

PRIMER NOTE

Development and characterization of microsatellite markers for two dioecious *Ficus* species

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Abstract

Microsatellite markers for *Ficus montana* and *Ficus septica* were developed using genomic libraries enriched for di-, tri- and tetranucleotide repeats. The subsets of five and three best scorable primer pairs were characterized on 24 *F. montana* and 36 *F. septica* individuals, respectively. For *F. montana*, loci showed five to 14 alleles per locus and expected heterozygosities ranged between 0.23 and 0.87. For *F. septica*, loci showed three to five alleles per locus and expected heterozygosities ranged between 0.36 and 0.49. Four primer pairs (two from each subset) cross-amplified in the other species, indicating transportability of the markers within the genus *Ficus*.

Keywords: dispersal, *Ficus montana*, *Ficus septica*, mutualism, pollinating fig wasps, simple sequence repeat (SSR)

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The genus *Ficus* (Moraceae) is one of the largest and most diverse genera of woody plants (Berg & Wiebes 1992), comprising approximately of 750 species distributed in tropical and subtropical zones. Except for some cases (Michaloud *et al.* 1996; López-Vaamonde *et al.* 2002; Molbo *et al.* 2003), most *Ficus* species rely on a specific wasp species (Hymenoptera: Agaonidae) for pollination. In turn, the pollinating fig wasp species reproduce only on a particular *Ficus* species. Although *Ficus* species and their pollinating wasps are dependent on each other for reproduction, there is a marked difference in gene flow mechanisms. Figs are eaten by many species of wide-ranging frugivores, and therefore seeds can be dispersed over long distances. Dispersal of wasps carrying pollen is wind mediated, and therefore mainly passive. The use of codominant polymorphic molecular markers is essential to determine the sources of gene flow, such as pollen and/or seed dispersal, in *Ficus* populations. Moreover, codominant highly polymorphic microsatellite markers would enable to determine the number of pollen donors to the individual fig or fig tree, reflecting patterns of wasp movements and their dispersal capabilities.

In this primer note, we report on the development and characterization of five microsatellite markers for *Ficus*

montana and three microsatellite markers for *Ficus septica*. Di-, tri- and tetranucleotide repeat-enriched libraries of *F. montana* and *F. septica* genomic DNA were constructed by a selective hybridization procedure (Karagoyozov *et al.* 1993) with the modifications described by Zavodna *et al.* (2002). Briefly, genomic DNA of each *F. montana* and *F. septica* (extracted from young leaves from three and two individuals, respectively) was digested with *RsaI* and *AluI*. Fragments in the size range of 300–1000 bp were isolated from agarose gel, ligated to a blunt adapter and enriched for microsatellite sequences by hybridization using Hybond-N+ membranes (Amersham). After the enrichment procedure, polymerase chain reaction (PCR) fragments were cloned using the TOPO-TA Cloning Kit (Invitrogen). Insert-containing colonies were transferred onto Hybond-N+ membranes and screened by hybridization to a mixture of the synthetic oligonucleotides. Out of 308 positive clones for *F. montana* and 223 positive clones for *F. septica*, 71 and 58 clones, respectively, were sequenced using PRISM dGTP BigDye Terminator Ready Reaction Kit (Applied Biosystems) and analysed on an ABI 3700 sequencer (Applied Biosystems). Primer pairs for the amplification of 12 (*F. montana*) and seven (*F. septica*) microsatellites were designed using the software package LASERGENE (DNASStar). The markers were tested using different PCR protocols (Table 1) on a PTC-200 thermocycler (MJ). The amplification reaction was performed in

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Table 1 Characterization of microsatellite markers for *Ficus montana* (FM) and *Ficus septica* (FS)

