtained in our study and based on a subset of markers used for *A. sibirica* (Table 4). The highest values of almost all parameters were found in the southern populations SH and BT, which are closely located to each other (Fig. 1).

Some of the loci demonstrated significant deviation from HWE, but it is likely due to relatively high self-pollination which is typical for *Abies* species that have a relatively high self-pollination rate among conifers [mean 11% for four populations of *A. balsamea* (Neale and Adams, 1985), 11% for *A. lasiocarpa* (Shea, 1987), 11% (Schroeder, 1989) or 24% (Kormuťák and Lindgren, 1996) for *A. alba*, mean 13% for seven populations of *A. amabilis* (Davidson, 1990), 6% for *A. borisii* (Fady and Westfall, 1997), and 10–13% for *A. sibirica* (authors' data, unpublished)], which

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Table 2. PCR multiplex panels, number and size range of alleles of the best 14 microsatellite loci used for population genetic genotyping of *Abies alba* and *Abies nordmanniana*

	A. alba		*A. nordmanniana*		PCR multiplex panel	
Locus	Range, bp	Number	Range, bp	Number	Fluorescent dye	#
As_1898225	241–307	12	247–307	6	HEX	1
As_1356647	263–268	2	268	1	6-FAM	
As_404145	181–211	6	181–217	4	6-FAM	
As_563614	156–162	4	180–240	6	6-FAM	
As_2612077	262–287	5	252–287	6	HEX	2
As_2631734	168–186	3	168	1	HEX	
As_3458593	240–255	4	245	1	HEX	
As_3602348	273–293	5*	No amplification	0	6-FAM	
As_154813	289–316	7*	292–295	2	HEX	3
As_2280886	220–264	10	224–272	9	6-FAM	
As_420232	179–191	2	179–191	2	6-FAM	
As_878430	260–275	4*	272	1	HEX	
As_1089795	273–339	9*	273–324	4	6-FAM	4
As_1970306	254–266	2	254–266	2	HEX	

*Loci with null alleles according to MICRO-CHECKER.

Table 3. Genetic variation parameters of the 10 microsatellite markers averaged for eight populations of *A. sibirica* and mean for all markers

Locus	N_a	N_e	H_o	H_e	*PIC*	F_{IS}	F_{ST}	$G'_{ST}H$	$G'_{ST}N$	G''_{ST}	*D*est
As_43741	3.2	2.6	0.67	0.59	0.56	−0.13	0.04***	0.07***	0.03***	0.07***	0.04***
As_99437	2.0	1.8	0.62	0.44	0.35	−0.41	0.01	0.00	0.00	0.00	0.00
As_420232	3.4	2.6	0.63	0.61	0.61	−0.04	0.10***	0.25***	0.10***	0.25***	0.17***
As_563614	4.9	3.7	0.71	0.73	0.73	0.02	0.05***	0.13***	0.03***	0.14***	0.10***
As_2687764	2.0	1.9	0.46	0.48	0.37	0.04	0.02	0.02	0.01	0.02	0.01
As_1356647	2.5	1.7	0.45	0.42	0.36	−0.09	0.03*	0.02*	0.01*	0.02*	0.01*
As_2288295	2.6	2.0	0.52	0.49	0.42	−0.07	0.03**	0.04**	0.02**	0.05**	0.02**
As_3458593	2.0	1.9	0.59	0.49	0.37	−0.22	0.02	0.01	0.01	0.01	0.01
As_404145	3.6	2.0	0.58	0.50	0.47	−0.16	0.04***	0.06***	0.03***	0.07***	0.04***
As_3666591	2.3	1.6	0.31	0.36	0.33	0.13	0.12***	0.18***	0.12***	0.19***	0.08***
Mean	2.9	2.2	0.55	0.51	0.46	−0.09	0.05***	0.07***	0.04***	0.08***	0.04***
Population	**N_a ± SE**		**N_e ± SE**		**H_o ± SE**			**H_e ± SE**		**F_{IS} ± SE**	
BB	2.6 ± 0.3		2.2 ± 0.2		0.55 ± 0.05			0.51 ± 0.04		−0.09 ± 0.07	
MO	2.5 ± 0.2		2.2 ± 0.2		0.62 ± 0.07			0.52 ± 0.04		−0.21 ± 0.09	
TK	2.6 ± 0.3		2.2 ± 0.2		0.48 ± 0.05			0.53 ± 0.03		0.10 ± 0.05	
MB	3.0 ± 0.4		2.3 ± 0.3		0.52 ± 0.05			0.51 ± 0.05		−0.05 ± 0.05	
IK	3.0 ± 0.3		2.1 ± 0.1		0.57 ± 0.05			0.51 ± 0.03		−0.14 ± 0.08	
KA	2.9 ± 0.3		2.0 ± 0.2		0.51 ± 0.05			0.46 ± 0.04		−0.10 ± 0.06	
SH	3.2 ± 0.4		2.3 ± 0.3		0.55 ± 0.04			0.52 ± 0.04		−0.08 ± 0.06	
BT	3.1 ± 0.3		2.4 ± 0.3		0.63 ± 0.07			0.54 ± 0.04		−0.15 ± 0.09	
Mean	2.9 ± 0.1		2.2 ± 0.1		0.55 ± 0.02			0.51 ± 0.01		−0.09 ± 0.03	

