PRIMER NOTE

# Development of microsatellite markers in *Abies nordmanniana* (Stev.) Spach and cross-species amplification in the *Abies* genus

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## Abstract

***Abies nordmanniana*** **(Stev.) Spach is a widely grown Christmas tree in Denmark, from where it is exported to most of Europe. An ongoing breeding programme is taking place, and as a tool for that, we report the development of five polymorphic nuclear microsatellite markers. To investigate the potential for transferring the markers to other species in the *Abies* genus, polymerase chain reaction amplification was tested in 19 other species. In general, amplification occurred in a very high proportion of the tested species.**

*Keywords*: *Abies nordmanniana*, inheritance, microsatellites, transferability

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*Abies nordmanniana* is a species with a limited distributional range in the Caucasian region, including Georgia, the southern part of Russia and the northeastern parts of Turkey (Liu 1971). Until recently, the species only had an ecological value, but now, it is widely grown for use as a high-value Christmas tree in many parts of Western Europe. A breeding programme was initiated in Denmark in the early 1990s (Nielsen 1993), and is still proceeding. To manage seed sources as well as to assist breeding activities and monitor gene flow, we decided to develop microsatellite markers, which are known to be highly polymorphic and informative molecular markers (Lefort *et al*. 1999).

Genomic DNA was extracted from current season needles of one individual standing in the Hørsholm Arboretum at the Royal Veterinary and Agricultural University, Denmark (Accesion no. 1983.0567). The DNeasy Plant Mini Kit from QIAGEN was used for DNA extractions.

An enriched library for dinucleotide repeats $(CA)_n$ and $(CT)_n$ was generated according to Edwards *et al*. (1996). The enriched DNA was cloned into pJV1 and transformed into DH10B cells. Sequencing was performed using either the MegaBACE DNA Analysis System from Amersham Biosciences or the CEQ 2000XL from Beckman Coulter.

Via the sequencing of several hundred clones, a total of 43 primer pairs were designed using the PRIMER 3 software (Rozen & Skaletsky 2000). Of the initial 43 pairs, 32 were resynthesized with WellRED dyes (Proligo Corp.). Fragment sizes of the amplified labelled simple sequence repeats (SSRs) were determined on the CEQ 2000XL Fragment analysis system. Following extensive testing, only five single loci SSRs were found to have easily interpretable polymorphic bands. The most common cause to abandon a primer pair was the occurrence of multibanding patterns.

Polymerase chain reaction (PCR) conditions for the five selected microsatellites were (final volume of 25 μL): 20 ng of DNA sample, 1× reaction buffer, 0.2 mM of each dNTPs (Proligo), 0.2 μM of each primer, 2.5 mM $MgCl_2$ and 1 U of *Taq* polymerase (TEMPase hotstart *Taq p*olymerase from Ampliqon®). Amplifications were carried out in PerkinElmer thermal cyclers (models 9700 and 2700) with the following thermal profile: initial 15 min of denaturation at 94 °C, then 35 cycles of denaturation at 94 °C for 1 min, annealing temperature (as in Table 1) for 1 min and extension at 72 °C for 1 min, with a final extension step at 72 °C for 8 min.

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**Table 1** Characterization of five *Abies nordmanniana* microsatellites

| Locus name (GenBank no.) | Primer sequence (5′–3′) F: forward primer R: reverse primer | Expected size (bp) | Anneal. temp. $T_a$ (°C) | Allele size range (bp) | $N$ | No. alleles | $H_O$ | $H_E$ | Null allele freq. | HWE test $P$ value | *Inheritance**: Mother genotype ($A_1$, $A_2$) No. of endosperm/genotypes |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NFF2 | F: GGGGTAGAGAGTTGGCTGCT | 113 | 58 | 105–148 | 160 | 31 | 0.669 | 0.906 | 0.149 | $P = 0.001$ | $A_1 = 113$ bp $A_2 = 119$ bp |
| (AY966493) | R: CATAAGGATGAGTGGCTTCCA | | | | | | | | | | 12/5 $A_1$: 7 $A_2$ ($P = 0.56$) |
| NFF3 | F: CCAATGGGTTGTCAGAGTGTT | 133 | 58 | 105–187 | 160 | 19 | 0.813 | 0.884 | 0.040 | $P = 0.698$ | $A_1 = 131$ bp $A_2 = 133$ bp |
| (AY966494) | R: GGCATTCGAGATTGCTTGAT | | | | | | | | | | 12/4 $A_1$: 8 $A_2$ ($P = 0.25$) |
| NFH15 | F: CGCCTCCCTCCATTACTTC | 113 | 58 | 98–138 | 160 | 15 | 0.731 | 0.822 | 0.059 | $P = 0.800$ | $A_1 = 101$ bp $A_2 = 113$ bp |
| (AY966492) | R: TCGTCTAGAGAGGCGAAATTCT | | | | | | | | | | 13/6 $A_1$: 7 $A_2$ ($P = 0.78$) |
| NFH3 | F: TTGCCATCAAATTAAAAATGCTT | 123 | 58 | 102–186 | 160 | 40 | 0.837 | 0.961 | 0.068 | $P = 0.222$ | $A_1 = 108$ bp $A_2 = 143$ bp |
| (AY966491) | R: CATCATTCTCTCTATCCCCATCA | | | | | | | | | | 14/6 $A_1$: 8 $A_2$ ($P = 0.59$) |
| NFF7 | F: CCCAAACTGGAAGATTGGAC | 165 | 57 | 107–173 | 145 | 47 | 0.455 | 0.950 | 0.352 | $P = 0.000$ | $A_1 = 147$ bp $A_2 = 151$ bp |
| (AY966495) | R: ATCGCCATCCATCATCAGA | | | | | | | | | | 14/9 $A_1$: 5 $A_2$ ($P = 0.29$) |

