Chapter 9

MICROSATELLITES IN *COFFEA ARABICA* L.

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Running title: Microsatellites in coffee

1. Introduction

DNA polymorphism has became a widespread tool in biotechnology; in fact, they are frequently used for a number of technical approaches as, for example, in agronomic traits identification, variety characterisation, and marker-assisted breeding programmes. *Coffea arabica* is expected to show polymorphic DNA sequences as any other species and indeed some polymorphisms have been described. However, it has been reported that restriction fragment length polymorphism (RFLP) (Lashermes *et al.*, 1996a) and polymorphism based on polymerase chain reactions (PCR, RAPD) (Orozco-Castillo *et al.*, 1994; Lashermes *et al.*, 1996b) have a relatively low degree of polymorphism (Paillard *et al.*, 1993, 1996). To-date, a high degree of polymorphism has been found only through AFLP (Lashermes *et al.*, 2000). As this species of coffee is autogamous and has a restricted genetic base, its heterozygosity is expected to be relatively low and the probability of finding a polymorphism is correspondingly reduced.

Microsatellites, also known as simple sequence repeats (SSRs), are produced by tandem repetition of sequences from 1 to 6 bp long and constitute highly informative markers. Indeed, they are abundant and are distributed uniformly and randomly in the euchromatin of eukaryotic genomes (Tautz and Renz, 1984; Wang *et al.*, 1994). They are inherited in a codominant Mendelian manner, are somatically stable and highly polymorphic. The polymorphisms are the result of the variation in the number of the

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repeated monomers. Primers can be designed on the single sequences flanking the microsatellite and then used to amplify, by PCR, the locus in various genotypes. Simple gel electrophoresis reveals polymorphic variations in the size of the amplification product.

Microsatellites are considered as the class of genetic elements with generally highly polymorphic sequences, which might provide a reasonable number of genetic markers. They have been found useful as genetic markers in a number of plants such as soy (Akkaya *et al.*, 1992; Morgante and Olivieri, 1993; Morgante *et al.*, 1994), *Arabidopsis* (Bell and Ecker, 1994), barley (Saghai-Maroof *et al.*, 1994), rice (Wu and Tanksley, 1993), Cucurbitaceae (Katzir *et al.*, 1996), corn (Ma *et al.*, 1996), and tomato (Broun and Tanksley, 1996).

We made an attempt to identify the polymorphic microsatellites in *C. arabica*, starting from two genomic libraries, one enriched in $(ATC)_n$ microsatellite and the other in $(TG)_n$, and describe the results in this article.

2. Experimental

2.1. PLANTS AND DNA EXTRACTION

DNA from *C. arabica* var. Caturra was used to construct the genomic libraries. To identify polymorphisms, the following genotypes were analysed: *C. arabica* variety Caturra, *C. arabica* ET-30 (Ethiopia), 12 plants belonging to F_2 generation (*C. arabica* var. ET-30 x *C. arabica* var. Caturra, IRD, Montpellier) and a number of plants of the cultivars Mundo Novo and Bourbon. DNA was extracted from lyophilised leaves following the method described by Murray and Thompson (1980) and Orozco-Castillo *et al.* (1994), as modified by Vascotto et al. (1999).

2.2. CONSTRUCTION AND SCREENING OF THE GENOMIC LIBRARIES

Two genomic libraries were constructed, one enriched in the microsatellite $(ATC)_n$ and one enriched in $(TG)_n$ (Morgante *et al.*, 1998; Rafalski *et al.*, 1996). It involved enrichment prior to cloning and the creation of fragments of DNA with known sequence extremities obtained by binding adapters to them (Karagyozov *et al.*, 1993; Kandpal *et al.*, 1994). The genome of *C. arabica* var. Caturra was partially digested with the enzyme *Tsp509I* (New England Biolabs, USA). Enrichment of the genomic libraries was obtained by selecting fragments containing a microsatellite on magnetic

beads covered by strepavidin (Boehringer), conjugated with the biotinilated oligonucleotides $(ATC)_{10}$, or $(TG)_{13}$. The fragments of 200-600 bp, thus selected were cloned at the site of *EcoRI* of the vector Lambda ZAP II (Stratagene, La Jolla, California).

