Molecular Ecology Resources (2008) **8**, 895–897 doi: 10.1111/j.1755-0998.2008.02101.x

PERMANENT GENETIC RESOURCES

Isolation and characterization of 19 microsatellite markers in a tropical and warm subtropical birch, *Betula alnoides* Buch.–Ham. ex D. Don

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Abstract

***Betula alnoides* is an ecologically and economically important species in the tropics and warm subtropics. Nineteen polymorphic microsatellite markers were isolated from this species, which displayed three to 12 alleles per locus. The observed heterozygosities ranged from 0.100 to 0.905, and the expected heterozygosities from 0.510 to 0.893. These markers would be useful tools in genetic resource assessment, molecular marker-assistant breeding, parentage analysis and genetic diversity studies for this species.**

Keywords: *Betula alnoides*, birch, microsatellite, primer development, SSR, tropics

Received 4 November 2007; revision accepted 3 December 2007

Betula alnoides Buch.-Ham. ex D. Don is distributed in the tropics and warm subtropics, distinct from other species of genus *Betula* which occur mainly in the temperate zone in the North Hemisphere (Chen 1994). It is indigenous to the northern part of India Peninsula, Myanmar, Indochina Peninsula and south China. *Betula alnoides* is fast growing and well formed, and is suitably used to grow large size timber; its wood is commonly used in high-quality furniture making and room decoration. *Betula alnoides* also plays important roles in water conservation, long-term maintenance of land fertility and biodiversity of forest ecosystems. However, the natural forests of this species have been heavily fragmented because of severe exploitation during last two decades. It is, thus, essential to assess quickly its genetic resources for making conservation strategies and breeding plan. It is also a suitable candidate for studying the dynamics of genetic diversity and population genetic structure under the fragmented habitats. Microsatellites are codominant markers and distribute abundantly and uniformly throughout the genome (Rossetto 2001); they are considered as powerful markers in genetic resources assessment, molecular markers-assistant breeding and studies on population genetics, molecular ecology and so on. Here, we develop 19 polymorphic microsatellite markers according to the protocol of Glenn & Schable (2005) and discuss their potential for transportable use in some other *Betula* species.

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Total DNA was extracted from dry leaves of a single tree according to the method of Zeng *et al*. (2002), and digested into 300–1000-bp fragments with *Rsa*I (New England Biolabs). The double-stranded SuperSNX (SuperSNX24 Forward: 5′-GTTTAAGGCCTAGCTAGCAGAATC-3′; Super SNX24 +4P Reverse: 5′-pGATTCTGCTAGCTAGGCCT-TAAACAAA-3′) were then ligated to these digested DNA fragments. From the adapter-ligated DNA libraries, the microsatellite-containing fragments were screened out and enriched by Dynabeads (Dynal Biotech) with 10 simple sequence repeat (SSR) probes including $(AG)_{12}$, $(AT)_{12}$, $(CG)_{12}$, $(GT)_{12}$, $(ACG)_{12}$, $(ACT)_{12}$, $(CCA)_8$, $(AACT)_8$, $(AAGT)_8$ and $(AGAT)_8$. To increase the amounts of the captured microsatellite DNA fragments, polymerase chain reaction (PCR) amplifications were further conducted on the PTC-100 thermal cycler (Bio-Rad) using the following programme: 95 °C for 2 min; 95 °C for 20 s, 60 °C for 20 s, 72 °C for 2 min (30 cycles); 72 °C for 30 min; and 15 °C for 10 min. The reaction mixture (25 μL) included 2.0 μL microsatellite-containing DNA template, 150 μM dNTPs, 2.0 μM $MgCl_2$, 0.5 μM SuperSNX24-F primer, 1× PCR buffer (Tiangen Biotech Ltd.) and 0.04 U/μL *Taq* DNA polymerase (Tiangen Biotech Ltd.). The PCR products were then transformed into pGEM-T easy vectors (Promega). After incubated, the positive (white) clones were picked and tested by PCR amplification as above to see whether the

