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PRIMER NOTE

Development and characterization of microsatellite markers for *Fagus crenata* Blume

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Abstract

Microsatellite markers can yield high-resolution genetic profiles for individual identification, and for parentage analysis, when evaluating gene dispersal in populations. *Fagus crenata* is an important dominant species in the cool temperate forests in Japan, and although many studies on the species have been conducted the patterns of gene dispersal via pollen and seeds are poorly understood. In order to be better informed about gene dispersal in *Fagus crenata*, we have developed 16 new microsatellite loci from an enriched library of genomic DNA. These 16 loci were highly variable, with 3–40 alleles per locus and an expected heterozygosity value of 0.11–0.98.

Keywords: beech, gene dispersal, genetic variation, mating system, parentage analysis, simple sequence repeat

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Fagus crenata Blume is monoecious, long-lived, and an outcrossing woody angiosperm that is wind-pollinated, with gravity and animal seed dispersal. It dominates the cool temperate deciduous broad-leaved forests of Japan. The species is considered to be important as a component of the ecosystem, and also for the conservation of biodiversity. The World Heritage listed Shirakami Mountains are occupied by *F. crenata* forests (UNESCO 2002). Many studies have been conducted on the ecological and population genetics of the species (e.g. Yamamoto *et al*. 1995; Tomaru *et al*. 1998; Takahashi *et al*. 2000), but thus far studies of gene dispersal within populations, via pollen and seed dispersal, have been inadequate, due to the lack of suitable genetic markers. Microsatellite markers are a powerful and effective tool for investigating gene dispersal and mating systems, using parentage analysis, in tree populations (e.g. Dow & Ashley 1996). Previously, nine microsatellite markers were developed for this species (Tanaka *et al*. 1999), but because the population possesses so many mature individuals parentage analysis could not be satisfactorily performed with this low number of pre-developed markers. There is a need to increase the number of markers available to achieve a satisfactory degree of genetic resolution. In this study, 16 microsatellite markers were developed from an enriched genomic $(CT)_{15}$ library of *F. crenata*.

Genomic DNA was extracted by a modification of the cetyltrimethyl ammonium bromide (CTAB) method of Murray & Thompson (1980), and was purified using equilibrium centrifugation in CsCl-ethidium bromide gradients. The genomic DNA was then digested with the restriction endonuclease (*Nde*II), and fragments of 300–1000 bp in length were fractionated. Approximately 500 ng of the DNA fragments were ligated to *Sau*3AI linkers (Takara), and nicks between the fragments and the linker sequences were repaired after the ligation. The DNA fragments with linkers were resolved in binding buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.5) and then hybridized to biotinylated $(CT)_{15}$ oligonucleotide probes after denaturation. The DNA molecules bound to the biotin-labelled probes were subsequently isolated by binding them to streptavidin-coated (M-280) Dynabeads® (Dynal Biotech). After rinsing the beads in two kinds of washing buffer (2 × saline sodium citrate buffer, 0.1% sodium dodecyl sulfate and 1 × saline sodium citrate buffer, 0.1% sodium dodecyl sulfate), the target DNAs were recovered by denaturing them in boiling water. The resulting fragments were reformed to double-strand conformation by polymerase chain reaction (PCR), and digested with *Nde*II to remove the linkers. The enriched fragments were ligated into

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Table 1 Primer sequences and characteristics of 16 polymorphic microsatellite loci in *Fagus crenata*

