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Development and characterization of microsatellite markers for a monoecious *Ficus* species, *Ficus insipida*, and cross-species amplification among different sections of *Ficus*

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Abstract

The animal-mediated pollination and seed-dispersal mutualisms of *Ficus* species give them key roles in tropical ecosystems, but may make them vulnerable to habitat fragmentation. The development of highly polymorphic markers is needed to analyse their genetic diversity and investigate the effects of fragmentation on gene flow. Of thirteen microsatellite loci isolated from *Ficus insipida*, a monoecious species in French Guiana, eleven were polymorphic (two to six alleles each). High levels of variation were found among loci; expected heterozygosities ranged from 0.151 to 0.715. All markers revealed a broad cross-species affinity when tested in 23 other *Ficus* species.

Keywords: *Ficus insipida*, genetic diversity, keystone species, microsatellite markers

The genus *Ficus* includes some 800 species of tropical and subtropical regions of the world. Well known for their species-specific pollination mutualisms (Anstett *et al.* 1997), *Ficus* spp. are also partners in generalized seed-dispersal mutualisms, their fleshy 'fruits' being consumed by a great diversity of frugivorous vertebrates (Shanahan *et al.* 2001). Figs are thus pertinent models for studying how forest fragmentation affects mutualist-mediated dispersal and gene flow, the demography and genetics of plant populations, and ultimately the structure and functioning of ecological communities. Khadari *et al.* (2001) developed the first polymorphic microsatellites for a dioecious fig, *Ficus carica*. More recently, Zavodna *et al.* (2005) developed five and three microsatellites, respectively, for two other dioecious species, *Ficus montana* and *Ficus septica*. Genetic variation was first examined in a few monoecious fig species from Panama using allozyme markers (Nason *et al.* 1996). That study documented extensive long-distance pollen-mediated gene flow and showed that breeding populations of the species studied were quite large. Since that study, however, no further work has been conducted on the population

genetics of other monoecious fig species. The aim of this study was to develop simple sequence repeat (SSR) markers for a monoecious fig species occurring in French Guiana, *Ficus insipida*, for studying the effects of forest fragmentation on both pollen- and seed-mediated gene flow. This species is a free-standing tree common in riverine forest. It belongs to the strictly neotropical section Pharmacosycea, the basal-most clade within the genus (Jousselin *et al.* 2003).

Leaves were collected from 63 trees located in three populations in French Guiana (Petit Saut, Roura and Cacao). DNA was extracted from dried leaves using the QIAmp Plant Kit (QIAGEN). The isolation of microsatellite loci from an enriched [(CT)_n and (GT)_n] library was performed following Dutech *et al.* (2000), using biotin-labelled microsatellite oligoprobes and streptavidin-coated magnetic beads, with the following modifications: (i) there was no size selection after digestion with the restriction enzyme; (ii) XL10 Gold ultracompetent cells (Stratagene) were used; (iii) recombinant clones were screened with (CT) and (GT) and AGE1 (AAACAGCTATGACCATGATTAC) or AGE2 (TTGTAAAACGACGGCCAGTG) oligonucleotides using a modified polymerase chain reaction (PCR) method (Waldbieser 1995); (iv) the PCR reagents were 1× reaction buffer (RedGoldstar Eurogentec), 1 μM of each primer,

Table 1 Characterization of 13 microsatellite loci in *Ficus insipida*. Repeat motif, primer sequences, annealing temperature, range of the allele size tested with 63 individuals, number of alleles per locus, observed (H_O) and expected (H_E) heterozygosities calculated on 63 adult trees from Petit Saut, Roura and Cacao in French Guiana, probability of exclusion calculated on the 43 adults from Petit Saut (Pe) and EMBL Accession no. Statistically significant deviations from Hardy–Weinberg expectations are indicated by * ($P < 0.05$) and *** ($P < 0.001$)

