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PRIMER NOTE

Characterization of microsatellite markers in *Fagus sylvatica* L. and *Fagus orientalis* Lipsky

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Abstract

Using an enrichment procedure, we cloned microsatellite repeats from European beech (*Fagus sylvatica* L.) and developed primers for the amplification of microsatellite markers. Six polymorphic loci were characterized which produced 3–21 alleles in 70 individuals from one Italian population, with an observed heterozygosity between 0.58 and 0.85. All six loci amplified fragments which were polymorphic in the closely related species, *Fagus orientalis*, also. Owing to their very high degree of variation, these markers should be very useful in gene flow studies of these species.

Keywords: *Fagus*, genetic diversity, inheritance, SSR

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Fagus sylvatica L. and *F. orientalis* Lipsky are two ecologically and economically very important broadleaves in the western parts of Eurasia. *Fagus* sp. is monoecious, wind-pollinated and allogamous. As a heavy-fruit tree species, long-distance gene flow by means of seeds is mediated through birds.

European beech (*F. sylvatica*) has been well studied on a range-wide scale using isozyme markers (Comps *et al*. 2001) and chloroplast DNA (cpDNA) markers (Demesure *et al*. 1996). These studies provided important information about migration history and distribution of diversity in European beech. However, no paternity analyses in *Fagus sylvatica* have been performed to date, because of the lack of suitable molecular markers. Microsatellites represent an ideal tool for gene flow studies, considering their high degree of polymorphism, as shown in other species of the Fagaceae (Streiff *et al*. 1999).

Microsatellite loci were isolated using an enrichment procedure (Karagyozov *et al*. 1993), modified by Van der Wiel *et al*. (1999) and Arens *et al*. (2000). Genomic DNA of *F. sylvatica* was extracted from freeze-dried leaves using a DNeasy Plant Mini Kit (Qiagen), digested with *Alu*I and *Rsa*I, and size-fractionated using agarose gel electrophoresis. DNA fragments between 300 and 1000 bp were recovered by electro-elution, enriched by hybridization to either synthetic GA-oligonucleotides, or one of two pools of synthetic tri- and tetranucleotides [$(TGT)_{10}$, $(GTG)_8$, $(GAG)_8$, $(GCT)_8$, $(TGTT)_8$ and $(GATA)_8$, or $(TCT)_{10}$, $(CGT)_8$, $(AGT)_9$, $(TGA)_9$ and $(GTAT)_8$], cloned in the PCR2.1-TOPO vector and transformed to *Escherichia coli* TOP10F′ (Invitrogen). Colonies were transferred onto Hybond N^+ membranes (Amersham) and screened by hybridization to the same mixtures of oligonucleotides. The positive clones were sequenced using a Ready Reaction Big Dye Terminator Cycle Sequencing Kit and an ABI 3700 sequencer (PE Biosystems).

Twenty-one sequences were selected for microsatellite primer development, focusing the attention in particular on simple and noninterrupted stretches: eight of them contained dinucleotide repeats $(GA)_n$ and 12 trinucleotide repeats [one $(CTT)_n$, four $(GGA)_n$, three $(TGA)_n$, two $(CCA)_n$, one $(GTT)_n$, one $(CAT)_n$]. In only one case was a compound stretch $(GCT)_n(GTT)_n(GCT)_n$ considered. Primers were designed using the software PRIMER Version 3.0 (Rozen & Skaletsky 1998). In order to optimize the simple sequence repeat (SSR) amplification conditions, two different $MgCl_2$ concentrations (1.5 and 2.5 mM) and 12 different annealing temperatures (from 54 to 65 °C) were tested using a Robo-Cycler GRADIENT96 (Stratagene). Of the 21 designed primers, 76% produced an amplified fragment of the expected size on a 1.4% agarose gel. These amplified fragments were then electrophoresed through a 6%, 20 cm long denaturing polyacrylamide gel (Reprogel, Amersham)

