

Isolation and characterization of microsatellite markers in the tetraploid birch, *Betula pubescens* ssp. *tortuosa*

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Abstract

The mountain birch, *Betula pubescens* ssp. *tortuosa* is a tetraploid tree species which forms the tree limit in northern Fennoscandia. We identified nine polymorphic microsatellite loci in order to characterize the genetic structure of populations at different elevations close to the treeline. The microsatellite loci were highly polymorphic, with 14–42 alleles per locus and an average expected heterozygosity of 0.73 ± 0.25 under random chromosome segregation and 0.68 ± 0.23 under random chromatid segregation.

Keywords: autopolyploidy, genetic structure, microsatellite markers, mountain birch, nonradioactive probes

The tetraploid mountain birch *Betula pubescens* Ehrh. ssp. *tortuosa* (Ledeb.) Nyman is a monoecious, wind-pollinated low tree or shrub, which often shows a typical polycormic growth form. It is a pioneer species that can quickly colonize open areas and is well adapted to marginal habitats (Atkinson 1992). The species forms stable natural forests in the subarctic zone and defines the altitudinal and latitudinal treeline in Fennoscandia. Expansion of the range of the species has recently been observed in the Swedish mountains associated with an elevation of the treeline, most likely the result of human-induced climate warming (Kullman 2002). Genetic diversity is the basis of the ability of organisms to adapt to changes in their environment through natural selection. Woody species, like forest trees, tend to have higher genetic diversity compared to herbaceous species, with the consequence that they are usually able to respond more quickly to environmental changes (Hamrick & Godt 1996). Microsatellites are commonly known to be highly variable and are therefore a powerful tool for detecting genetic variation within species. We developed nine microsatellite markers in *B. pubescens* ssp. *tortuosa* in order to characterize the genetic structure and infer the

pattern of gene flow between populations at different elevations close to the treeline in northern Sweden.

Microsatellite regions were isolated following the classic approach of colony transfer on nylon membranes (Estoup & Martin 1996). DNA was extracted from dried buds collected in the Abisko area in northern Sweden, using the DNeasy plant mini-kit (Qiagen). DNA was digested with three restriction enzymes, *Hae*III, *Alu*I and *Rsa*I (Boehringer Mannheim) and size-selected fragments (400–1000 bp) were inserted into plasmid pUC18. Ultracompetent cells (Stratagene) were then transformed and the recombinant colonies were transferred on nylon membranes (Boehringer Mannheim) and subsequently hybridized with digoxigenin-labelled oligonucleotide probes — namely (AG)₁₂, (AC)₁₂, (CTC)₈ and (AAT)₈, obtained with the DIG oligonucleotide tailing kit (Roche Molecular). Twenty-two positive clones were detected out of 8000 recombinants using the DIG luminescent Detection Kit (Roche Molecular) and used as PCR (polymerase chain reaction) templates for amplification using M13 forward and reverse universal primers. The PCR products were then sequenced using the same primers on a ABI 377 Perkin Elmer automated sequencer following the manufacturer's protocol (Applied Biosystem). Seventeen sequences contained microsatellites (GenBank AY423607–623). These sequences were developed complementary to 33 microsatellites previously identified in *Betula pendula* (GenBank AF310845–877) by Kulju *et al.* (2004). Using the

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software OLIGO 6 (MBI), we designed primers for the sequences that contained suitable flanking regions and at least five repeats. We were able to design primers for 21 sequences, 15 out of 33 from *B. pendula* and six out of 17 from *B. pubescens*.

To characterize the microsatellite loci, DNA was extracted from dry leaves collected in the Abisko area, using the DNeasy plant mini-kit (Quiagen). PCR reactions were performed in a 10 μ L volume containing 1 ng of template DNA, 1 μ L buffer 10X (100 mM Tris-HCl, 500 mM KCl, 0.8% Nonidet P40, Fermentas), 1.25 mM MgCl₂, 4 μ g BSA, 0.075 mM of each dNTP, 0.4 μ M of each primer, the forward being 5' labelled with an infrared dye (IRD), and 0.4 U *Taq* polymerase (Fermentas). Multiplexing was achieved with 0.4 μ M of each primer in the same final volume of 10 μ M. Amplification was undertaken as follows: a first step at 94 °C for 3 min followed by 30 cycles, each consisting in 30 s denaturing at 94 °C, 30 s annealing at specific T_a and 45 s extension at 72 °C. The last cycle was ended by an extra 5 min at 72 °C to complete extension. PCR products were loaded on a 6% Long Ranger polyacrylamide gel, which was run for 3 h on a LI-COR DNA sequencer 4200 (LI-COR, Inc.). Isolated bands were scored according to the sizing standard IRD-infrared dye 50–350 (LI-COR, Inc.) in the software GENE IMAGIR 4.03 (Scanalytics). Because it is a tetraploid genome, one to four bands can be detected at each locus. Allelic dosage was resolved by estimating the number of alleles in a band according to the relative intensity peak of the band. Primers pairs that gave consistent, specific products were tested for polymorphism on 452 individuals, from three populations at different elevations close to the treeline.