Table 1 The characteristics of 19 polymorphic micosatellite markers in *Betula alnoides*

Locus	Repeat motif	Primer sequence (5′–3′)	T_a (°C)	Size range (bp)	*A*	H_O	H_E	*Bl*	*Bf*	EMBL-Bank Accession no.
BAG01	$(TG)_8(GA)_{13}$	F: CAAGGTGCTCAGGGTGTTT R: TATCCTCTTTGGCATTGAA	52	265–277	6	0.905	0.807	+	+	EU193153
BAG02	$(TC)_{15}(CA)_9$	F: CCTTGCTCTGCGTGATGTATTT R: CAGAATCCTCTTATTTCCACAG	52	206–238	6	0.579	0.764	+	+	EU193154
BAG03	$(CT)_{17}$	F: TGCGTATGCTTCCAACAC R: ACCCAGAACCTGTCCACC	52	332–352	5	0.727	0.796	+	+	EU193155
BAG04	$(TC)_{21}$	F: TCTTTGCCTTAATGATTGTTGC R: GGCTGACGGACACTGTTTGA	52	209–233	6	0.476	0.807	+	+	EU193164
BAG06	$(CG)_5(CA)_8$	F: TCGCACATACACTCACACATT R: ATGCTGCACACTTTTGATTTA	50	166–176	5	0.857	0.810	+	+	EU193156
BAG09	$(AT)_5G(TG)_8$	F: TCGTTCAGGCAACTCTATCAAT R: ATTTGTAAAGATGGATGGAGAA	54	255–271	4	0.636	0.710	+	+	EU193158
BAG10	$(AG)_5 \ldots (GA)_{25} \ldots (GA)_8$	F: ACCAACTCCTAAACCACCATACC R: GGGAGGATTTCAACGGCATTTA	54	201–265	12	0.333	0.893	+	+	EU193159
BAG12	$(TCG)_5$	F: GTCCTCCTTCACTATTCCTTTGT R: CTTTCAGGGTAATTCTGATTTGG	52	139–166	7	0.818	0.804	+	–	EU193161
BAG14	$(TC)_7(CT)_9$	F: CATTGCTTCATTTTGGCTTCTA R: TTCTGATGTGAAATACTGCTGG	50	236–268	9	0.619	0.882	+	–	EU193162
BAG16	$(TG)_8(AG)_8T(AG)_6$	F: CACTAACCGCAACCAATAATCC R: ATTTGCCTTCCTACCAACTCTG	54	313–333	7	0.682	0.840	–	+	EU193163
BAG17	$(AG)_{15}$	F: GTTGGTAGTTGTGTGATGGA R: AGTTGTATGGATAGAAAAGT	52	171–183	4	0.227	0.680	+	+	EU193165
BAG18	$(GGT)_5$	F: GGATGGGTGACTAAGAGGGAGGA R: GCAATGGTAGACAAAGTCACAGC	50	245–251	3	0.381	0.553	+	–	EU193166
BAG20	$(TG)_9TT(AG)_{13}$	F: TTCTCCCACAACCCTTGATGC R: CCAGGCATCCAGTCCCTTATTA	50	235–261	5	0.550	0.673	+	+	EU193167
BAG22	$(TC)_{15}$	F: CGACGACGATTACAACGAGT R: TGGGTTGACATTCTAAGGGTGCT	52	257–275	5	0.318	0.684	+	–	EU193168
BAG24	$(TC)_{21}$	F: GAATTATTCGTGTTCAAAGTCT R: CATACAAGCAGTCAACCCATCT	52	262–284	6	0.762	0.789	–	–	EU193170
BAG25	$(CT)_5$	F: GATCTCGCCGTCGTCTCAAC R: CCAACAGGCAACAGATACAGAGT	52	257–269	4	0.318	0.532	+	+	EU193171
BAG27	$(TC)_{10}A(CA)_{10}$	F: ATGTGGAAGAGAGGATAGAATCT R: TGGAGTTAAGAGAACACAATAGA	50	317–343	7	0.455	0.835	+	+	EU193172
BAG28	$(AC)_7$	F: ACCTATTACATTGAATTCTATGA R: ATCATCTGTTAGCTTATCATAAT	52	298–312	4	0.100	0.510	+	+	EU193173
BAG29	$(AC)_{10}$	F: AGGCTTTGGCTTCCCTTATT R: CGGACACTTGTTCCGATTTT	52	157–183	9	0.409	0.855	+	+	EU193174