In the case of the genomic library enriched in the microsatellite $(ATC)_n$, the phage plaques were transferred on a nylon membrane Biodyne Plus (Pall) and screened by a ^{33}P- $(ATC)_{10}$ probe in a solution of 5xSSC, 1x blocking reagent (Boheringer Mannheim), 0.1% laurilsarcosina, 0.02x SDS. Two washing (5 min each) with 0.5x SSC and 0.1% SDS at room temperature were carried out, followed by further two washing for 15 min each with the same solution at 45°C. In the case of the TG-enriched genomic library, the phage plaques were screened by a 5'-$(DIG)_3(TG)_{13}$ (Oswel) probe in the same solution as the other probe. Two washing of 5 min each with 2x SSC and 0.1% SDS at room temperature were carried out, followed by further two washing for 15 min each with 1xSSC and 0.1% SDS at 60°C.

2.3. DNA SEQUENCING AND DESIGN OF PRIMERS

The positive clones were sequenced using an automatic sequencer, ABI 373A (Perkin Elmer), following a full scan method. A Thermo Sequence dye terminator cycle sequencing pre-mix kit (Amersham Pharmacia Biotec) was used for the sequencing, following the manufacturer's instructions. Primers were designed on the single sequences flanking the microsatellites using either the Primer3 programme (Whitehead Institute for Biomedical Research of Cambridge, Massachusetts, USA) or *Primers!* for the world wide web (Williamstone Enterprises). Where ever possible, all primers were designed in such a way as to have a T_m of around 58°C, a dimension of 20-22 bp as to obtain an amplification product of 100-300 bp. The primers were designed with the same characteristics in order to amplify all the sequences containing microsatellites under the same amplification conditions. A constant KS tail (5'-TCGAGGTCGACGGTATC-3') was added to one of the two primers for each primer pair. The primers were synthesised by Genset.

2.4. PCR AMPLIFICATION OF THE MICROSATELLITES

Each microsatellite was amplified with a "touchdown" PCR to increase the reaction specificity (Don *et al.*, 1991; Hecker and Roux, 1996; Mellersh and Sampson, 1993). The amplification product was fluorescently labelled via a 3-primer system: primer, 1) was locus specific primer; 2) was the other locus specific primer with the KS constant

tail primer; 3) was the KS primer whose 5' end was conjugated to a fluorescent label (either 6-FAM, 6-carbossifluoresceina, or JOE, 2',7'-dimetossi-4',5'-dicloro-6-carbossifluoresceina). During the first cycles of amplification the pair of locus-specific primers amplified the microsatellite containing sequence and inserted the KS complementary tail, which was primed by the fluorescent KS primer during the successive amplification cycles.

The amplification reaction mix consisted of primer, 100 nM each; fluorescent KS primer, 22 nM; dNTP 200 _M each, Taq-polymerase (Genenco) 0.625 U, 50 ng of genomic DNA, buffer 1x (Tris-HCl 100 mM pH 9.0, KCl 500 mM, TritonX-100 1%), $MgCl_2$ 1.5 mM in a total volume of 25 $^{-1}$. Amplification conditions were: 6 cycles of denaturation 45 sec. at 94°C; elongation for 45 sec at 72°C; annealing for 45 sec, gradually reducing the temperature by 1°C every cycle from 60 to 55°C, followed by a further 34 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec elongation at 72°C for 30 sec. The reaction ended with an elongation at 72°C for 8 min. The PCR was carried out in a PTC-200 (MJ Research) thermocycler.