Locus	Repeat motif	Forward and reverse primer sequences	Annealing temperature (°C)	Size range	No of alleles† (bp)	H_O	H_E	Pe	GenBank Accession no.
FinsI12	(TC) ₅ (CT) ₁₁	NED-L: GAACCTTCAACCTCAATCAA R: CTCCCCTTTCCTAGTCCCTTA	55	144–157	4	0.217	0.269	0.289	AM039801
FinsJ10	(TC) ₁₁	FAM-L: AGGTGGAATGAGGAGAGAGT R: AAACATCCTTCTCTGGACTTG	54	163–170	2	0.339***	0.484	0.264	AM039802
FinsK9	(CT) ₁₃	HEX-L: ACGCACTTAACCCCTTTCAG R: TTCGAGTCAACGAAACAAA	55	99–118	3	0.091*	0.239	0.112	AM039803
FinsM5	(TC) ₁₃	HEX-L: ATGAATGGTGAAATCCTGAA R: CATGGCTCAACTTAGAAAC	55	176–182	1	—	—	—	AM039804
FinsN1	(TA) ₂ (CA) ₁₀ (TA) ₇ CATA(TG) ₂	HEX-L: AGGGCTGAGATAGGTTGATT R: TAAGTTGGTGTGTGGCATC	55	150–160	6	0.127***	0.151	0.171	AM039805
FinsN3	(GA) ₁₀	FAM-L: AAAACTTCTCCTCTGTATTTT R: TTTGCTTCTTCTCTGTTTGAC	54	165	1	—	—	—	AM039806
FinsP8	(CA) ₈	NED-L: TGAAGAAAACGGAGCTTG R: CTAAATCTGACGGTTCAAAA	54	157–160	2	0***	0.433	—	AM039807
FinsQ5	(GA) ₁₂	FAM-L: CATGTCAGGAGGTGTCTAGG R: CTCCAAATGGGTATGTCAAG	55	149–156	2	0.200***	0.406	0.251	AM039808
FinsQ6	(TG) ₇ AG(TG) ₅	HEX-L: TTCTCCAATTAACCTCCAA R: CATGAAATCACCTTCCTCAT	55	98–107	4	0.069***	0.335	0.257	AM039809
FinsT7	(TA) ₁₁ (TG) ₁₆	HEX-L: GAATCTGGAGGTGGAATAAAC R: AAAGATCGCTCGTCAACC	55	193–210	6	0.517	0.611	0.467	AM039810
FinsU9	(AG) ₁₆	NED-L: CGTGTATTGATGTGTGTGTG R: TCACCTCCTCCTTCTTTTG	54	148–156	5	0.458	0.553	0.455	AM039811
FinsA1	(CT) ₁₂	NED-L: AATCCCGTACTTCACTTG R: AGAACTTATTGCACGGACAG	55	194–222	5	0.560***	0.603	0.551	AM039812
FinsH5	(AT) ₅ GTAT (GT) ₁₁	HEX-L: GACCGTATAGATGATTTGGG R: CATCCTGTGAACGACACTT	54	262–272	5	0.656	0.715	0.621	AM039813
Average					3.3	0.249	0.369	0.989†	

†Cumulative probability of exclusion.

1.875 mM MgCl₂, 1 mM dNTP and 0.75 U of *Taq* DNA polymerase (RedGoldStar Eurogentec) and (v) the PCR program was 2 min of denaturation at 94 °C, 27 cycles at 94 °C for 30 s, 60 °C for 1 min and 72 °C for 2 min and 5 min of elongation at 72 °C. A total of 344 clones were screened and 198 gave a positive signal. Fifty-two positive clones were sequenced using ABI PRISM 310 DNA sequencer (Applied Biosystems) or by Genaxis. Thirteen nonredundant sequences were obtained. Primers were then designed with use of PRIMER 3 (Rozen & Skatlefsky 1997). Amplification reactions were carried out separately for each locus in 20 µL including 0.1 µL of each 10 µM fluorescent-labelled primer and unlabelled primer (Table 1), 2.5 µL of 10× manufactured reaction buffer, 1.5 µL of 25 mM MgCl₂, 0.63 µL of 20 mM dNTP mix, 0.2 µL of 5 U/µL *Taq* DNA polymerase (RedGoldStar Eurogentec) and 1 µL of genomic DNA. The PCRs were performed using a PTC 100 thermocycler (MJ research) in the following conditions: 3 min of denaturation at 94 °C and 35 cycles of 30 s of initial denaturation at

94 °C, 45 s of annealing at 55 °C, 45 s of extension at 72 °C and 5 min of elongation at 72 °C. One microlitre of diluted PCR product (1/20) was pooled in 15 µL of deionized formamide and 0.2 µL of GeneScan-500XL ROX size standard and analysed on an ABI PRISM 310 DNA sequencer.

