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Characterization of microsatellite loci in *Coffea arabica* and related coffee species

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Coffee trees (family Rubiaceae) are classified in two genera, *Coffea* and *Psilanthus*. Particular attention has been paid to the genus *Coffea* which includes two cultivated species of economic importance, *C. arabica* L. and *C. canephora* Pierre. *C. arabica* ($2n = 4\times = 44$) is an amphidiploid (Lashermes *et al.* 1999) while other *Coffea* species are diploid ($2n = 2\times = 22$). Molecular phylogenies of *Coffea* species have been successfully established (Cros *et al.* 1998). Those analyses suggest several major clades, which present a strong geographical correspondence (i.e. west Africa, central Africa, east Africa and Madagascar). Further steps would be to study the genetic structure of populations and gene flow between species.

The aim of this present paper is to develop a set of molecular genetic markers, known as simple sequence repeats (SSRs) or microsatellites (Weber & May 1989), suitable for genetic studies of coffee species. Eleven primer pairs that reliably detect microsatellite loci are described. We assessed their potential as genetic markers in the discrimination of *C. arabica* and *C. canephora* genotypes, and examined cross-amplification in various coffee species.

The plant material (55 individuals) resulted from several collecting missions in Africa and Madagascar. The species *C. arabica* are represented by 32 individuals sampled from different locations in Ethiopia and Yemen, while *C. canephora* are represented by 10 individuals collected in the Central African Republic, Congo and Côte-d'Ivoire. A total of 13 *Coffea* taxa were surveyed. The closely related genus *Psilanthus* was also represented by two species, *P. ebracteolatus* and *P. travencorensis*. DNA was isolated from lyophilized leaves through a nuclei isolation step as described by Agwanda *et al.* (1997).

DNA clones from a partial genomic library (*C. arabica* var. Caturra) enriched for $(TG)_{13}$ motifs (Vascotto *et al.* 1999) were sequenced using automated fluorescent technology (ABI sequencer). Oligonucleotide primers complementary to flanking regions of identified repeats were designed for 11 sequences using the computer program Primer3 (Whitehead Institute for Biomedical Research). SSR assays were carried out by means of the polymerase chain reaction (PCR). Reaction mixtures for the PCR amplification of SSR loci contained 25 ng of genomic DNA, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 1.5 mM of $MgCl_2$, 0.2 pmol of each primer, 0.2 mM of dCTP, dGTP, dTTP, 0.01 mM of dATP, 0.8 mCi of [α^{33}P]-dATP (Amersham Pharmacia), and 0.5 U of *Taq* DNA polymerase (Promega) in a 25-μL final volume. Reactions were performed in a PTC-200 thermocycler (MJ Research). The amplification cycle consisted of an initial 2 min denaturation at 94 °C, followed by 5 cycles of denaturation at 94 °C for 45 s, 1 min primer annealing at 60 °C with decreasing temperature of one degree at each cycle, and 1 min 30 s elongation at 72 °C. Then, 30 cycles of 45 s at 90 °C, 1 min at 55 °C and 1 min 30 s at 72 °C were performed and followed by a final 8 min elongation at 72 °C. Amplification products were electrophoresed on 6% denaturing polyacrylamide gel with 8 M urea and 1 × TBE. Radioactively labelled 10-bp ladder DNA was used as a size standard.

The 11 primer pairs were successful in amplification of variable-length fragments (Table 1). Only five of the 11 microsatellite loci appeared to be polymorphic in *C. arabica*. This result illustrated the very low genetic diversity present in *C. arabica* as a consequence of its origin, reproductive biology, and evolution (Lashermes *et al.* 1999). On the other hand, the microsatellite loci showed a broad range of genetic diversity across the accessions of *C. canephora*. The mean heterozygosity values were 0.04 and 0.47 in the predominantly autogamous *C. arabica* and the self-incompatible species *C. canephora*, respectively.