N_a, average number of alleles; N_e, effective number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; PIC, polymorphic information content; F_{IS}, fixation index; F_{ST}, coefficient of differentiation between populations; $G'_{ST}N$, Nei's standardized G_{ST}; $G'_{ST}H$, Hedrick's standardized G_{ST}; G''_{ST}, Hedrick's standardized G_{ST} further corrected for bias when the number of populations is small; *D*est, Jost's estimate of differentiation between populations; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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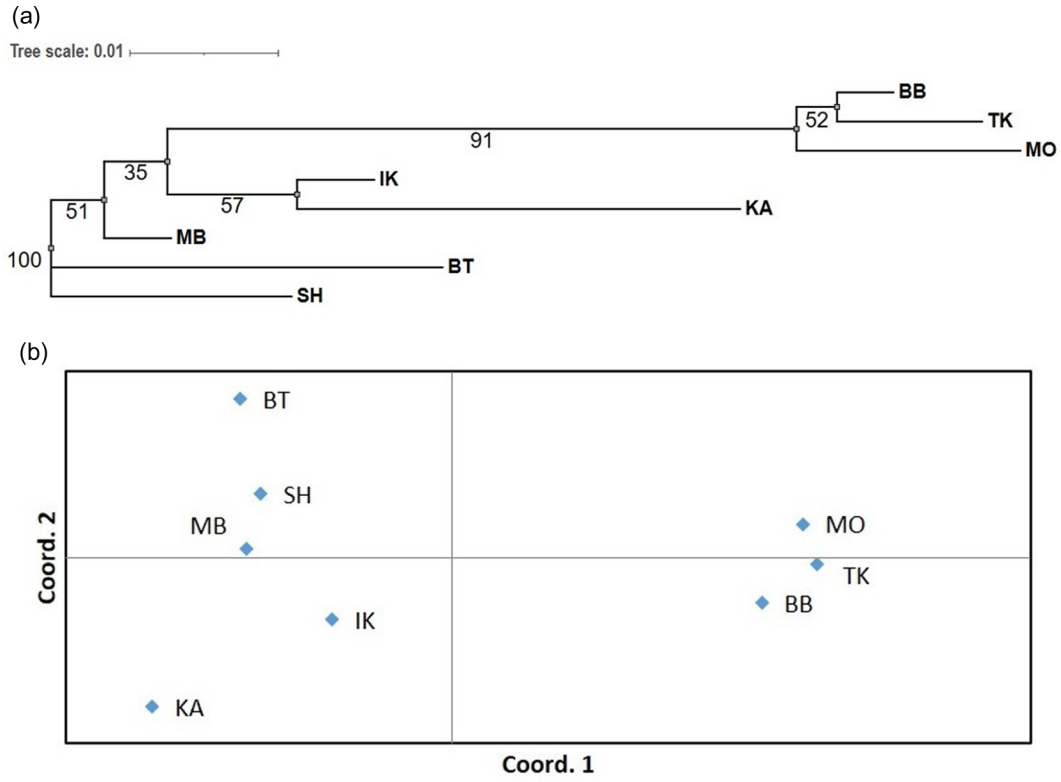

