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Use of nuclear microsatellite loci for evaluating genetic diversity among selected populations of *Abies alba* Mill. in the Czech Republic

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ABSTRACT: The simple sequence repeats (SSR) method of DNA analysis was used to determine genetic diversity within and among silver fir (*Abies alba* Mill.) populations. Total genomic DNA was extracted from 250 *Abies alba* adult individuals collected from 8 important populations growing in different parts of the Czech Republic. Samples were screened using eight selected polymorphic nuclear microsatellite markers. PCR products were separated by capillary electrophoresis. Identified genetic loci were verified as reliable and polymorphic and were used for comparative genetic analyses of silver fir populations. The genotypic data from the adult trees implies genetic differences among the studied populations. The populations' genetic diversity expressed as Shannon's information index values was highest in the JD06 population from Hochwald. The lowest genetic diversity was in the JD02 population from Velké Polčané, Morávka. The longest genetic distance appeared between the JD02 (Velké Polčané, Morávka) and JD07 (Hojsova Stráž) populations. The closest genetic distance was between the JD01 (Babín) and JD03 (Vodslivy) populations.

Keywords: simple sequence repeats; silver fir populations; genetic variability and differentiation; genetic distance; gene reserves; DNA analysis

Abies alba Mill. is a coniferous species belonging to the family Pinaceae. It plays a very important ecological role as a stabilizing element in the forest ecosystem. This species is located mainly in the mountain forests of southern and central Europe. It is generally associated with beech (*Fagus sylvatica* L.) at lower and middle altitudes and with spruce (*Picea abies* L. Karst) at high altitudes (Liepelt et al. 2009). Silver fir is a species native to the Czech Republic, where it grows in all marginal and inland mountains. From the foothills and mountainous areas, this species also descends to the uplands regions, but it never extends beyond the upper edge of the forest and seldom exceeds an altitude of 1,100 m a.s.l. (Úradníček et al. 2009). Over the past two centuries, however, trees of this species have decreased in number, and especially from the forests of the middle parts of the northern region of its occurrence. A catastrophic decline occurred as a consequence of environmental stress factors and silvicultural preferences for other conifers, mostly Norway spruce, in the second half of the 20th century. Diminishing silver fir brings not only environmental losses but also economic losses, because fir is one of the most productive European forestry species (Musil, Hamerník 2007). The hypothesis of Larsen (1986) about *Abies alba* decline is insufficient intrapopulation genetic variability resulting in a decrease of the adaptation ability of this species compared with other forest species. *Abies alba* is more sensitive to changes in temperature, water availability and air pollution (Postolache et al. 2013).

In order to reintroduce this species in larger proportions, it is important to acquire more detailed knowledge about the dynamics of genetic diversity

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within and among silver fir populations. For silver fir, several genetic surveys have been conducted using terpenes, isozymes, mitochondrial DNA markers, chloroplast and nuclear microsatellites (simple sequence repeats) (Konnert, Bergmann 1995; Sagnard et al. 2002; Cremer et al. 2006; Liepelt et al. 2009; Piovani et al. 2010; Gömöry et al. 2012; Postolache et al. 2013). Microsatellite markers are commonly used in population genetic studies for analyses of gene flow, parentage analyses, and studies on genetic diversity (Pfeiffer et al. 1997). Inasmuch as nuclear microsatellites are highly polymorphic, selectively neutral and codominant markers, they are best suited for the analysis of small-scale genetic diversity (Cremer et al. 2006). The aim of this study was to provide an initial insight into the levels of genetic diversity and differentiation of *Abies alba* populations in the Czech Republic using selected nuclear microsatellite loci.

MATERIAL AND METHODS

Needle samples were collected in spring from eight indigenous populations growing in the Czech Republic. The sample sites were selected to cover the areas of important *Abies alba* populations in the Czech Republic. The populations are designated as JD01 from Babín in Natural Forest Area 16 (Bohemian-Moravian Highlands), JD02 from Velké Polčané, Morávka in Natural Forest Area 40 (Moravian-Silesian Beskids), JD03 from Vodslivy in Natural Forest Area 10 (Central Bohemian Hills), JD04 from Kraslice and Klášterec in Natural Forest Area 1 (Krušné hory Mts.), JD05 from Vsetín in Natural Forest Area 41 (Hostýn-Vsetín Uplands and Javorníky Mountains), JD06 from Hochwald in Natural Forest Area 28 (Hrubý Jeseník Mountains), JD07 from Hojsova Stráž in Natural Forest Area 13 (Bohemian Forest), and JD08 from Kraví hora, Boubín in Natural Forest Area 14 (Nové Hrady Mountains). These populations are growing in gene reserves (gene conservation units) with the exception of the JD04 population, the samples of which were collected from residual stands. The minimum distance between two sampled individuals was 100 m. Locations of the populations are illustrated in Fig. 1 and geographical coordinates are recorded in Table 1.