Microsatellite marker	Primer sequence (5'–3')	Amplification conditions*	Repeat†	Expected product length (observed range)	Number of alleles	H_O/H_E	EMBL Accession No.
FM4-15	F: ATCTTCGTCGGTATTGCTTTTCACT R: GGAAGAGAACCCTTTTGTATTGG	LT60/27	(TCC) ₇ (TCA) ₁₀ (CCATCA) ₅	217 (204–282)	14	0.57/0.87‡	AJ854072
FM4-18	F: CGTATGGCCCATGCTTGACTCAC R: CTCGGATTGCCACGTTAGGTTG	LT55/27	(ATC) ₁₉	211 (174–216)	5	0.17/0.23	AJ854073
FM1-27	F: GTGATTTGCGATGGCGTGGTTTA R: TCTTCGCTTGTCTCGTCAGTGTCC	LT55/32	(GA) ₁₉	314 (245–311)	13	0.91/0.87	AJ854074
FM3-64	F: GATGGTGTGTGTGTCGATGGTTCAT R: GCGGCTCGGTGGAACCTTGAG	LT55/27	(CAA) ₈	281 (260–287)	5	0.50/0.46	AJ854075
FM4-70	F: CAGATGAGGTTGACGATGTTATTG R: TAAACCCTCTTCAAATTCACCTCTC	LT55/32	(GAA) _{20–1}	228 (202–232)	7	0.63/0.78‡	AJ854076
FS4-11	F: AAGGCAACGGGGATAAAGTATPCA R: CTCGAGAGCAACTCCATCACG	LT55/30	(CGA) ₆	277 (272–293)	5	0.31/0.46‡	AJ854077
FS3-31	F: CATCATCCCCGTGAGAAAGTGAGG R: TGAGGCGGTGATGGTGAAT	LT60/35	(GCA) ₁₂	246 (228–246)	3	0.40/0.36	AJ854078
FS3-37	F: CAAACGAGGAACAACACATACAGC R: GATGCACAGGAGTTAACGGGAATG	LT60/27	(CAA) _{20–2}	215 (208–214)	3	0.51/0.49	AJ854079

*LT60/35: 1 cycle 3 min at 94 °C, 35 cycles (15 s at 94 °C, 45 s at 60 °C, 2 min at 72 °C), 20 min at 72 °C; LT60/27: 1 cycle 3 min at 94 °C, 27 cycles (15 s at 94 °C, 45 s at 60 °C, 2 min at 72 °C), 20 min at 72 °C; LT55/32: 1 cycle 3 min at 94 °C, 32 cycles (15 s at 94 °C, 45 s at 55 °C, 2 min at 72 °C), 20 min at 72 °C; LT55/30: 1 cycle 3 min at 94 °C, 30 cycles (15 s at 94 °C, 45 s at 55 °C, 2 min at 72 °C), 20 min at 72 °C; LT55/27: 1 cycle, 3 min at 94 °C, 27 cycles (15 s at 94 °C, 45 s at 55 °C, 2 min at 72 °C), 20 min at 72 °C.

†The number after hyphen denotes the number of mismatches from a perfect repeat; ‡statistically significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

20- μ L volume containing 75 mM of Tris-HCl (pH 8.8), 1.5 mM of MgCl₂, 20 mM of (NH₄)₂SO₄, 0.01% (v/v) Tween 20, 100 μ M of each dNTP, 200 nM of each primer, 2 ng of template DNA and 0.3 unit GoldStar DNA polymerase (Eurogentec). Amplification products were visualized by silver staining as described by Zavodna *et al.* (2002). In order to assess the allele sizes, a sequencing reaction on pGEM-3Zf(+), using the pUC/M13 forward 24-mer (Promega) as primer, accompanied the samples.

The five and three best scorable polymorphic loci were further characterized on 24 individuals of *F. montana* and 36 individuals of *F. septica*, respectively. Four to six individuals originating from each of the Sumatra, Sebesi, Krakatau, and Java islands (Indonesia) were analysed. DNA was extracted from a single leaf following the hot cetyltrimethyl ammonium bromide (CTAB) method of Rogstad (1992) with the modifications as described in Parrish *et al.* (2004). For *F. montana*, loci showed five to 14 alleles per locus and expected heterozygosities ranged between 0.23 and 0.87 (Table 1). For *F. septica*, loci showed three to five alleles per locus and expected heterozygosities ranged between 0.36 and 0.49 (Table 1). Deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were tested using the software TFGA version 1.3 (available at <http://bioweb.usu.edu/mpmbio/>) and FSTAT version 2.9.3.2 (available at <http://www2.unil.ch/izea/software/fstat.html>). All loci for *F. septica* were in linkage equilibrium, however,

three out of 10 pairwise exact tests rejected linkage equilibrium between some loci of *F. montana* (FM4-15–FM1-27; FM4-15–FM4-70; FM1-27–FM3-64; $P < 0.004$; adjusted $\alpha = 0.005$). Three loci (FM4-15, FM4-70, FS4-11) were not in HWE, with observed heterozygosity lower than the expected values (Table 1). The locus FM4-18 also showed a lower than expected heterozygosity, albeit nonsignificant (Table 1). Deviation from HWE could be caused by several factors, such as the presence of null alleles, a Wahlund effect, or the mating system affected by pollen carrying wasp dispersal (inbreeding). Considering the fact that the tested individuals originated from different islands, population subdivision (Wahlund effect) can be expected.

We also performed cross-amplification with five *F. montana* primer pairs and the three *F. septica* primer pairs. The amplification reactions and PCR conditions were carried out as described for the focal species without further optimization. Four primer pairs (FM3-64, FM4-70, FS4-11, and FS3-31) yielded PCR products in the other species, indicating a high transportability of the markers.

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