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PRIMER NOTE

Identification and characterization of nuclear microsatellite loci in *Abies alba* Mill.

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Abstract

Eleven polymorphic nuclear microsatellite markers for *Abies alba* Mill. were developed from an enriched genomic library. An average of 5.2 alleles per locus and a mean expected heterozygosity of 0.532 were found in a sample of 24 *Abies alba* individuals from different populations within Europe. These loci can be used in studies of genetic diversity for parentage analysis and for estimation of gene flow in silver fir populations. Moreover, successful amplifications were obtained for eight other Mediterranean *Abies* species, suggesting that these loci may be useful for similar applications in other fir species.

Keywords: *Abies*, cross-species amplification, SSR

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Abies alba Mill., a conifer species belonging to the family of Pinaceae, is a key species of the mountainous forest ecosystems in Europe. In Central Europe, a decline of *A. alba* has occurred as a consequence of environmental stress factors and silvicultural preferences for other conifers, mostly Norway spruce. Due to its important ecological role as a 'stabilizing' tree species, a reintroduction of larger proportions of silver fir into the mountainous forests has been promoted by forest managements. For this, it is necessary to have a more detailed knowledge about the dynamics of genetic diversity within and among silver fir populations. Population genetic studies so far relied on isozyme gene markers (e.g. Hussendörfer *et al*. 1995) and chloroplast microsatellite markers (e.g. Ziegenhagen *et al*. 1998; Vendramin *et al*. 1999). Nuclear microsatellites as highly polymorphic, selectively neutral and codominant markers are best suited for the analysis of small-scale genetic diversity. So far, they were not available for silver fir. Here, we present a set of novel nuclear microsatellite markers developed in *A. alba* and demonstrate the transferability to other *Abies* species.

An enriched genomic library for di- (GA, GT, AT, GC), tri- (CAA, ATT, GCC) and tetranucleotide (GATA, CATA, ATAG) was constructed and screened for microsatellite sequences, following the protocol of Edwards *et al*. (1996). The plasmids containing the microsatellite candidates were screened by a polymerase chain reaction (PCR). DNA fragments larger than 250 bp were sequenced with an Amersham MegaBACE 1000 automated sequencer using the DYEnamic ET Terminator Sequencing Kit (Amersham Biosciences). For sequencing, primers flanking the polycloning site of the pBlueScriptII vector (Stratagene) were designed external to the M13 universal primers.

A total of about 170 clones were sequenced. More than 90% of the clones contained the microsatellite stretches; however, it was possible to design primers, using PRIMER 3 software (Rozen & Skaletsky 2000), only in 44 cases (about 30%) because in several cases the stretches were too close to the end of the clone and/or too long. Fourteen primers (about 32%) yielded distinct PCR products of the expected size.

The 14 loci were checked for variability analysing needles from 17 to 24 *A. alba* individuals collected in Bulgaria, France, Germany and Switzerland. Total DNA was extracted as described by Dumolin *et al*. (1995). PCRs were performed in a 25 μL reaction volume containing 30 ng of DNA, 1× PCR buffer (Promega), 2 μM of each primer (forward primer fluorescence labelled), 5 mM of each dNTP, 2.5 mM $MgCl_2$ (A) or 1.5 mM $MgCl_2$ (B), 1 U Go *Taq* polymerase (Promega) and 0.8% BSA. The amplification was carried

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Table 1 Characteristics of 14 nuclear microsatellite loci developed for *Abies alba*

Locus name (Accession no.)	Primer sequence (5′–3′)	Repeat motif	PCR product size (bp)	PCR profile	*N*	*A*	H_O	H_E
SF 1 (DQ218453)	F: HEX-TTGACGTGATTAACAATCCA R: AAGAACGACACCATTCTCAC	$(CCG)_9$	221–226	A	24	3	0.333	0.598*
SF b4 (DQ218454)	F: FAM-GCCTTTGCAACATAATTGG R: TCACAATTGTTATGTGTGTGG	$(GT)_{16}$	166–186	A	17	5	0.294	0.599**
SF b5 (DQ218455)	F: FAM-AAAAAGCATCACTTTTCTCG R: AAGAGGAGGGGAGTTACAAG	$(CT)_{15}$	143–155	A	24	5	0.625	0.593n.s.
SF g6 (DQ218456)	F: FAM-GTAACAATAAAAGGAAGCTACG R: TGTGACACATTGGACACC	$(AC)_9$	107–113	A	22	3	0.136	0.129n.s.
SF 50 (DQ218457)	F: FAM-CATTTGGTGCGGTTCATTTC R: AGTGGCATTTCACTTATTGG	$(GT)_{11}(GC)_{10}$	96–102	B	23	4	0.087	0.475***
SF 78 (DQ218458)	F: FAM-CATTGTTGTCTTTGTTTCACA R: TGCACCGTTTTGTTTTTCC	$(CGCA)_8(CA)_{15G\backslash CA}(_)_8$	158–244	B	22	15	0.682	0.883***
SF 83 (DQ218459)	F: FAM-AGCAGCATAACCAAGGGTCAA R: TCTGAATTTCTAAAGGCGGC	$(CTT)_3\ldots(GCC)_5$	197–206	A	24	2	0.042	0.041n.s.
SF 239 (DQ218460)	F: HEX-GCTCTGTGCACTGCCTGT R: TTCGGAGACTAACGCATCTCA	$(TG)_{11}$	108–122	B	21	6	0.286	0.642**
SF 324 (DQ218461)	F: FAM-TTTGAACGGAAATCAAATTCC R: AAGAACGACACCATTCTCAC	$(CCG)_8$	110–116	A	24	3	0.333	0.348n.s
SF 331 (DQ218462)	F: FAM-TGTAATGCTTTTCATGGGCAA R: TTACATGGGAAAACCATCCA	$(GT)_{11}$	106–116	C†	19	5	0.211	0.747***
SF 333 (DQ218463)	F: FAM-ATTTGTTCATTTTGGTCCTG R: ACACAGGAAAAAGTCGGTAA	$(CA)_{12}(TA)_4$	168–178	A	23	6	0.391	0.792**
SF 2 (DQ218464)	F: FAM-TGTTGGATTTATGGTTACCTC R: GACAAAACCCTTTTGCAAAAC	$(CA)_4GA(CA)_4GA(CA)_3$	166	A	24	1	—	—
SF 8 (DQ218465)	F: FAM-TACAGCAGCCTGTAGGTATG R: GAGTGGTCGATACACACAAA	$(GCC)_6$	132	A	24	1	—	—
SF g36 (DQ218466)	F: FAM-CACAAGAAAAAGCTGGTAAA R: TAGGAGTTTGGGACTTCAGA	$(TG)_9(CG)_6\ldots(GT)_5$	99	A	19	1	—	—