Total genomic DNA was extracted using a DNeasy Plant Mini Kit (QIAGEN, Venlo, Netherlands) from 20 mg of dry needles collected from 250 *Abies alba* adult individuals. DNA concentration was measured spectrophotometrically. The SSR method is based on polymerase chain reaction (PCR) with specific primers. PCR was optimized with the tested primers, whose oligonucleotide sequences had been developed by Hansen et al. (2005) and Cremer et al. (2006) (Table 2). Eight nuclear microsatellite markers were chosen to assess genetic diversity of the *Abies alba* populations, and they were assembled

Fig. 1. Locations of the populations

Table 1. The geographic coordinates of *Abies alba* populations

Populations	Geographic coordinates	
	N	E
JD01 – Babín	49.54373–49.5476	15.88387–15.8937
JD02 – Velké Polčané, Morávka	49.51575–49.53122	18.56762–18.60737
JD03 – Vodslivy	49.84817–49.86392	14.83783–14.86120
JD04 – Kraslice and Klášterec	50.34653–50.44971	12.93909–13.26551
JD05 – Vsetín	49.26001–49.72778	18.1302–18.18492
JD06 – Hochwald	49.93938–49.94438	17.21695–17.23575
JD07 – Hojsova Stráž	49.1728–49.18003	13.19633–13.2228
JD08 – Kraví hora, Boubín	48.70062–48.73422	14.68562–14.75827

into two multiplexes (A and B) with specific primers labelled fluorescently (FAM, VIC, NED). Amplification reaction conditions for each sample were in a final volume of 15 µl containing 1 µl of template DNA (≈ 20 ng/µl), 1.5 µl of 10 × PCR buffer (minus Mg), 0.2 mM of each dNTP (Takara Bio), 2 mM $MgCl_2$, 0.37 U of Platinum® Taq DNA polymerase (Invitrogen by Life Technologies), and primer combinations of the forward and reverse primers in two multiplex reactions. The primers and their concentrations in multiplex A were SF b4 (4 µM), NFH3 (2 µM), NFF3 (1 µM), and SF g6 (1 µM). In multiplex B, these were SF 78 (1 µM), SF 1 (1 µM), NFH15 (2 µM), and NFF7 (0.5 µM). The reaction mixtures were supplemented with sterile water for molecular biology (Sigma-Aldrich, St. Louis, USA). The PCR profile was as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing temperature at 58°C for 30 s and extension at 72°C for 45 s, with a final extension step at 72°C for 20 min. Amplifications were carried out in a Veriti Thermal cycler (Applied Biosystems, Foster City, USA). PCR products were separated by capillary electrophoresis using the Applied Biosystems 3500 genetic analyser. PCR amplifications and sizing of amplification products were repeated twice or three times to confirm the fragment lengths. Alleles were sized using GeneMapper® 4.1 software (Applied Biosystems). Calculations of genetic diversity parameters were performed using the statistical programs GenAlEx 6.5 (Peakall, Smouse 2006, 2012) and CERVUS (Kalinowski et al. 2007).

RESULTS

The eight *Abies alba* populations were evaluated separately. Clear, reproducible PCR products were produced for all eight microsatellite loci. The primer sequences of the eight selected markers and their genetic diversity parameters estimated using GenAlEx 6.5 are recorded in Table 2. There were 151 differ-

Table 2. Characteristics of selected nuclear microsatellite loci across the eight investigated *Abies alba* populations