Figure 2. Neighbour-joining tree with bootstrap values at the nodes (**A**) and principal coordinates analysis (PCoA) (**B**) of eight populations of *A. sibirica* based on the standard Nei's genetic distances (D_N). See Fig. 1 for the full names of the populations and their geographic location.

is comparable to other conifers [2–17% (Adams and Birkes, 1991) or mean ~15% (Table l in Muona, 1990; Mitton, 1992)].

Based on clustering presented in Fig. 2 phylogenetic relationships between populations of *A. sibirica* reflect their geographic location in general (Fig. 1). Cluster analysis clearly separated the populations BB, TK and MO located in the northern regions with a high degree of confidence (bootstrap value of 99%) from the distant populations located to the south, among which the southern cluster of populations MB, BT and SH is also distinguished, but with less support (bootstrap value of only 60%).

However, according to the results of the Mantel test, no significant correlation was found between genetic and geographic distances, which may indicate that, in addition to geographic isolation, the genetic differentiation in the studied area of this species could be affected by the ecological heterogeneity of the habitat at different altitudes above sea level related to soil fertility, water availability and mineral nutrition. The obtained results are consistent with similar data obtained for *A. sibirica* using other genetic markers (Larionova *et al.*, 2007; Semerikova and Semerikov, 2016).

Abies alba and Abies nordmanniana

In general, the variation of 14 markers developed in this study for *A. alba* was similar to the variation of 11 microsatellite markers developed for this species by Cremer *et al.* (2006): $N_a = 5.2$ *vs* 4.5 in our study (Table 4), $H_o = 0.31$ *vs* 0.46, $H_e = 0.53$ *vs* 0.56, and similar to $N_a = 9.2$, $H_o = 0.43$, and $H_e = 0.49$ obtained in Postolache *et al.* (2014) based on 24 markers including 16 EST-SSRs (calculated from Table 1 in Postolache *et al.*, 2014), but slightly less than $H_e = 0.75$ based on 11 microsatellite markers used by Hrivnák *et al.* (2017), including four markers developed by Cremer *et al.* (2006), and values obtained for many other populations of *A. alba* (H_o and $H_e = 0.5–0.8$, see Dalmaris *et al.*, 2022 for references). The lower variation observed in our study can be explained by intentionally not using microsatellite loci with dinucleotide repeats that are mostly used in other studies but notoriously known for typical difficulty regarding consistent and reliable genotyping.

Although very preliminary, variation observed in a small sample of *A. nordmanniana* was less than variation observed in much larger sample in Hansen *et al.* (2005) which was based only on five highly polymorphic markers: $N_a = 30.4$ (calculated from Table 1 in Hansen *et al.*, 2005) *vs* 3.5 in our study (Table 4), $H_o = 0.70$ *vs* 0.43 and $H_e = 0.90$ *vs* 0.40.

The null alleles detected in a few loci of both *A. sibirica* and *A. alba* can potentially lead to an underestimation of allelic richness and heterozygosity for these loci in populations and introduce biases in measures of population differentiation by reducing the observed number of alleles and heterozygosity estimates, but it is unlikely that they would significantly affect these parameters based on all markers, because only a few loci were affected. Moreover, to mitigate the impact of null alleles, we used MICRO-CHECKER program to statistically correct genetic diversity and differentiation parameters for null allele presence.