Results of cross-species amplifications with the 11 primer pairs are reported in Table 2. Although designed from sequences

Table 1 Oligonucleotide primer sequences, repeat motifs, PCR product sizes, allele number, and heterozygosity level of 11 microsatellite loci isolated from *Coffea arabica*

Locus	EMBL Accession no.	Repeat motif*	PCR primer sequence (5′ → 3′)	Product size (bp)	Allele number/ Heterozygosity†	
					C. arabica	*C. canephora*
M2a	AJ250250	$(GT)_8/(GT)_6/(GT)_7$	F: AGTGGTAAAAGCCGTTGGTG R: GCGGTTGTTGGTGAGTTGAA	205–218	2/0.00	3/0.40
M3	AJ250251	$(CA)_6/(CA)_3/(CA)_3/(CA)_3/(CA)_4/(CA)_3/(CA)_3/(CA)_3$	F: ATTCTCTCCCCCTCTCTGC R: TGTGTGCGCGTTTTCTTG	248–258	2/0.00	3/0.20
M11	AJ250252	$(GT)_4/(GA)_4/(GT)_4/(GT)_6$	F: ACCCGAAAGAAAGAACCAAG R: CCACACAACTCTCCTCATTC	140–146	1/0.00	2/0.20
M20	AJ250253	$(GA)_5(GT)_8TT(GT)_4TT(GT)_7(GA)_{11}(TC)_2(CT)_3GT$	F: CTTGTTTGAGTCTGTCGCTG R: TTTCCCTCCCAATGTCTGTA	240–270	5/0.00	6/0.70
M24	AJ250254	$(CA)_{15}(CG)_4CA$	F: GGCTCGAGATATCTGTTTAG R: TTTAATGGGCATAGGGTCC	132–166	6/0.13	10/0.80
M25	AJ250255	$(GT)_5CT(GT)_2/(GT)_{12}$	F: CCCTCCCTGCCAGAAGAAGC R: AACCACCGTCCTTTTCCTCG	160–170	3/0.03	3/0.30
M27	AJ250256	$(GT)_{11}$	F: AGGAGGGAGGTGTGGGTGAAG R: AGGGGAGTGGATAAGAAGG	118–134	2/0.00	4/0.60
M29	AJ250257	$(CTCACA)_4/(CA)_9$	F: GACCATTACATTTCACACAC R: GCATTTTGTTGCACACTGTA	103–122	3/0.00	3/0.20
M32	AJ250258	$(CA)_3/(CA)_3/(CA)_{18}$	F: AACTCTCCATTCCCGCATTC R: CTGGGTTTTCTGTGTTCTCG	89–135	7/0.10	6/0.30
M42	AJ250259	$(GT)_3/(GT)_7$	F: ATCCGTCATAATCCAGCGTC R: AGGCCAGGAAGCATGAAAGG	72–103	2/0.00	5/0.70
M47	AJ250260	$(CT)_9(CA)_8/(CT)_4/(CA)_5$	F: TGATGGACAGGAGTTGATGG R: TGCCAATCTACCTACCCCTT	100–132	7/0.10	10/0.80

*Microsatellite motifs are sequenced alleles (*C. arabica* var. Caturra).
†Estimated among samples of 32 and 10 individuals of *C. arabica* and *C. canephora*, respectively.

Table 2 Cross-species amplification using primers designed for *Coffea arabica*. Assays producing amplification are indicated by '+', no amplification is indicated by '–'. One sample tree of each species was tested

Species	Geographical distribution*	Locus										
		M2a	M3	M11	M20	M24	M25	M27	M29	M32	M42	M47
C. liberica	WC	+	+	–	+	+	+	+	+	+	+	+
C. congensis	WC	+	+	+	+	+	+	+	+	+	+	+
C. eugenioides	C	+	–	+	+	+	+	+	+	+	+	+
C. sp. Moloundou	C	+	+	–	+	+	+	+	+	+	+	+
C. pseudozanguebariae	E	–	–	+	+	+	+	+	+	+	+	+
C. salvatrix	E	–	–	–	+	+	–	+	–	+	+	+
C. sessiliflora	E	–	+	+	+	+	+	+	+	+	+	+
C. resinosa	M	–	–	+	+	+	+	+	+	+	+	+
C. perrieri	M	–	+	–	+	+	+	+	+	+	+	+
C. bertrandi	M	–	+	+	+	+	+	+	+	+	+	+
C. dolychophylla	M	–	–	+	+	+	+	+	+	+	+	+
P. travencorensis	I	+	+	–	+	+	+	+	+	+	+	+
P. ebracteolatus	W	–	+	–	+	+	+	+	+	+	+	+