Locus	Primer sequence (5'–3')	PCR product size	N_a	I	H_o	H_e
SF b4	F: GCCTTTGCAACATAATTGG R: TCACAATTGTTATGTGTGTGG	147–191	19	1.869	0.922	0.792
NFH3	F: TTGCCATCAAATTAA AAATGCTT R: CATCATTCTCTCTATCCCCATCA	93–133	19	2.069	0.582	0.834
NFF3	F: CCAATGGGTTGTCAGAGTGTT R: GGCATTCGAGATTGCTTGAT	107–143	13	1.571	0.805	0.755
SF g6	F: GTAACAATAAAAGGAAGCTACG R: TGTGACACATTGGACACC	96–118	11	1.329	0.309	0.647
SF 78	F: CATTGTTGTCTTTGTTTCACA R: TGCACCGTTTTGTTTTTCC	153–260	48	2.627	0.846	0.902
SF 1	F: TTGACGTGATTAACAATCCA R: AAGAACGACACCATTCTCAC	201–222	5	0.723	0.473	0.451
NFH15	F: CGCCTCCCTCCATTACTTC R: TCGTCTAGAGAGGCGAAATTCT	82–132	17	2.028	0.835	0.826
NFF7	F: CCCAAACTGGAAGATTGGAC R: ATCGCCATCCATCATCAGA	121–159	19	2.033	0.761	0.816

N_a – number of different alleles, I – Shannon's information index, H_o – observed heterozygosity, H_e – expected heterozygosity

Table 3. Numbers of alleles in *Abies alba* populations at individual loci

Populations/loci	SF b4	NFH3	NFF3	SF g6	SF 78	SF 1	NFH15	NFF7
JD01	6	10	5	6	17	3	11	9
JD02	7	13	9	4	17	3	13	14
JD03	11	13	9	6	25	3	12	14
JD04	11	10	6	8	18	3	9	11
JD05	12	14	8	6	16	3	11	14
JD06	12	13	7	6	23	3	11	13
JD07	11	9	6	4	19	3	12	11
JD08	12	11	7	8	21	3	11	12

ent alleles detected at the 8 loci in the 250 silver firs, ranging between 5 and 48 alleles and with an average of 18.9 alleles per locus. Expected heterozygosity ranged from 0.451 to 0.902 with a mean value of 0.753. Shannon's information index calculated for allelic and genetic diversity ranged from 0.723 (at locus SF 1) to 2.627 (at locus SF 78). These values correspond to the highest and lowest number of different alleles at the same loci. Possible null alleles were detected using the CERVUS program. Null allele frequency estimates were at locus SF b4 (–0.0653), NFH3 (0.1991), NFF3 (–0.0319), SF g6 (0.3498), SF 78 (0.0447), SF 1 (0.0167), NFH15 (0.0081), NFF7 (0.0556). Micro-checker software (Oosterhout et al. 2004) for identifying and correcting genotyping errors in microsatellite data was used. It was found that there is no evidence for large allele dropout. In most cases there is also no evidence for scoring error due to stuttering but there are some exceptions. In populations JD01, JD03, JD04 they were discovered at loci SF g6 and NFH3 that stuttering might have resulted in scoring errors, as is indicated by the highly significant shortage of heterozygote genotypes with alleles of one repeat unit difference.

The numbers of alleles in the eight investigated *Abies alba* populations at individual loci are recorded in Table 3. The highest number of alleles (25) was found in the JD03 population at locus SF 78. This is probably a consequence of this population's having the largest sample size.

Additional genetic characteristics for each population are shown in Table 4. The genetic diversity expressed as Shannon's information index values was highest in the JD06 population from Hochwald (1.908), even though this population's sample size was smaller (36 individuals) than was that of the JD03 population from Vodslivy (59 individuals). The lowest genetic diversity was 1.629 in the JD02 population from Velké Polčané, Morávka (also with 36 examined samples).

Estimates of differentiation among populations are presented in Table 5, which displays a pairwise comparison of populations. F_{ST} values ranged from 0.015 to 0.036.

Genetic distances among populations were calculated based on Nei's standard genetic distance (Nei 1972). Pairwise genetic distance coefficients for each pair of populations are shown in Table 6. The longest genetic distance (0.232) appeared between the JD02 (Velké Polčané, Morávka) and JD07 (Hojsova Stráž) populations. The closest genetic distance (0.091) was between the JD01 (Babín) and

Table 4. Mean values for genetic characteristics of the eight investigated *Abies alba* populations from eight selected nuclear microsatellite loci

