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Isolation of microsatellite loci for paternity testing in *Phillyrea angustifolia* L. (Oleaceae)

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Male and hermaphrodite individuals co-exist within the same population of *Phillyrea angustifolia* and hermaphrodites produce viable pollen (Lepart & Dommée 1992). However, effective male function of hermaphrodites in populations of *P. angustifolia* is still debated and a direct estimation of male reproductive success via paternity analysis is necessary. Microsatellite loci have been proved to be useful for paternity testing in trees (Chase *et al*. 1996). Here we report 10 primer sets designed to amplify $(GA)_n$ and $(ATT)_n$ microsatellites in *P. angustifolia*.

Nuclear DNA was extracted (Pillen *et al*. 1992) from one individual, digested with the restriction enzyme *Asc*I, and size-fractionated on agarose gel according to Mörchen *et al*. (1996). DNA fragments ranging from 0.3 to 1.5 kb were ligated into *Eco*RI-cut dephosphorylated pUC19 cloning vector. After transformation into DH5α competent cells (Clontech Laboratories), 13 500 recombinant clones were transferred onto nylon membranes with a Biomek 1000 robot (Beckman) and hybridized with 13 ^{32}P-labelled oligonucleotide probes as described by Epplen (1992).

We obtained a total of 317 positive clones: 90 were revealed by the $(TC)_8$ probe, 81 by $(AAC)_5$, 49 by $(TAA)_6$TA, 16 by $(CA)_8$, 14 by $(GATA)_4$, 13 by $(AT)_{12}$, 12 by $(ACG)_5$, 10 by $(GGAT)_4$, 9 by $(GAA)_5$, 8 by $(CAC)_5$, 8 by $(CT)_4(CA)_5$, 4 by $(GCC)_5$, and 3 by $(TAC)_5$. We selected 74 clones and sequenced them on a Li-Cor automated DNA sequencer 4000L, using the Sequitherm Long-Read Cycle Sequencing kit (Epicentre Technology). Eighteen clones contained repeats of at least nine di- or six trinucleotides. Primer pairs were designed using PRIMER3 (Rozen & Skaletsky 1998) with the following parameters: melting temperatures (50–60 °C), GC content (25–60%), and no. 3′ GC clamps.

Total DNA was extracted from frozen leaves using a SDS–PVP method (Saumitou-Laprade *et al*. 1999). Polymerase chain reactions (PCR) were carried out in a total volume of 15 µL containing 10 ng of template DNA, 3.5 mM $MgCl_2$, 200 µg/mL of BSA, 100 µM dNTP, 0.2 µM each primer (one of which was fluorescence labelled either with IRD-700 or IRD-800), 20 mM Tris-HCl (pH 8.3), 50 mM KCl, and 0.4 units of AmpliTaq DNA polymerase (Perkin-Elmer). PCR was carried out on a Perkin-Elmer Gene-Amp system 9600 (95 °C, 5 min, followed by 94 °C, 45 s, annealing temperature (Table 1), 45 s, 72 °C, 30 s, for 30 cycles, final extension 72 °C, 2 min. Amplification products, loaded on 7% denaturing polyacrylamide gels, were analysed on a Li-Cor automated DNA sequencer 4200. For each locus, the allele size was determined relative to the sequenced positive clone using a pUC19 sequence as one nucleotide size standard.

Amplification and polymorphism were tested on a sample of 16 individuals from Languedoc-Roussillon (southern France). Of the 18 primer sets tested, 10 revealed a polymorphic locus (Table 1). These 10 loci were analysed on 33–43 individuals collected in two small populations in Les Salins de Giraud (Camargue, southern France). Number of alleles and allele size range were scored, and values of H_E and H_O were determined using FSTAT software version 2.3 (Goudet 1995). Of the 10 loci presented, seven exhibited more than five alleles, and five loci showed significant differences between expected and observed heterozygosity. These differences were always due to an excess of homozygotes. In addition, we also investigated whether the primers amplify in four other Oleaceae species: *Olea europea*, *Ligustrum vulgare*, *Jasminum fruticans*, and *Fraxinus excelsior*. With the exception of one locus, amplification was successful at least in one of the tested species (Table 2).

Table 1 Primers and characteristics of the polymorphic microsatellite loci in *Phillyrea angustifolia*. Allele size range in base pairs (bp) and number of alleles, gene diversity (H_E), observed frequency of heterozygotes (H_O), and test for Hardy–Weinberg deviation (*P*-values) within the two Salins populations