2.5. ANALYSIS TO IDENTIFY POLYMORPHISMS

The amplification products were analysed with an ABI 373A automatic sequencer using the GENESCAN 672 (Perkin Elmer) programme on sequencing gel containing 4.75% polyacrylamide (acrylamide/bisacrylammide 19:1), urea 8.3 M and TBE 1x. The run lasted 10 hours. The volume of the sample analysed in the gel varied from 1-15^{-1} depending on the efficiency of the amplification

3. Results

3.1. ANALYSIS OF THE TWO GENOMIC LIBRARIES

Approximately 3,400 clones of the ATC genomic library were screened, 189 clones were positive (c. 6%) and 111 were sequenced. As regards (TG)n microsatellites, approximately 4,400 clones were screened and 503 found positive, of which 236 were sequenced. A total of over 100 Kbp was sequenced. Approximately 70% of the clones contained a microsatellite sequence. The genome of *C. arabica* contained microsatellite sequences just as any other species.

A total of 69 $(ATC)_n$ microsatellites and 180 $(TG)_n$ microsatellites were found. The ATC microsatellites had an average length of seven repetitions, and a maximum length

of 14 repetitions (Table 1). With regard to the structural characteristics of these microsatellites, $(ATC)_n$ repetitions were rarely compound [in these cases the sequence repeated in tandem adjacent to the $(ATC)_n$ microsatellite was GAC, GCC, GAA and ATT], and generally there was a prevalence of sequences repeated in perfect tandem. The average length of the microsatellites tended to increase as the length of the repeated unit decreased. The average length of the $(TG)_n$ microsatellites was found to be 10 repetitions with a maximum of 27. The most frequent sequence repeated in tandem adjacent to the $(TG)_n$ microsatellite was TC.

Table 1 shows the main characteristics of the microsatellites sequenced. Only 20% of the clones were suitable for primer design: 22 and 24 pairs of primers for $(ATC)_n$ and $(TG)_n$ microsatellite loci respectively. The primers were all initially tested on genomic DNA of *C. arabica* var. Caturra to confirm that they were able to give an amplification product of the desired size.

Table 1. Main characteristics of the ATC and the TG microsatellites.

Library	Number of repeats				Simple		Compound	
	≤ 6	7-10	11-14	>14	Perfect	Imperfect	Perfect	Imperfect
ATC	27	37	5	-	33	25	9	2
TG	21	58	38	63	78	59	24	19

Table 2 reports the sequences of the microsatellites and the size of the amplification products obtained. The two genomic libraries differed greatly in amplification efficiency: only a third of the primers gave an amplification product in the ATC genomic library whereas the primers designed for the TG library gave positive results in approximately 85% cases.

3.2. IDENTIFICATION OF POLYMORPHISMS

Two criteria were applied to distinguish the real alleles from any aspecific amplification product:

a) consideration was given only to clearly defined peaks of high intensity which,
b) showed an effective segregation in F_2. Table 3 shows the sequences of the primers, the number of alleles and of genotypes recorded for each polymorphic locus. The number of alleles and genotypes should, however, be considered provisional. The analyses of other cultivars should lead to further alleles and genotypes being identified. Almost all the polymorphic loci analysed showed one or two alleles per plant (Fig.1); presumably they were homozygotes, or heterozygotes, respectively. Only the locus E12-3CTG showed a profile with 3 or 4 peaks, three always being

present while one was variable. It is possible that in this case the pair of primers amplified a number of independent loci. Overall, $(TG)_n$ microsatellite loci proved to be more polymorphic than $(ATC)_n$; 12 out of 20 $(TG)_n$ loci proved to be polymorphic whereas only one out of 12 $(ATC)_n$ loci analysed was polymorphic.

Table 2. Microsatellite repeat and expected size of the amplification product.