Eleven of the 13 loci were polymorphic and 46 allelic variants were identified (Table 1). The average number of alleles per locus was 3.3 (2–6) and mean expected heterozygosity was 0.369 (0.151–0.715). Six loci showed significant deviation from Hardy–Weinberg equilibrium. Their heterozygote deficiency could be due to the presence of null alleles or to population substructuring. The latter was the case for the FinsP8 locus, in which different alleles were fixed in different populations. Tests of pairwise linkage disequilibrium showed significant values for two loci, FinsQ5 and FinsQ6. The combined value for probability of paternity exclusion calculated using the program FAMOZ (Gerber *et al.* 2003) was 0.989. Using 11 polymorphic loci enabled us to distinguish all the 43 adults in Petit Saut.

Table 2 Cross-amplification of *Ficus insipida* microsatellite primers in 23 species of the genus *Ficus*. The section is indicated for each species

<i>Ficus</i> species	No. of individuals tested	I12	J10	K9	U9	H5	Q6	A1	Q5	M5	N3	T7	P8	N1
Section Pharmacosycea														
<i>F. insipida</i>	63	++	++	++	++	++	++	++	++	++	++	++	++	++
<i>F. maxima</i>	1	+	+	-	+	++	+	++	-	+	+	+	+	+
<i>F. piresiana</i>	1	+	+	-	+	+	+	+	-	-	+	+	+	+
Section Conosycea														
<i>F. beddomei</i>	5	+	+	++	-	+	+	-	++	++	+	-	-	+
<i>F. talboti</i>	1	++	+	-	-	++	+	+	-	+	+	-	+	+
Section Galoglychia														
<i>F. ovata</i>	1	++	++	-	-	+	+	++	-	++	+	++	+	++
<i>F. lutea</i>	1	+	++	-	-	+	+	++	-	++	+	++	+	++
<i>F. thomingii</i>	2	++	-	-	-	++	++	++	-	++	++	++	++	+
Section Americana														
<i>F. nymphaeifolia</i>	15	++	++	-	-	++	++	++	++	++	++	++	++	++
<i>F. broadwayi</i>	1	+	-	-	-	+	+	+	+	+	+	+	+	+
<i>F. guianensis</i>	1	+	+	-	-	+	+	+	+	+	+	+	+	+
<i>F. leiophylla</i>	1	+	+	-	-	+	-	+	-	-	+	+	+	+
<i>F. maronensis</i>	1	+	+	-	-	+	-	+	+	+	+	+	+	+
Section Malvanthera														
<i>F. platypoda</i>	1	++	++	-	-	+	++	+	-	+	+	-	+	+
Section Oreosycea														
<i>F. nervosa</i>	1	+	++	-	+	+	+	+	-	+	+	+	+	+
Section Urostigma														
<i>F. tsahela</i>	5	-	+	+	-	+	+	-	+	+	+	+	-	+
<i>F. ingens</i>	1	+	+	-	-	-	+	+	-	+	+	+	+	++
Section Sycomorus														
<i>F. sur</i>	1	+	+	-	-	++	+	+	-	+	+	-	++	+
<i>F. sycomorus</i>	1	+	+	-	-	+	+	++	-	+	+	-	++	+
<i>F. racemosa</i>	20	+	++	-	-	-	++	++	+	-	++	-	+	++
Section Sycocarpus														
<i>F. hispida</i>	5	++	+	-	-	+	-	-	+	+	++	-	++	++
Section Sycidium														
<i>F. exasperata</i>	5	+	++	++	-	++	+	-	+	++	-	-	++	++
<i>F. montana</i>	5	++	+	-	-	++	+	-	++	++	++	-	-	+
<i>F. parietalis</i>	1	++	+	-	-	-	+	++	-	++	-	-	-	+
No. of species with PCR products		23	22	4	4	21	21	19	11	20	22	14	20	24

These 13 loci were also tested with the same PCR conditions on one to 20 samples of 23 other species of *Ficus* representing 10 of the 17 sections of the genus. Except for loci FinsK9 and FinsU9, the *F. insipida* microsatellites gave an amplification product in most of the species tested, independently of their phylogenetic proximity to *F. insipida* (Table 2), demonstrating the wide potential applicability of these markers. We are currently using these markers to investigate the impact of fragmentation of the Petit Saut population, due to the construction of a dam and the impoundment of a large lake, creating islands of various sizes, on gene flow of *F. insipida*.

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