Characteristic	JD01	JD02	JD03	JD04	JD05	JD06	JD07	JD08
N	20	36	59	18	29	36	24	28
N_a	8.375	10.000	11.625	9.500	10.500	11.000	9.375	10.625
N_e	4.672	4.315	5.017	6.015	5.024	6.378	5.783	5.800
I	1.634	1.629	1.742	1.842	1.837	1.908	1.808	1.849
Private alleles	0.250	1.250	0.500	0.375	0.625	0.250	0.250	0.875
H_o	0.675	0738	0.686	0.694	0.659	0.708	0.719	0.652
H_e	0.726	0.718	0.733	0.770	0.767	0.782	0.763	0.764
uH_e	0.745	0.729	0.740	0.792	0.781	0.793	0.779	0.778

N – sample size, N_a – number of different alleles, N_e – number of effective alleles, I – Shannon's information index, Private alleles – number of private alleles, H_o – observed heterozygosity, H_e – expected heterozygosity, uH_e – unbiased expected heterozygosity

Table 5. Pairwise population FST values determining genetic differentiation among populations

	JD01	JD02	JD03	JD04	JD05	JD06	JD07	JD08
JD01	0							
JD02	0.025	0						
JD03	0.015	0.020	0					
JD04	0.027	0.027	0.018	0				
JD05	0.029	0.018	0.028	0.023	0			
JD06	0.019	0.024	0.016	0.016	0.019	0		
JD07	0.024	0.036	0.019	0.019	0.029	0.016	0	
JD08	0.022	0.032	0.022	0.013	0.021	0.016	0.015	0

JD03 (Vodslivy) populations. Nei's standard genetic distances are graphically illustrated in Fig. 2. The dendrogram was produced by the UPGMA clustering technique using the PHYLIP 3.696 software (Felsenstein 2005). Generated clusters (identified genetic distance) correspond with the geographical location of individual populations.

Polymorphic information content (PIC) of selected microsatellite loci was obtained using CERVUS. Table 7 presents the resulting PIC values and numbers of heterozygotes and homozygotes. All observed loci were highly polymorphic with the exception of SF 1, which had a much lower PIC value than did the others. Markers were classified as informative when PIC was ≥ 0.5 (Sharma et al. 2010). This

Table 6. Results of the pairwise population matrix of Nei's genetic distance analysis

	JD01	JD02	JD03	JD04	JD05	JD06	JD07	JD08
JD01	0							
JD02	0.136	0						
JD03	0.091	0.113	0					
JD04	0.186	0.172	0.122	0				
JD05	0.180	0.112	0.192	0.171	0			
JD06	0.127	0.136	0.099	0.121	0.133	0		
JD07	0.164	0.232	0.122	0.142	0.202	0.123	0	
JD08	0.152	0.193	0.150	0.097	0.130	0.121	0.110	0

Table 7. Polymorphic information content (PIC) of the investigated microsatellite loci from all studied *Abies alba* samples and numbers of heterozygotes and homozygotes

Locus	PIC	No. of	
		heterozygotes	homozygotes
SF b4	0.814	231	19
NFH3	0.847	145	105
NFF3	0.729	202	48
SF g6	0.638	80	170
SF 78	0.927	213	37
SF 1	0.385	115	135
NFH15	0.842	210	39
NFF7	0.835	189	60

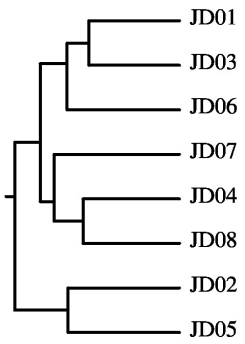

Fig. 2. UPGMA dendrogram based on Nei's genetic distance between *Abies alba* populations

assessment revealed there to be excesses of homozygotes at the SF g6 and SF 1 loci. This divergence is most likely due to the presence of null alleles.

DISCUSSION

Genetic variation in *Abies alba* Mill. has been investigated using various DNA markers: for example chloroplast microsatellites (Vendramin et al. 1999; Parducci et al. 2001), the mitochondrial *nad5-4* locus (Gömöry et al. 2012), and nuclear microsatellites (Hansen et al. 2005; Cremer et al. 2006, 2012).