	TG Library			ATC Library	
Locus	Repeat	Product (bp)	Locus	Repeat	Product (bp)
14-2CTG	$(CA)_7$	130	10-1CATC	$(ATC)_6$	187
17-2CTG	$(TC)_{14}(CA)_{11}(CA)_{16}$	217	2-1CATC	$(ATC)_8$	205
25-2CTG	$(CT)_{16}(TG)_{10}$	138	6-1CATC	$(GAT)_6$	195
28-2CTG	$(CA)_{15}$	155	A2-2CATC	$(GAT)_7$	152
30-2CTG	$(CA)_{11}$	232	B4-2CATC	$(ATC)_7$	199
32-2CTG	$(CA)_{12}$	128	B7-2CATC	$(ATC)_8$	246
38-2CTG	$(TG)_{11}(GA)_6$	100	B9-2CATC	$(GAT)_6$	290
4-1CTG	$(TG)_8$	117	C2-2CATC	$(ATC)_{14}$	234
7-1CTG	$(TG)_{20}$	191	C4-2CATC	$(ATC)_6$	304
E10-3CTG	$(CA)_7$	136	D10-2CATC	$(GAT)_8$	286
E11-3CTG	$(CA)_8$	175	D4-2CATC	$(ATC)_8$	192
E12-3CTG	$(CA + TA)_{38}$	150	D9-2CATC	$(GAT)_{10}$	307
E5-3CTG	$(CA)_{13}$	211	E1-2CATC	$(GAT)_6$	421
E6-3CTG	$(TG)_{16}$	341	F6-2CATC	$(ATC)_6$	126
E7-3CTG	$(CA)_{10}$	215	F7-2CATC	$(ATC)_4$	182
E8-3CTG	$(CA)_{14}$	198	F8-2CATC	$(GAT)_6$	233
F1-3CTG	$(CA)_{10}$	201	F9-2CATC	$(ATC)_{14}$	172
F9-3CTG	$(CT)_7(CA)_{11}$	137	H10-2CATC	$(ATC)_{14}$	221
G1-3CTG	$(TG)_{13}$	198	H12-2CATC	$(GAT)_6$	236
I2-3CTG	$(TG)_{17}$	173	H6-2CATC	$(ATC)_6$	220
I5-3CTG	$(TG)_{17}$	150	H9-2CATC	$(ATC)_9$	223
I6-3CTG	$(TG)_{15}$	139	I3-2CATC	$(GAT)_{11}$	217
I7-3CTG	$(TA)_5(TG)_{17}$	142			
I9-3CTG	$(TG)_{21}$	212			

Table 3. Primer sequences and alleles

Locus	Foreward primer (5'→3')	Reverse primer (5'→3')	N.of alleles	N. of genotypes
C2-2CATC	CTCTCCCTCAGTCAATTCCA	CTTGGTCTCCCTCCTTTTTC	3	3
4-1CTG	AAAAAGCTGGTCCATGTCAA	GGGGCGTTCAGTTATAAACA	2	3
14-2CTG	TTTTCTTGCTAATCTTTGAGGA	ACTCTAATGGGGTCATGTGG	3	3
17-2CTG	AGGCCTTCATCTCAAAAACC	AGCGTTACTTGAGGCAAAGA	3	2
32-2CTG	AAGGGGAGTGGATAAGAAGG	GGCTGGATTTGTGCTTTAAG	4	4
E6-3CTG	CTGGGTTGGTTCTGATTTTG	GGTTCCCAGAGATTCTCTCC	5	4
E7-3CTG	TGACATAGGGGGCTAAATTG	TTAATGGTGACGCTTTGATG	4	3
E8-3CTG	CACTGGCATTAGAAAGCACC	GGCAAAGTCAATGATGACTC	2	2
E10-3CTG	ATGCCAAGTCGGAAAAGAA	GGCAAGCTCTAGCCTTTGA	2	3
E11-3CTG	AGTGATCTTCGCAGCCATT	TCTTTTTGTGACTGGGCTTC	2	2
E12-3CTG	TGCTTAGGCACTTGATATAGGA	CACGTGCAAGTCACATACTTTA	4	2
G1-3CTG	TGTTGCTGAACTGTGTTGCT	TCCAGAGAAATGTCGGAAGT	2	2
I9-3CTG	TGGCCGTGATAATAAACAGC	ATGTGGCAATCTAAAGCCAA	3	3

4. Discussion

The results reported here were obtained from two DNA genomic libraries of *C. arabica* var. Caturra, enriched in the microsatellite $(ATC)_n$ or $(TG)_n$. They are the first genomic libraries of this type constructed for this species. From the results of the library screening, it is not possible to estimate the frequency of the $(ATC)_n$, or the $(TG)_n$ microsatellites in the *C. arabica* genome. Studies have been conducted on genomic libraries in which the inserts of DNA fragments were not random. The enrichment was certainly substantial but, in the absence of a non-enriched reference genomic library, a precise estimate was not possible.