*Endemic geographical distribution of species is indicated by the following letters: 'E' (east Africa), 'M' (Madagascar), 'WC' (west and central Africa), 'C' (central Africa) and 'I' (India).

isolated in *C. arabica*, these primers worked well for most of the diploid coffee species. Representatives of the *Psilanthus* genus consistently yielded amplification products from the primer pairs supporting the hypothesis that species belonging to the two genera, namely *Coffea* and *Psilanthus*, are closely related (Cros *et al.* 1998).

Altogether, the primers described in this paper can provide useful markers to investigate levels of genetic variation in coffee species with respect to germplasm management and genetic study in natural plant populations.

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Microsatellite loci from the marine fish *Nemadactylus macropterus* (Perciformes: Cheilodactylidae)

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Nemadactylus macropterus is a commercially exploited marine fish of New Zealand and southern Australia. Population genetic studies employing allozymes and mitochondrial DNA (mtDNA) did not differentiate *N. macropterus* populations within southern Australia, and identified only slight but significant differentiation between Australia and New Zealand (Elliott & Ward 1994; Grewe *et al.* 1994). Microsatellite analyses conducted on marine taxa have often identified population differentiation not evident from other molecular characteristics (Bentzen *et al.* 1996; O'Connell *et al.* 1998; Shaw *et al.* 1999). Consequently, seven polymorphic microsatellite loci were developed for the study of *N. macropterus*.

Genomic DNA was extracted from frozen liver tissue and purified following a CTAB phenol–chloroform protocol (Hillis *et al.* 1990), and the presence of high molecular weight (> 20 kb) fragments was confirmed by agarose gel electrophoresis. DNA was then digested with *Dpn*II and 400–600 bp fragments were gel purified using the Geneclean Spin Kit (BIO 101, Inc.). A pUC19 vector was similarly digested with *Bam*HI, dephosphorylated with calf intestinal alkaline phosphatase, and gel purified. Ligations were performed in equal molar ratios at 14 °C using T4 DNA ligase. Approximately 100 ng of ligated DNA was transformed into 100 µL of XL1-Blue heat competent cells (Stratagene). Cells were grown on LB plates containing ampicillin, X-gal and IPTG.

Oligonucleotide $[AC]_{16}T$ was 3′ end-labelled with digoxygenin-11-ddUTP using the DIG Oligonucleotide 3′ End-Labelling Kit (Roche). Colony lifts employed Hybond-N nylon membranes (Amersham), and were treated following the instructions for the DIG Luminescent Detection Kit (Roche). Hybridization was conducted at 50 °C for 6–12 h with a probe concentration of 10 pmol/mL, and chemiluminescent detection employed CSPD substrate. Plasmids from 31 positive colonies were miniprep purified (Sambrook *et al.* 1989). Insert sequences were polymerase chain reaction (PCR) amplified using M13 primers, gel purified and sequenced with the ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit (Perkin-Elmer).

Seven PCR primer pairs (Table 1) were designed for microsatellite amplification using PrimerSelect (DNASTAR). One primer for each locus was 5′ end-labelled with FAM or HEX dye. The 3′ thymine-rich primer for locus *Nma106* produced a nonspecific product of approximately 180 bp, and therefore the opposite primer was labelled. *Nma187* and *Nma245* were developed from the same clone. Other sequences useful for the design of microsatellite-amplifying PCR primers are available in GenBank (accession nos AF125121–AF125138).