T_a, annealing temperature (°C); *A*, the number of alleles per locus; H_O, observed heterozygosity; H_E, expected heterozygosity; *Bl* and *Bf*, transportable use for *Betula luminfera* and *Betula fujianensis*, respectively.

objective DNA fragments were inserted. If successful, the clones were sequenced on the ABI 3730 automated sequencer (Applied Biosystems). The primers were designed with PRIMER PREMIER version 5.0 (Premier Biosoft International). A total of 274 positive (white) clones were picked, 58 different microsatellite-containing fragments were obtained, and 25 primers were designed.

The polymorphisms of the isolated microsatellite loci were analysed with 22 individuals of *Betula alnoides* from some patches of this species within a heterogeneous landscape in Guangxi, China. The PCR condition was 94 °C for 3 min; 94 °C for 30 s, the annealing temperature for 30 s, 72 °C for 30 s (30 cycles); 72 °C for 10 min; and 15 °C for 10 min. The reaction mixture (10 μL) containing 5 ng DNA template, 150 μM dNTPs, 2.0 μM $MgCl_2$, 0.5 μM primer, 1× PCR buffer (Tiangen Biotech Ltd.) and 0.04 U/μL *Taq* DNA polymerase (Tiangen Biotech Ltd.). Genotypes of 22 individuals were determined by electrophoresis on 6% denaturant polyacylamide gels with pUC18 DNA/*Msp*I (Tiangen Biotech Ltd.) as allelic ladder and visualized by silver staining.

Twenty-two loci were successfully amplified, of which three (BAG07, BAG11 and BAG23, shown in GenBank) were monomorphic and 19 were polymorphic, and the allele number of polymorphic loci ranged from three to 12 (Table 1). POPGENE version 1.31 (Yeh & Yang 1999) was used to calculate the observed and expected heterozygosities,

and to test Hardy–Weinberg equilibrium and linkage disequilibria. The observed and expected heterozygosities ranged from 0.100 to 0.905 and from 0.510 to 0.893, respectively (Table 1). Thirteen loci showed significant ($P < 0.01$) deviation from Hardy–Weinberg equilibrium. Therefore, MICRO-CHECKER version 2.2.3 (van Oosterhout *et al*. 2004) was further used to detect the presence of null alleles and to retest the significance for departure from Hardy–Weinberg equilibrium of loci in which null allele might exist. It was shown that there probably occurred null allele in seven loci (99% confidence): BAG10, BAG14, BAG17, BAG22, BAG27, BAG28, BAG29, and after corrected by this method, six loci (BAG02, BAG06, BAG09, BAG12, BAG18, BAG25) showed significant ($P < 0.01$) deviation from Hardy–Weinberg equilibrium. This was also due perhaps to population substructuring because the samples were collected from several patches. No significant linkage disequilibrium was detected for all pairwise analyses of loci. Thus, these markers could be used to assess genetic resources, to conduct molecular marker-assistant breeding and to study genetic diversity and mating system for this species.

Additionally, possibility of using these primers in *Betula luminifera* and *Betula fujianensis* of section Betulaster was tested with one individual for each species. It was shown that of the 19 loci 17 were present in *Betula luminifera* and 13 in *Betula fujianensis* (Table 1).

Acknowledgements

This work is financially supported by National Natural Science Foundation of China (30400345) and State Forestry Administration, P. R. China (2006BAD01A1603-03).

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