Although the two libraries allowed the design of almost the same number of primers (22 and 24 pairs of primers), approximately only 50% of the primer pairs tested for amplification of an $(ATC)_n$ microsatellite gave an amplification product. Much better results were obtained with the $(TG)_n$ microsatellites in which 85% of the primers gave an amplification product. Trinucleotide repetitions seemed to be more difficult to analyse, similarly to what was found in rice (Panaud *et al.*, 1996). The cause has still to be ascertained; it might be partially due to the use of a single set of amplification conditions for all the microsatellites and it could be possible that by varying the reaction conditions, an amplification product might be obtained from at least some of these primers. We considered it more important to maintain the same amplification conditions rather than to optimise amplification reactions, which would the development of multiplex, i.e. simultaneous amplification and analysis of several loci.

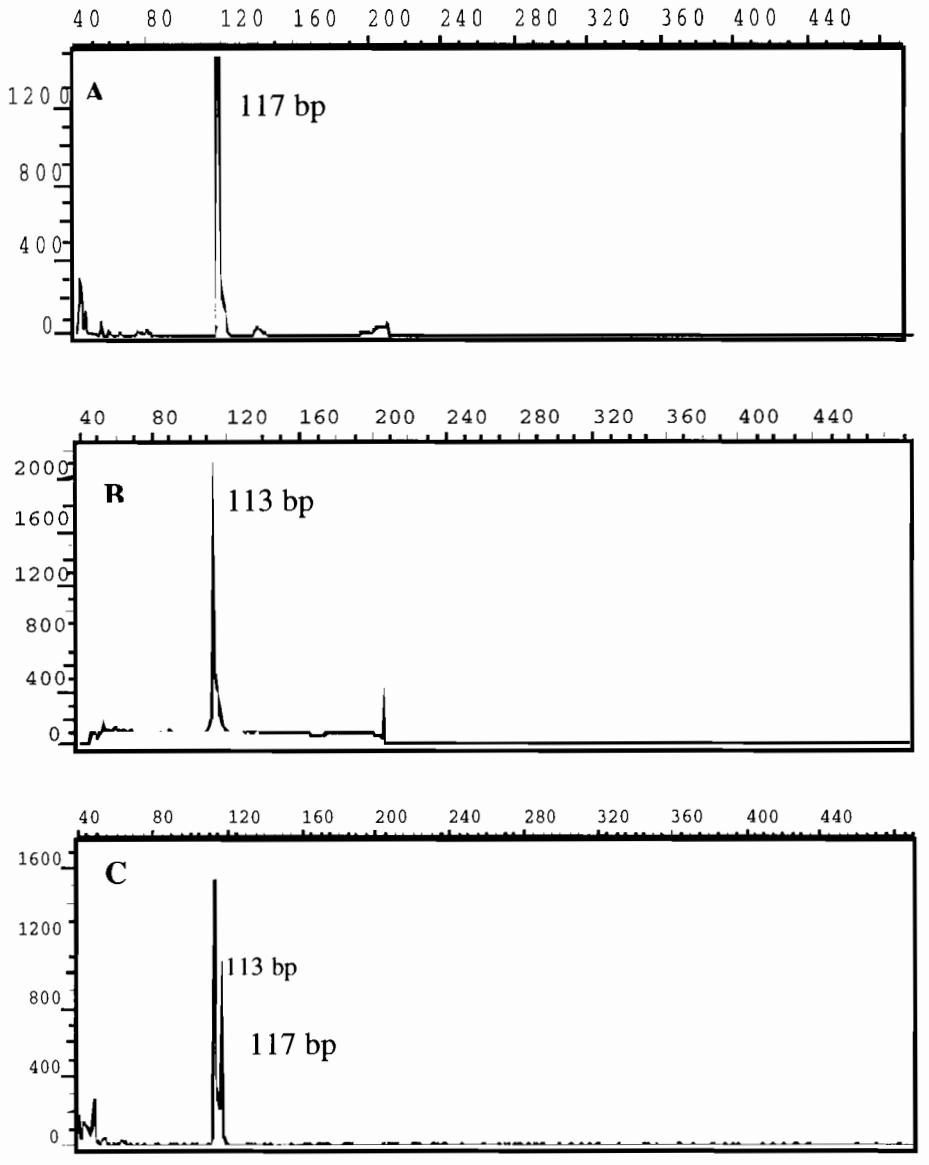

Figure 1. Electropherograms of three segregating plants for the 4-1CTG locus: Pane A: F_2-19; pane B: F_2-8; pane C: F_2-13. Both the parental Caturra and ET-30 showed the same alleles as for F_2-13. The numbers close to the peaks indicates the allele expressed in bp.

The amplification product of each locus gave sometimes several bands of variable intensity, most probably because of the Taq-polymerase slippage during the amplification reaction (Levinson and Gutman, 1987; Schlotterer and Tautz, 1992). However, on the basis of the intensity of the fluorescent signal and of its reproducibility, it was possible to identify the alleles. The segregation of the peaks in the F_2 plants greatly simplified the identification of real alleles.

As *C. arabica* is a tetraploid species, one particularly interesting question concerns the total number of alleles which may be recorded in one single plant, following the amplification of a single microsatellite. In theory, from one (in homozygotes) to two alleles (in heterozygotes) per plant might be expected, if our primers were locus specific for one of the two ancestral genomes. We might have been able to obtain even four bands per plant, if one pair of primers had simultaneously amplified both the pairs of ancestral chromosomes. In reality, the majority of the polymorphic systems analysed