$N$ = number of individuals genotyped.
*First line shows the two alleles in a heterozygote mother individual in the seed orchard ($A_1$ and $A_2$). Second line shows the number of haploid megagametophytes (= endosperms) genotyped and how the two possible alleles were distributed. Finally comes the $P$ value for a $\chi^2$ goodness-of-fit test, assuming a 1 : 1 segregation.

The characteristics and polymorphism of the microsatellite loci were investigated using 40 individuals from each of four different populations, including two populations from the middle of the distribution area (Ambrolauri and Borshomi/Nedzvi in Georgia), one from the northern part (Zelenczuk/Arkhyz in Russia) and one from the southern part (Savsat/Yayla in Turkey).

Polymorphism, as well as null allele frequency estimates was assessed with CERVUS 2.0 (Marshall *et al*. 1998). Exact tests of Hardy–Weinberg proportions as well as tests for linkage disequilibrium (LD) within each of the four populations were done in GENEPOP (http://wbiomed.curtin.edu.au/genepop; Raymond & Rousset 1995). Mendelian inheritance was investigated by genotyping one individual in a seed orchard heterozygote for the SSRs and 12–15 megagametophytes derived from it. The megagametophytes in conifers are haploid and of maternal origin, and are therefore expected to have a 1 : 1 segregation assuming simple codominant Mendelian inheritance.

Number of alleles and expected heterozygosity range between 15 and 47, and 0.822 and 0.960, respectively. The estimated frequency of null alleles was higher than 5% for four of the five loci (Table 1). Results from exact test of Hardy–Weinberg proportions within the Ambrolauri population are reported in Table 1 (results were very alike for all four populations). Significant deviation from Hardy–Weinberg expectations ($P < 0.05$) was observed for NFF7 and NFF2. This deviation is most likely to be due to the presence of null alleles. Significant evidence ($P < 0.05$) of LD between loci was found between NFF2 and NFH3 as well as between NFF3 and NFH3 in one of the four populations (Zelenczuk/Arkhyz, Russia), otherwise not. No significant segregation distortion was observed at any loci.

The development and optimization of microsatellites is an expensive and time-consuming process, particularly in conifers, which are characterized by large and repetitive genomes (Echt *et al*. 1999). Therefore the possibility for transferring SSRs to related conifer species is of potential interest, particularly in the genus *Abies* where no SSRs are available so far. Accordingly, the SSRs were tested on 19 other *Abies* species, collected in the Hørsholm Arboretum. The species were chosen equally from three geographical centres of origin: Asia (6 species), the European/Mediterranean area (6 species) and North America (7 species). Cross-amplification was generally tested on two individuals from each species. The resulting fragment sizes, in case of successful amplification, are listed in Table 2.

These SSRs are currently being used to monitor gene flow in *A. nordmanniana* clonal seed orchards. In addition, they will be used in controlling the genetic uniformity of the grafted clones. Encouragingly for possible interspecies transfer of the developed SSRs, cross-species amplification was successful in most of the tested species, regardless of geographical centre of origin.

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**Table 2** Cross-species amplification of SSRs in some *Abies* species. Fragments are in bp