†C: 4 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 50 °C, 30 s at 72 °C and a final extension at 72 °C for 7 min.
N, sample size; *A*, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.
*, ** and ***: significant departure from Hardy–Weinberg equilibrium at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. n.s. = not significant.

out in a thermal cycler (T1, Biometra) with following conditions: 5 min at 94 °C followed by 10 touchdown cycles of 30 s at 94 °C, 30 s at 60 °C (A) or at 65 °C (B) (1 °C lower per cycle), 40 s at 72 °C and 25 cycles of 30 s at 94 °C, 50 s at 50 °C (A) or 55 °C (B), 40 s at 72 °C with a final extension time of 7 min at 72 °C. The PCR products were separated by capillary electrophoresis using the Amersham MegaBACE automated sequencer (Amersham Biosciences). Alleles were sized using the size standard MegaBACE ET400-R (Amersham Biosciences) and the MEGABACE FRAGMENT PROFILER version 1.2 (Amersham Biosciences).

Genetic diversity parameters were estimated using GENALEX version 6 (Peakall & Smouse 2005). Eleven loci were polymorphic, and three loci were monomorphic (Table 1). For the polymorphic loci, between two and 15 alleles were detected with an average of 5.2 alleles per locus. The expected heterozygosity ranged from 0.041 to 0.883 with a mean value of 0.532. Significant deviation from expected heterozygote frequencies at Hardy–Weinberg equilibrium was observed for SF 1 ($P < 0.05$), SF b4, SF 239, SF 333 ($P < 0.01$) and SF 50, SF 78, SF 331 ($P < 0.001$). These loci showed deficiencies of heterozygotes, which might be due to a sampling effect (small sample size, different population genetic structures (Wahlund effect), absence of natural out-crossing mating system). We cannot exclude the presence of null alleles, either. Those alleles, however, that could be checked in controlled crosses followed Mendelian segregation (data not shown). All loci were tested for linkage disequilibrium (LD) using the software GENEPOP (Raymond & Rousset 1995). This test did not reveal any significant cases of LD.

Cross-species amplification of the 14 primer pairs was tested in eight other Mediterranean *Abies* species with two to five individuals each (Table 2). One hundred and seven of 112 combinations of loci and species (96%) were able to amplify products. All amplification products obtained from the eight species ranged within the sizes of the respective *A. alba* microsatellite loci. Most of the loci were polymorphic,

Table 2 Transferability test to additional *Abies* species

Species	Locus name													
	SF 1	SF b4	SF b5	SF g6	SF 50	SF 78	SF 83	SF 239	SF 324	SF 331	SF 333	SF 2	SF 8	SF g36
Abies borisii regis	+ m	(+)	+	+	+	+	+	+	+	+	+	–	+	+
Abies bornmuelleriana	+ m	+	+	+	+	+	+	+ m	+	+	+	+	(+)	+
Abies cephalonica	+ m	–	+	+	+	+	+	+ m	+	+	+	+	+	+
Abies cilicica	+ m	+	+	+	+	+	+	+ m	+	+	+	+	(+)	+
Abies equitrojani	+ m	+	+	+	+	+	+	+ m	+	+	+	+	+	+
Abies marocana	+ m	+	+	+	+	+	+	+	+	–	+	+	–	+
Abies numidica	+ m	–	+	+	+	+	+	+ m	+	+	+	+	+	+
Abies pinsapo	+ m	+	+	+	+	+	+	+ m	+	+	+	+	(+)	+

+, PCR amplification; –, no PCR amplification; m, multibanding pattern; (+), weak amplification.

suggesting that these markers are also useful for similar applications in other *Abies* species.

Overall, these results illustrate the utility of the newly developed microsatellite loci for assessing spatial patterns of genetic diversity for parentage analysis, for gene flow studies and for individual identification in *A. alba* populations. Using the newly developed markers in ongoing studies, we are comparing the dynamics of genetic diversity in silver fir between closed stands and large-sized forest gaps.

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