PCR amplifications of microsatellites were conducted individually in 20 µL volumes comprising 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg/mL gelatin, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 units *Taq* F1 DNA polymerase (Fisher Biotech), 0.4 mM of each oligonucleotide primer, and approximately 20 ng of DNA. Thermal cycling conditions comprised 35 cycles of 94 °C/30 s, 62.5 °C/30 s, and 72 °C/60 s, using a Perkin-Elmer 9600 machine. An initial denaturation of 94 °C/5 min and a final extension of 72 °C/10 min were employed. PCR products were mixed in appropriate ratios (Table 1) to achieve even peak heights when multiplexed on an ABI 377 (Perkin-Elmer), and alleles were scored relative to the GS500 size standard. The PCR primers were tested on the following cheilodactylids and successfully amplified homologous microsatellites: *N. douglasii*, *N. valenciennesi*, *N. bergi*, *Nemadactylus* sp., *Acantholatris monodactylus*, and *A. gayi*.

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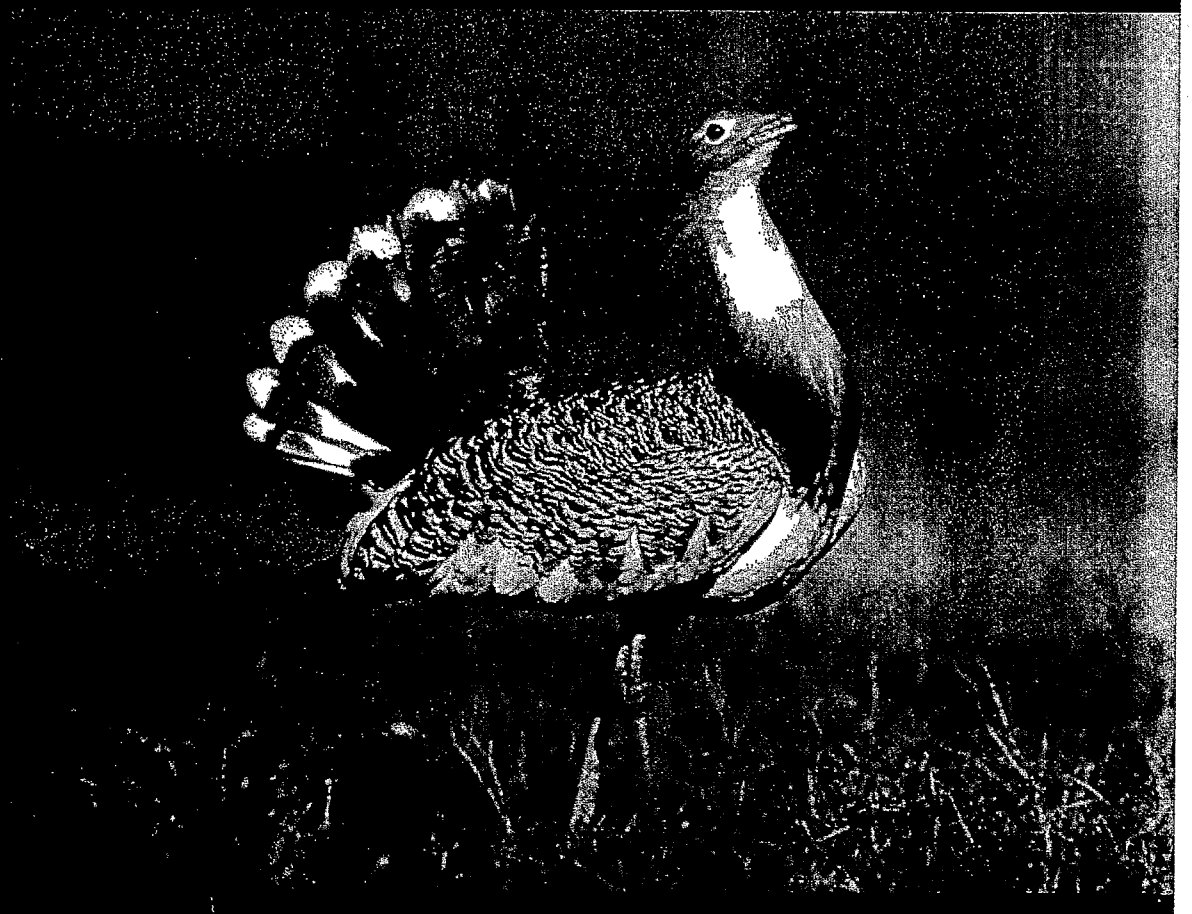

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