Accession nos	Locus	Repeat sequence	Primer sequence (5′ to 3′)	Annealing temp. (°C)	Size range (bp)	No. of different alleles	H_E	H_O	*P*-values	No. of individuals tested
AF145338	PA(GA)$_{1}$	(AG)$_{32}$	F: TTTTGAGGATCATTAGCAGCC R: AAATTGGAAATGGATTCCTCT	56	83–117	12	0.809	0.725	0.061	40
AF145339	PA(GA)$_{2}$	(GT)$_{5}$(GA)$_{17}$	F: AAGGAAGATGGGTCTTTGGG R: ATTCAGCTCCTCCACCTTCA	60	111–115	3	0.497	0.558	0.906	43
AF145340	PA(GA)$_{4}$	(AG)$_{25}$	F: CGACGGTCGTAGAATTGGA R: CAATCTACCCACGCCCTAAC	65	58–114	17	0.92	0.636	0.001	33
AF145341	PA(GA)$_{5}$	(GA)$_{12}$	F: TCTTTGCTTCGTTGCTTTTG R: TCTTGCTCCCTCGACATTTT	56	114–156	12	0.88	0.860	0.281	43
AF 145342	PA(GA)$_{6}$	(GT)$_{14}$(GA)$_{13}$	F: AGAGAGAGTTGGGAAAGGGG R: AGATTCAGATGCCGAGGATG	56	103–137	14	0.881	0.814	0.13	43
AF145343	PA(GA)$_{8}$	(AG)$_{30}$	F: TTGTAGTTGGCCATCATATACATTC R: TCACATTGAGATGATAATACGAAAGTT	55	129–178	15	0.904	0.559	0.001	34
AF145344	PA(GA)$_{9}$	(GA)$_{11}$	F: CAACACTCAACAGCCACCAC R: GGACCGTCATTATGTGAGGC	61–56*	144–166	10	0.831	0.595	0.001	42
AF145345	PA(GA)$_{11}$	(GA)$_{13}$	F: TTCCAAAACTCGTTCTCTGATTC R: GGTTACAGCAGTAGTGAGGAGC	56	106–117	5	0.466	0.561	0.991	41
AF145346	PA(ATT)$_{1}$	(TTA)$_{11}$	F: TTCACCCCGTTCAGTTTTTC R: AGAAGCCGAGTAATGAAATTGC	55	71–147	20	0.928	0.690	0.001	42
AF145347	PA(ATT)$_{2}$	(TAA)$_{6}$	F: CACCTCCCGGTTAACAAGAA R: TGACGCGGTTATTTTGTGAA	60	115–136	3	0.193	0.116	0.025	43

* Touch down.

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Table 2 Cross-species amplification with the 10 pairs of *Phillyrea angustifolia* primers. An individual of each species was tested

Species	$PA(GA)_1$	$PA(GA)_2$	$PA(GA)_4$	$PA(GA)_5$	$PA(GA)_6$	$PA(GA)_8$	$PA(GA)_9$	$PA(GA)_{11}$	$PA(ATT)_1$	$PA(ATT)_2$
Olea europea	1	1	m	1	m	0	m	1	m	1
Ligustrum vulgare	m	1	0	1	m	0	2	1	0	0
Jasminum fruticans	0	0	1	2	m	0	0	2	2	0
Fraxinus excelsior	1	2	1	2	1	0	1	2	1	1

0, 1, 2: no, one, or two PCR products detected; m, multiple banding pattern.

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Variable microsatellite loci for the leafcutter ant *Acromyrmex echinatior* and their applicability to related species

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Ants of the tribe Attini are limited to the Neotropics. The 12 known genera comprise approximately 200 species which are all special among the Formicidae because of their ectosymbiotic interaction with a mutualistic fungus (Cherrett *et al*. 1989). This 'unholy alliance' is a profound evolutionary and ecological success story. Leafcutter ants are dominant herbivores in many neotropical ecosystems and genetic studies of their phylogeny and social organization have recently advanced considerably (e.g. Schultz 1999). The genera *Acromyrmex* and *Atta* cover a diverse array of social organization, with some species having multiple queens in at least some of their colonies (Bekkevold *et al*. 1999), whereas other species exhibit the highest level of queen mating so far known in ants (Fjerdingstad *et al*. 1998; Boomsma *et al*. 1999). The recently estimated degree of multiple mating in *Acromyrmex* (Boomsma *et al*. 1999) was determined with genetic markers of low variability (2–3 allozymes and one moderately variable microsatellite marker). This produced accurate estimates of relatedness but could not avoid relatively high nondetection errors of multiple paternity (Boomsma & Ratnieks 1996). To increase the resolution of such studies in the future and to be able to test hypotheses for the evolution of multiple queen-mating more accurately, we describe here five polymorphic microsatellite markers for *Acromyrmex echinatior* and report on their applicability and polymorphism in two other sympatric *Acromyrmex* species, *A. octospinosus* and *A. insinuator*. *Acromyrmex insinuator* is a recently described social parasite of *A. echinatior*, a host species that was previously known only as a form of *A. octospinosus* (Schultz *et al*. 1998).

DNA was extracted by high-salt purification from *A. echinatior* workers preserved in ethanol. The genomic library was constructed by ligating size-selected fragments (200–500 bp) from a *Sau*3A1 digest into the plasmid vector pBluescript II SK+ which was cut with *Bam*HI. Vectors were transformed into competent *Escherichia coli* cells and recombinants were identified by blue–white selection. A total of 2000 recombinant clones was transferred onto nylon membranes which were hybridized with a mixture of three DIG-labelled oligonucleotide probes (CT_{10}, GT_{10} and ATT_{10}). This screening yielded 56 clones with a positive signal. Inserts of the putative microsatellite-containing clones were amplified with T7 and reverse primers. The amplification products were run on a 1%