Locus	Repeat sequence	Fluorescent label	Primer sequence (5′–3′)	T_a (°C)	Size range (bp)	A	H_O	H_E	F	Accession no.
sfc0007–1	$(AAAT)_4$	FAM	F: GCATTCAATTAGAACAAGAGG R: TCAATGTTTGCGACAATAAC	60	136–170	15	0.85	0.87	0.017	AJ586309
sfc0007–2	$(AG)_{24}$	HEX	F: TGTCGCAAACATTGACAAGG R: GTGGATGTGAGGTCGTTGG	60	149–157	3	0.29	0.51	0.422	AJ586309
sfc0018	$(AG)_{17}$	HEX	F: GAAGCAGAGCATTGTATTGG R: CATCTGTTTCAGTTCTGTAAAGG	60	161–191	11	0.88	0.80	−0.103	AJ586310
sfc0036	$(TC)_{23}$	HEX	F: CATGCTTGACTGACTGTAAGTTC R: TCCAGGCCTAAAAACATTTATAG	60	96–142	17	0.82	0.91	0.101	AJ586311
sfc0109	$(GA)_{27}$	FAM	F: TTGGTGGTCAACATCAC R: TGACCATTAAGTCAACAATC	55	93–175	30	0.68	0.96	0.302*	AJ586312
sfc0146	$(TC)_{17}$	FAM	F: TCGATTTCAGACGTGATG R: TCCGCCAATTTGGTATG	55	130–202	30	0.44	0.94	0.535*	AJ586313
sfc0161	$(AG)_{22}$	FAM	F: AAGCTCCACGATTCATTC R: GCTGGAGTTGCTCTAAGTC	60	77–165	40	0.97	0.98	0.010	AJ586314
sfc0195–2	$(TC)_7$	NED	F: CCAGCCTCTCGTCTATTATC R: AATGGAATGCTTGTTCAAC	55	175–187	6	0.50	0.65	0.230	AJ586315
sfc0289–1	$(AG)_8$	FAM	F: GGAAAGCTTGGTACTATTAGAG R: AAGAGAAGCTTAGTCATGTACAC	60	142–186	9	0.24	0.73	0.680*	AJ586316
sfc0305	$(GA)_{24}$	HEX	F: CCAATGGACTTGTTATACCAATC R: GCACCAGTTGCTTACAGAATAG	60	159–203	17	0.74	0.92	0.201*	AJ586317
sfc360–2	$(AG)_6$	HEX	F: ATGCTTTGCTGTTCAAGATG R: TTTGCATAAACTCACTCTCAGTC	55	105–109	3	0.12	0.11	−0.031	AJ586318
sfc0378	$(AG)_{14}$	NED	F: CCTAAAATTCAGTGATGATTATG R: TGGCTTTGAGTCTGAGATG	58	223–249	13	0.88	0.89	0.012	AJ586319
sfc0488	$(GA)_{15}$	HEX	F: TCTCGATTTATAGTGTTTCTG R: CATCCTTGTACTTCTCTAACAG	55	129–153	11	0.68	0.88	0.237*	AJ586320
sfc1063	$(CT)_{13}$	NED	F: TTTCCAACTACAACTTCATTG R: AGTGCTCGCATCGTATG	55	188–222	15	0.79	0.86	0.080	AJ586321
sfc1105	$(TC)_{31}$	HEX	F: TCGTCTCTTCCGTCATCAC R: CAGCGTATACCTAATCAATTCC	58	120–180	18	0.79	0.79	−0.007	AJ586322
sfc1143	$(AG)_{21}$	FAM	F: TGGCATCCTACTGTAATTTGAC R: ATTCCACCCACCATCTGTC	58	96–136	17	0.91	0.93	0.017	AJ586323

A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F, fixation index.
*Significant departure from HWE ($P < 0.05$).

plasmid vectors, pUC118*Bam*HI (Takara), and cloned into *Escherichia coli* DH5 competent cells. Plasmid DNAs from these clones were purified using a Wizard®SV96 Plasmid DNA Purification System (Promega), and sequenced using an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems) with a BigDye® Terminator sequencing kit (Applied Biosystems). Out of 591 sequences, 501 were positive, 256 of which had sufficient flanking regions to design primers. Based on the sequences containing microsatellites, 96 microsatellite primer pairs were designed using the program OLIGO (National Biosciences). After PCR optimization, a forward primer of each successful pair was fluorescently labelled. PCR amplifications were carried out using a GeneAmp PCR System Model 9700 (Applied Biosystems), in a total volume of 10 µL, containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer, 1–10 ng of template DNA, and 0.25 U of *Taq* DNA Polymerase (Invitrogen), with the following temperature profile: 3 min denaturation at 94 °C; followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing (Table 1), and 30 s extension at 72 °C; with a final extension at 72 °C for 5 min. To evaluate the polymorphism of the microsatellite regions, the DNAs of 34 *F. crenata* individuals, sampled from 17 locations scattered throughout the entire distribution, were used. These DNAs were also extracted by the modified CTAB method and genotypes were determined by using ABI PRISM® 310 Genetic Analyser (Applied Biosystems). The number of alleles per locus (A), observed heterozygosity (H_O) and expected heterozygosity (H_E) for each locus was calculated, and deviations from Hardy–Weinberg equilibrium (HWE) were assessed with fixation index (F) values, using the program FSTAT (Goudet 2001). Of the 96 microsatellite primer pairs, 16 showed single-locus polymorphism. Polymorphism

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detected in the 34 individuals gave values for A ranging from 3 to 40, with an average of 15.9, and H_E of 0.11–0.98 with an average of 0.80. Five out of the 16 loci showed significant deviations from HWE (Table 1). These deviations may be due to the presence of null alleles and population differentiation. We suggest that these markers will be useful in parentage analysis and for an understanding of gene dispersal and the mating system of *F. crenata*.

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