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using an ALF Express automatic sequencer (Pharmacia) at 35 W constant power for ≈ 80 min, to check for the quality of the fragments generated by the best amplification protocols. Only primer pairs producing well-scorable bands (no or weak stutter bands, quality 1 or 2 in Smulders *et al*. 1997) were considered. Six microsatellites (38%) met this requirement (Table 1). In addition to this set of six microsatellites, one primer pair for the amplification of a microsatellite developed in *F. crenata* (Tanaka *et al*. 1999) was successfully tested in *F. sylvatica* (FCM5, see Table 1). The polymerase chain reaction (PCR) conditions for the seven selected SSRs (final volume of 25 μL) were: 10 ng of template DNA, 10× Pharmacia reaction buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris–HCl pH 9.0), $MgCl_2$ as in Table 1 and 0.2 mM of each dNTPs (Pharmacia), 0.4 μM of each primer, 1 U of *Taq* DNA polymerase (Pharmacia). Amplification was carried out in a PE thermal cycler model 9700 with the following thermal profile: 5 min denaturation at 95 °C followed by 30 cycles of 1 min denaturation at 95 °C, 1 min annealing (Table 1), 1 min extension at 72 °C, and a final extension step of 72 °C for 8 min.

To test inheritance and to obtain preliminary information about the polymorphism of each SSR, a progeny of 87 individuals originated from a controlled cross and 70 individuals sampled in an Italian natural population of were analysed. For sizing microsatellite alleles, an automatic sequencer Pharmacia ALF Express was used, according to the conditions reported above. External and internal molecular mass standards (50, 100, 150, 200, 250 and 300 bp) were used in conjunction with FRAGMENT MANAGER Version 1.2 (Pharmacia) to size the amplified fragments. Microsatellites were amplified separately; for analysis, they were multiplexed by size.

Mendelian inheritance was confirmed for all seven microsatellites: no significant deviation from the expected ratios was observed. Analysis of the full sib progeny revealed the presence of null alleles at two loci (*FS1-25* and *FS4-46*). All seven loci displayed a high level of polymorphism. The total number of alleles in *F. sylvatica* ranged from 3 to 21, averaging 12 per locus. The most polymorphic primer pairs were *FS1-03* and *FCM5*, and they amplified 21 and 18 different alleles each in only 70 individuals. The observed heterozygosity ranged between 0.58 and 0.85, the expected heterozygosity was comparable (Table 1).

All seven loci amplified fragments of the expected sizes also in the closely related species, *F. orientalis* (45 individuals of 3 different populations tested), often consisting of new alleles (Table 1).

Table 1 Characterization of seven polymorphic microsatellite markers in *Fagus sylvatica* and the related species *F. orientalis*

Microsatellite locus	Primer sequences 5′–3′	Anneal. temp. (°C)	$MgCl_2$	Repeat	*Fagus sylvatica*				*Fagus orientalis*				GenBank Accession no.
					Obsd allele size range (bp)	No. alleles	H_O	H_E	Obsd allele size range (bp)	No. alleles	H_O	H_E	
FS1-15	TCAAACCCAGTAAATTTCTCA GCCTCAATGAACTCAAAAAC	60	2.5	$(GA)_{26}$	95–135	11	0.61	0.72	83–133	16	0.89	0.89	AF528095
FS1-25	GACCCATACCTCTCAGCTTC AGAGATCATTGCAACCAAAC	65	1.5	$(GA)_{23}$	78–126	14	0.65	0.70	80–118	4	0.71	0.71	AF528093
FS1-03	CACAGCTTGACACATTCCAAC TGGTAAAGCACTTTTTCCCACT	60	1.5	$(GA)_{18}$	90–132	21	0.83	0.82	86–112	10	0.60	0.53	AF528090
FS1-11	TGAATTCAATCATTTGACCATTC GGAAGGGTGCTTCAATTTGG	63	2.5	$(GA)_{15}$	98–120	10	0.74	0.81	98–120	9	0.71	0.84	AF528091
FS3-04	AGATGCACCACTTCAAATTC TCTCCTCAGCAACATACCTC	60	1.5	$(GCT)_5(GTT)_3$ $(GCT)_6$	201–207	3	0.58	0.54	192–204	4	0.55	0.52	AF528092
FS4-46	GCAGTCCTCCACCATTACTA TACAACAGCAGGCTATCCAT	60	1.5	$(TGA)_{23}$	209–371	10	0.85	0.83	176–272	10	0.68	0.83	AF528094
FCM5	ACTGGGACAAAAAAACAAAA GAAGGACCAAGGCACATAAA	60	1.5	$(AG)_{10}$	274–334	18	0.83	0.85	272–338	15	0.74	0.86	(1)

H_O, observed heterozygosity; H_E, expected heterozygosity estimated using GENALEX software (Peakal & Smouse 2001). (1) from Tanaka *et al.* (1999).

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