For comparing the genetic characteristics of eight important *Abies alba* populations in the Czech Republic, we first tested nuclear microsatellite markers reported previously (Hansen et al. 2005; Cremer et al. 2006) and then selected for our samples eight SSR loci giving interpretable polymorphic patterns. Although the markers NFH3, NFF3, NFH15 and NFF7 had been developed initially for *Abies nordmanniana* (Stev.) Spach (Hansen et al. 2005), the possibility of transferring SSRs has been tested for related species of the genus *Abie*s (including *Abies alba*) because the development of microsatellite markers is a costly and time-consuming process. The earlier authors had reported values of expected (H_e) and observed (H_o) heterozygosity of geographically distant *Abies nordmanniana* populations from Georgia, Russia and Turkey for selected markers, as follows: NFH3 (H_o – 0.837, H_e – 0.961), NFF3 (H_o – 0.813, H_e – 0.884), NFH15 (H_o – 0.731, H_e – 0.822), and NFF7 (H_o – 0.455, H_e – 0.950). In comparing these heterozygosity values with our results from eight Czech *Abies alba* populations, we realized that there were no major differences. Only the locus NFF7 gave a much higher value (0.761) of observed heterozygosity. The markers SF b4, SF g6, SF 78, and SF 1 were selected from 14 loci developed for *Abies alba* by Cremer et al. (2006) after

PCR testing with our silver fir samples. Cremer et al. (2006) had checked the variability of these loci among *Abies alba* populations in Bulgaria, France, Germany and Switzerland. Their values of expected and observed heterozygosity were as follows: SF 1 (H_o – 0.333, H_e – 0.598), SF b4 (H_o – 0.294, H_e – 0.599), SF g6 (H_o – 0.136, H_e – 0.129), and (SF 78 H_o – 0.682, H_e – 0.883). Comparing these values with our results, we found much higher values for locus SF b4 (H_o – 0.922, H_e – 0.792), in which the heterozygotes numbered 231 and homozygotes 19. Another greater difference was found for locus SF g6, and particularly in expected heterozygosity.

Woo et al. (2008) had investigated genetic variation using ISSR markers in 248 individuals of *Abies nephrolepis* Max. representing eight natural populations in Korea. They had a similar number of samples and the same number of populations as in our work. Genetic distances among Korean populations calculated based on Nei's genetic distance were smaller (average 0.027) than were distances among the eight Czech populations, which displayed genetic distances ranging from 0.091 to 0.232. Shannon's diversity index for the Korean populations also ranged within lower values from 0.336 to 0.396. This might be due to the lower level of polymorphism for ISSR markers, which have a dominant character. The statistics for ISSR data can underestimate the genetic variation, because ISSR does not allow the recognition of heterozygotes. The small mean value of genetic distances reveals that eight Korean populations are closely related and there is a free gene exchange among them (Woo et al. 2008).

Cremer et al. (2012) reported genetic diversity parameters similar to our results at nuclear microsatellite loci SF 1 and SF 78 of *Abies alba* populations from south-western Germany. Only allelic richness at locus SF 78 was higher in the Czech populations.

In the present work, we demonstrated the feasibility of using nuclear microsatellite markers to study genetic diversity and differentiation among *Abies alba* populations. Genotyping data from 250 *Abies alba* adult trees within eight important populations located in different parts of the Czech Republic imply genetic differences among the studied populations. Based on these results, it is justified to follow the principles of zoning in the transmission of silver fir reproductive material. The current opinion about the history of silver fir during the last glacial and postglacial periods is that the sources of recolonization were located in the southern Balkans (Greece), north-western Balkans (Croatia, Bosnia) and Apennines (Gömöry et al. 2012). The natural distribution area of silver fir is located mainly in Europe's mountainous regions, ranging north to south from Poland to the northern border of Greece and west to east from the western Alps to Romania and Bulgaria (Wolf 2003). Although variation at nuclear loci is generally much more continuous (Gömöry et al. 2012), our genetic distance results graphically illustrated in Fig. 2 show closer distribution of the JD04, JD07 and JD08 populations from south-western Czech Republic (Bohemian Massif) and the JD02 and JD05 populations from eastern Moravia growing in the Carpathian region. This could be influenced by distribution of refugia during the last glaciation and the subsequent recolonization processes.

The initial insight into the levels of genetic diversity and differentiation of *Abies alba* populations in the Czech Republic using selected nuclear microsatellite loci implies genetic differences among the studied populations. Using these markers, we compared the dynamics of genetic diversity in populations of silver fir which are localized in a relatively small area of the Czech Republic. Acquired knowledge can contribute to reforestation management strategies of this species.

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