Twelve microsatellites successfully amplified out of 21. Of these 12, one showed multiple stutter bands and two had a large number of null alleles, probably resulting from a mutation on one of the primer sites. The remaining nine loci were polymorphic and codominant (Table 1). We calculated heterozygosities considering the species as autopolyploid, using the software, AUTOTET (Thrall & Young 2000). The existence of multiple heterozygous states in polyploids makes the estimation of observed heterozygosity (H_O) from the proportion of full homozygotes excessively conservative, because this class is considerably less sensitive to inbreeding than it is in diploids (Bever & Felber 1992). We therefore used a more rigorous calculation of H_O , called path analysis, where the five possible classes of genotypes are being weighted inversely to the probability of any of their alleles being identical by descent. Furthermore, in autopolyploids, sister chromatids can sort in the same gamete resulting in double reduction, with the consequence to increase the production of homozygous gametes compared to what is expected under random mating (Bever & Felber 1992). Expected heterozygosity (H_E) was therefore computed separately under either random chromosome segregation (RC_eS) or random chromatid segregation (RC_dS) assuming maximum double reduction ($\alpha = 1/7$; Wricke & Weber 1986). Fixation indices F were calculated as $1 - (H_O/H_E)$. Departure from Hardy–Weinberg equilibrium (HWE) was tested with χ^2 goodness-of-fit tests for observed-to-expected genotype frequencies, under either RC_eS or RC_dS assuming maximum double reduction ($\alpha = 1/7$), using the software AUTOTET (Thrall & Young 2000). The χ^2 test is

Table 1 Microsatellites markers in *Betula pubescens* ssp. *tortuosa*

Locus	GenBank ref.	Repeat	Size (bp)	Primer sequence (5'–3')	T _a (°C)	MgCl ₂ (mM)	Allele number	H _O	H _E (C _e)	F (C _e)	H _E (C _d)	F (C _d)
L2.3	AF310847†	(AG) ₁₆	208–252	CGGGAAGATATGCACTGTTT TTGGCGGGTGAAGTAGAC	58	1.25	18 (6)	0.15	0.25*	0.42	0.24*	0.37
L2.5	AF310848†	(CT) ₉	94–128	CTATATTGGCTCCAAGCAC ACACCCCACTGACAGATAA	55‡	1.25	17 (10)	0.63	0.70*	0.11	0.66*	0.04
L3.1	AF310851†	(CT) ₁₃ A(TC) ₆	134–166	CACACTGCTGCCTGA TCATAAAACCCCTCAAAGAAT	48	1.25	14 (10)	0.38	0.39	0.02	0.36	–0.05
L1.10	AF310856†	(AG) ₄ AA (AG) ₁₁	152–206	TTTCCAACGCTTCTTGATG TGGATAAGGAAGGCATGTC	55‡	1.25	28 (19)	0.89	0.93	0.04	0.87	–0.02
L5.4	AF310862†	(TC) ₂₆	134–188	GAAAGCATGAGACCCGCTCTT AACCTAAACAGCCTGCCAAA	50	1.25	27 16	0.90	0.91	0.00	0.84	–0.07
L021	AF310877†	(CT) ₁₄	168–236	TCTACGCTGTGACCAGTC AGAATCCTAGCCTTTTCAAT	55	1.25	42 17	0.64	0.78*	0.18	0.73*	0.12
Bo.F394	AY423608	(TC) ₁₃	128–194	AATGCAGCATCTCTTACC CACGCAATAATATGGAAA	48	1.25	30 16	0.78	0.86*	0.10	0.81	0.03
Bo.F330	AY423611	(TC) ₁₄	172–210	TGGCAGCACGAAAGT TGGGAATGAGAGAACAAG	48	1.25	38 (30)	0.85	0.95	0.11	0.89	0.05
Bo.G182	AY423617	(TC) ₁₆ (AC) ₅	120–160	TGTGTGGCTCCTTATCTCTTA GGCAACAAATATGAGGTAG	45	1.875	23 (16)	0.78	0.80	0.02	0.75	–0.05
Mean \pm S.D.							26.3 \pm 9.3	0.67 \pm 0.25	0.73 \pm 0.25	0.09 \pm 0.05	0.68 \pm 0.23	0.02 \pm 0.05

†Sequences isolated by Kulju *et al.* (2004); ‡Amplification was carried out by multiplexing L2.5 and L1.10. †Values in parentheses are remaining number of alleles after pooling the rare alleles (frequency < 0.01) into a single class. T_a, annealing temperature of the primer pair; H_O, observed heterozygosity; H_E, expected heterozygosity; F, fixation index; C_e, chromosome segregation; C_d, chromatid segregation; For H_E (C_e) and H_E (C_d), significance for deviation from Hardy–Weinberg equilibrium (χ^2 goodness-of-fit test): *P < 0.05.

known to give suspect results when expected frequencies of some genotypic classes are low. Consequently, we pooled into a same class all alleles except the most common one, according to Swofford & Selander (1989).

The three populations were not significantly differentiated. Overall, the nine microsatellite loci were highly polymorphic, with 14–42 alleles and a mean H_E of 0.73 ± 0.25 under RC_eS and 0.68 ± 0.23 under RC_dS (Table 1). They showed a large number of rare alleles, with an average of 40.8% of alleles with a frequency $< 1\%$. These results are concordant with the relative conservancy of the tetrasomic genome, in which rare alleles are eliminated much more slowly than under strict disomic inheritance and allelic richness is usually higher compared with related diploids (Bever & Felber 1992). All loci were following HW expectations except $L2.3$, $L2.5$ and $L021$ under RC_dS . HW departure as well as heterozygote deficiency and subsequent high F -value observed in $L2.3$ suggest the presence of null alleles at that locus.

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