Conclusions

Based on whole genome sequencing data, 10 polymorphic microsatellite markers were developed for *A. sibirica*, 14 for *A. alba* and 13 for *A. nordmanniana*. Among them, at least five (*As_1356647*, *As_404145*, *As_563614*, *As_3458593* and *As_420232*) can be used for all three species, but potentially more. The markers were tested

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Table 4. Genetic variation and differentiation parameters of the 14 microsatellite markers averaged for four populations of *A. alba* (A, B, C and D) and one population of *A. nordmanniana* and mean for all markers

Locus	N_a	N_e	H_o	H_e	*PIC*	F_{IS}	F_{ST}	$G'_{ST}H$	$G'_{ST}N$	G''_{ST}	*Dest*
As_1898225	10.0	6.6	0.84	0.85	0.85	0.00	0.02	0.01	0.00	0.02	0.01
As_1356647	2.0	1.7	0.34	0.40	0.34	0.14	0.08**	0.13**	0.09**	0.14**	0.06**
As_404145	4.8	2.3	0.54	0.56	0.54	0.04	0.05*	0.08*	0.04*	0.09*	0.06*
As_563614	3.3	2.1	0.34	0.51	0.45	0.33	0.06*	0.10*	0.05*	0.11*	0.06*
As_2612077	5.8	4.0	0.31	0.75	0.75	0.58	0.05*	0.14*	0.03*	0.15*	0.12*
As_2631734	2.5	1.3	0.28	0.22	0.22	−0.26	0.09***	0.11***	0.10***	0.13***	0.03***
As_3458593	2.8	1.9	0.50	0.47	0.41	−0.06	0.08**	0.14**	0.08**	0.15**	0.08**
As_3602348	4.0	2.5	0.21	0.59	0.60	0.65	0.04	0.04	0.02	0.04	0.03
As_154813	5.8	3.1	0.66	0.67	0.66	0.01	0.04*	0.10*	0.03*	0.10*	0.07*
As_2280886	8.5	4.7	0.78	0.78	0.80	0.00	0.05***	0.22***	0.05***	0.23***	0.19***
As_420232	1.8	1.3	0.21	0.20	0.20	−0.03	0.07**	0.08**	0.08**	0.10**	0.02**
As_878430	3.8	2.7	0.46	0.62	0.65	0.26	0.03	0.03	0.01	0.03	0.02
As_1089795	5.5	3.0	0.41	0.67	0.68	0.38	0.01	−0.06	−0.02	−0.06	−0.04
As_1970306	2.3	2.0	0.60	0.50	0.38	−0.22	0.01	−0.01	−0.01	−0.01	−0.01
Mean	4.5	2.8	0.46	0.56	0.54	0.17	0.05***	0.07***	0.03***	0.08***	0.04***
Population	**N_a ± SE**		**N_e ± SE**		**H_o ± SE**			**H_e ± SE**		**F_{IS} ± SE**	
A	4.3 ± 0.6		2.7 ± 0.3		0.45 ± 0.07			0.56 ± 0.05		0.16 ± 0.10	
B	4.8 ± 0.9		3.0 ± 0.4		0.43 ± 0.06			0.58 ± 0.05		0.25 ± 0.10	
C	4.9 ± 0.7		2.8 ± 0.4		0.44 ± 0.07			0.57 ± 0.06		0.19 ± 0.09	
D	3.9 ± 0.6		2.7 ± 0.4		0.44 ± 0.06			0.53 ± 0.06		0.14 ± 0.10	
Mean	4.5 ± 0.3		2.8 ± 0.2		0.44 ± 0.03			0.56 ± 0.03		0.18 ± 0.05	
*Abies nordmanniana**	3.5 ± 0.7		2.4 ± 0.4		0.43 ± 0.11			0.40 ± 0.09		−0.10 ± 0.11	

*Based on 13 loci (*As_3602348* with no amplification was excluded).

on samples from eight natural populations of *A. sibirica* and four populations of *A. alba*, and preliminary data on the level of population genetic variation and differentiation were obtained. These markers can potentially be used also in other species in genus *Abies* with the ability to use simple gel electrophoresis, which is very convenient in field research. For genetic laboratories equipped with devices for capillary gel electrophoresis, we have developed multiplex panels of 14 loci. It should be noted that only eight tree samples were studied for *A. nordmanniana*; therefore, the data for this species are preliminary, and it is necessary to significantly increase the number of samples and accessions for more accurate estimates of the genetic variability of this species.

The proposed list of nuclear microsatellite loci will be very useful for studying the variability of natural and artificial populations of different fir species helping to address different problems and questions related to conservation, restoration and reproduction of fir forests.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S1479262123000552.

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