| | | | | NFF2 | | NFF3 | | NFH15 | | NFF7 | | NFH3 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | Country | ID no. | Origin | Amp. | Fragments (bp) | Amp. | Fragments (bp) | Amp. | Fragments (bp) | Amp. | Fragments (bp) | Amp. | Fragments (bp) |
| *A. firma* | Japan | A.fir-1 | A | X | 116 | X | 105 | X | 102 | X | 115 | X | 106 |
| *A. firma* | Japan | A.fir-2 | A | X | 109,116 | X | 107 | X | 102,104 | 0 | | X | 106,116,121,133 |
| *A. holophylla* | South Korea | A.hol-1 | A | X | 109 | X | 107 | X | 97 | 0 | | 0 | |
| *A. holophylla* | South Korea | A.hol-2 | A | X | 109 | X | 107,123 | X | 95,97 | 0 | | 0 | |
| *A. homolepis* | Japan | A.hom-1 | A | X | 109 | X | 105 | X | 93 | X | 144 | X | 116,142 |
| *A. homolepis* | Japan | A.hom-2 | A | X | 108 | X | 105 | X | 93,136 | X | 150 | X | 119,140 |
| *A. koreana* | South Korea | A.kor-1 | A | X | 113,116 | 0 | | 0 | | X | 137 | X | 92,116,135 |
| *A. koreana* | South Korea | A.kor-2 | A | X | 109 | 0 | | X | 104,116 | X | 137,154 | X | 82,100 |
| *A. veitchii* | Japan | A.vei-1 | A | X | 111 | X | 127,129 | X | 103 | X | 143 | X | 137,151 |
| *A. veitchii* | Japan | A.vei-2 | A | X | 109 | X | 105,127 | X | 104 | X | 143,151 | X | 135 |
| *A. nephrolepis* | South Korea | A.nep-1 | A | 0 | | X | 107 | X | 114 | X | 145 | X | 114,142 |
| *A. nephrolepis* | China | A.nep-2 | A | 0 | | X | 105 | X | 114 | X | 143,161 | X | 108,120 |
| *A. alba* | Poland | A.alb-1 | M | X | 119 | X | 131,133 | X | 112,118 | X | 145,155 | X | 118,149 |
| *A. alba* | Poland | A.alb-2 | M | X | 107 | X | 133,135 | X | 112,121 | X | 141,155 | X | 116,143 |
| *A. bornmuelleriana* | Turkey | A.bor-1 | M | X | 111,140 | X | 137,141 | X | 97,101 | X | 121,147 | X | 171 |
| *A. cephalonica* | Greece | A.cep-1 | M | X | 135,162 | X | 124,133 | X | 94,120 | X | 133,149 | X | 112,145 |
| *A. cephalonica* | Greece | A.cep-2 | M | X | 119,125 | X | 135,143 | X | 118 | X | 143,153 | X | 128 |
| *A. cilicica* | Turkey | A.cic-1 | M | 0 | | X | 103,137 | X | 103,116 | X | 143,153 | X | 102,133 |
| *A. nebrodensis* | Italy | A.neb-1 | M | X | 108 | X | 133,139 | X | 124 | X | 131,133 | X | 120 |
| *A. nebrodensis* | Italy | A.neb-2 | M | X | 109 | X | 133 | X | 124 | X | 137 | X | 120,128 |
| *A. pinsapo* | Morocco | A.pin-1 | M | X | 105 | X | 149,175 | X | 104 | X | 139,157 | X | 120,128 |
| *A. pinsapo* | Morocco | A.pin-2 | M | X | 109 | 0 | | X | 86,104 | X | 139,151 | X | 116,142 |
| *A. balsamea* | USA | A.bal-1 | N | X | 107 | X | 121 | X | 116 | 0 | | X | 120,142 |
| *A. balsamea* | USA | A.bal-2 | N | X | 109 | X | 113,115 | X | 116,118 | 0 | | X | 114,130 |
| *A. concolor* | USA | A.con-1 | N | X | 109,117 | X | 111 | 0 | | X | 141 | X | 107 |
| *A. concolor* | USA | A.con-2 | N | X | 109,120 | X | 113 | 0 | | 0 | | X | 95 |
| *A. fraseri* | USA | A.fra-1 | N | X | 109 | X | 131,137 | X | 103 | 0 | | X | 114,122 |
| *A. fraseri* | USA | A.fra-2 | N | X | 110 | X | 129 | X | 103,116 | 0 | | X | 122,126 |
| *A. grandis* | Canada | A.gra-1 | N | X | 109 | X | 113 | X | 126 | X | 125,127 | X | 130,137 |
| *A. grandis* | Canada | A.gra-2 | N | X | 109 | X | 113,124 | X | 126 | X | 125,127 | X | 114,127 |
| *A. lasiocarpa* | USA | A.las-1 | N | 0 | | X | 111 | X | 108,128 | 0 | | X | 131,137 |
| *A. lasiocarpa* | USA | A.las-2 | N | 0 | | X | 103,111 | X | 112,130 | X | 135,139 | X | 129 |
| *A. magnifica* | USA | A.mag-1 | N | 0 | | X | 120,134 | X | 83,108 | X | 125,153 | X | 88,121 |
| *A. procera* | USA | A.pro-1 | N | X | 110 | X | 109,134 | X | 112 | X | 152,154 | X | 90,108 |
| *A. procera* | USA | A.pro-2 | N | X | 110 | X | 109,137 | 0 | | X | 153,165 | X | 108,116 |

Origin: A, Asia; M, European/Mediterranean area; N, North America; Amp, Amplification: X, PCR amplification; 0, No PCR amplification; bp, base pairs.

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