showed single bands in at least one plant out of the 12 F_2 plants. A typical example was the 4-1CTG locus in which both the parental lines had two alleles (113bp and 117bp) whereas one F_2 plant showed the 113bp allele only, two F_2 plants gave the 117bp allele only an and all the others gave both alleles. In this case, it is clear that the 4-1CTG locus shows a diploid-type segregation and a similar behaviour has been observed in a further 6 polymorphic loci. In the remaining polymorphic genetic systems it was not possible to distinguish with certainty between diploid and tetraploid behaviour.

It is also interesting to compare the two genomic libraries in relation to the number of bands simultaneously present in the single plants regardless of polymorphisms. The vast majority of ATC loci was monomorph and revealed a single band, whereas a substantial proportion of TG loci revealed two bands, which were present in all the plants analysed even for loci classified as monomorphs. Given the limited number of cultivars analysed we cannot say whether these latter cases were heterozygotes for 1 locus or homozygotes for 2 loci. This doubt should be resolved by extending the analysis to a reasonable number of different varieties.

5. Conclusion and perspectives

The genome of *C. arabica* proved to carry polymorphic microsatellite sequences, as expected. Furthermore some of the primer pairs here described appeared to amplify single loci of the homologous chromosome pair; *i.e.* they were able to discriminate between the two chromosome sets derived from the diploid donor ancestral plants. This result, when confirmed by a consistent behaviour of other loci, could offer an interesting key for studying the origin of the two sets of chromosomes, the actual contribution of

the two diploid progenitors as well as the possible functional evolution of the homologous loci within this organism.

The apparent diploid behaviour of some of the microsatellites introduces an optimistic note in the project of constructing a first genetic map of *C. arabica*. Indubitably, this task would be facilitated if a single locus could unambiguously be attributed to a single linkage group. The hypervariability and co-dominant Mendelian heredity of the microsatellites described here could offer a number of advantages in marker assisted breeding programmes.

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