Progeny Analysis by Microsatellites in Crosses of *Coffea arabica* L.

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SUMMARY

Genetic polymorphisms are a fundamental tool for studying the rules governing inheritance as well as for tracing specific genes in breeding programmes. In the last decade it was discover that DNA itself is a major source of polymorphism. *Coffea arabica* is expected to show polymorphic DNA sequences as any other species even if, this species could show a low level of genetic variability because of the restricted genetic base of the cultivated varieties and because of self-fertility. Nevertheless we could identify some DNA sequences which show a high degree of polymorphism, namely microsatellites.

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. They are inherited in a codominant Mendelian manner and they are somatically stable. The polymorphisms are the result of the variation in the number of the repeated monomers. Primers can be designed on the single sequences flanking the microsatellite and then used to amplify the locus by PCR. Simple gel electrophoresis reveals polymorphic variations in the size of the amplification product. Microsatellites have already been found to be useful as genetic markers in a number of plants such as soy bean, *Arabidopsis*, barley, rice, corn, tomato and other plants.

Here we report the analysis of two *C.arabica* crosses with some microsatellite to show their Mendelian inheritance and their use in molecular assisted breeding programmes.

In the first cross we analysed 51 microsatellite and the parents showed different alleles for 4 systems. All the progeny had the same alleles as expected by Mendelian inheritance. For instance, the analysis of the E10-3CTG microsatellite gave the following results: the mother was homozygote for the 135bp allele and the father was homozygote for the 137bp allele, all the progeny was heterozygote 135/137. This cross was performed with the aim of selecting plants resistant to nematodes and we should be able to follow the inheritance of the resistance gene/s when more polymorphisms become available.

The second cross was analysed with 30 microsatellites, two of which proved to be homozygote in the parents while all the progeny was heterozygote as reported in the table below.

Microsat.	PARENTS		F_1 PROGENY					
	Icatuai	IAPAR-59	I-30-1-1	I-30-1-2	I-30-2-1	I-30-2-2	I-30-3-1	I-30-3-2
34-6CTG	108/108	112/112	108/112	108/112	108/112	108/112	108/112	108/112
37/6CTG	121/121	119/119	119/121	119/121	119/121	119/121	119/121	119/121

More microsatellite should be developed and their association to interesting gene should be established for a practical use in breeding programmes.

INTRODUCTION

The cultivated varieties of *Coffea arabica* show a very low level of genetic diversity (Bertraud and Charrier, 1988) due to autogamy and the limited number of original plants from which the main cultivars were derived. Consequently, there are very few allelic variants, making it difficult to find polymorphisms. Numerous techniques have been applied to study polymorphisms in *C. arabica*, such as RFLP (Lashermes et al., 1996a), RAPD (Orozco-Castillo, 1994; Lashermes et al., 1996b), and AFLP (Lashermes et al., 2000), and positive results have in fact been obtained. Nonetheless, the same techniques applied to other species have provided a greater number of polymorphic loci.

Another approach to studying variability in *C. arabica* and identifying polymorphism is the analysis of microsatellites. These highly polymorphic repeated sequences are very informative molecular markers because they are codominant and therefore, in contrast to the abovementioned techniques, enable the heterozygous samples to be distinguished from the homozygous. Due to these characteristics microsatellites are powerful tools for following specific genes in assisted cross programmes.

In this paper we describe the analysis by microsatellites of different crosses of *Coffea arabica* and of its progeny, F_1 or F_2.

Furthermore, we begun a project of RFLP analysis of different varieties of *C. arabica* and of *C. canephora* for coding sequencies. The aim of this is twofold: firstly, to find markers for the construction of a low density genetic map and secondly, to create the possibility of finding differences within the genes of *Coffea arabica*, that is, polymorphisms capable of marking the expressed genes

MATERIALS AND METHOD

Amplification of the microsatellites

The microsatellites used for the analysis of the crosses were identified and amplified as described in Rovelli et al. (2000), beginning with two genomic libraries of *C. a.* var Caturra enriched in di- tri- nucleotides TG and ATC.

Identification of the polymorphic microsatellites

The primers which amplified the microsatellites were tested on DNA of the parent samples of three crosses; if the microsatellites presented different alleles from their parents, we proceeded with the analysis of the progeny.

The amplification products were analysed on sequencing gel with an ABI automatic sequencer and the length of the fragments containing microsatellites was calculated with the GENESCAN 672 (Perkin Elmer) programme.

Samples

The samples examined were: 1) *C. a.* var Caturra x *C. a.* var Ethiopica ET-30 cross, and 96 plants of the F2 population, originating from IRD (Montpellier, France); 2) *C. a.* introgressed genotype Catimor x *C.a.* var Icatual cross, and 6 plants of the F1 population, originating from IAPAR (Londrina, Brazil); 3) *C. a.* introgressed genotype Sarchimor x *C. a.* var Ethiopica ET-6 cross and 17 plants of the F1 population, originating from CATIE (Turrialba, Costa Rica).

The DNA was extracted with a modification of the method of Murray and Thompson (1980) and Orozco-Castillo et al. (1994).

RFLP analysis

The fragments of DNA examined are traits of genomic DNA amplified with primers designed on EST sequences; the EST were derived from a genomic library of radical meristems of *C. a.* var Bourbon red.

For the RFLP analysis we chose those fragments which provided an amplification from genomic DNA of greater length than the corresponding cDNA, and which therefore presumably contained introns.

RESULTS

Analysis of the crosses

The *C. a.* var Caturra x *C. a.* var Et-30 cross was analysed with 59 microsatellites. Only five of these proved to be polymorphic in the parental samples. The F_2 population of 96 plants was analysed with 5 microsatellites to examine the distribution of the alleles. Table 1 summarises the results.

Three out of 28 microsatellites analyses in the introgressed genotype Catimor x *C. a.* var Icatual cross demonstrated different alleles in the parents, and therefore we analysed the 6 plants of the F_1 population. Table 2 shows the alleles, expressed in bp, relative to the three polymorphic microsatellites.

The introgressed genotype Sarchimor x *C. a.* var Et-6 cross was analyses with 51 microsatellites and four were polymorphic in the parents, and of these we analysed the 17 F_1 plants. The alleles of these are shown in Table 3.
In total 8 polymorphic microsatellites were identified.

RFLP analysis

48 EST sequences were amplified of which 13 produced a genomic amplification with a length greater than the corresponding EST. We therefore analysed these 13 genomic loci, presuming that they also contained introns, where mutations are more likely to accumulate. Table 4 shows the names of the genes with which the EST sequences were the most homologous in the database.

Varieties of *C. arabica* (Ethiopica, Caturra, Mundo Novo, Laurina and a wild variety) and *C. canephora* were examined.

These traits of genomic DNA were analysed with 8-11 restriction enzymes (AciI, AluI, BamHI, EcoRI, FokI, HindIII, HinfI, HphI, MboI, MnlI, MspI, Tsp45I, Tsp509I). The analysis of the restriction patterns to date have not uncovered differences between the varieties of *C. arabica* in the size and number of bands.

Table 1.

Microsatellite	Alleles (expressed in bp)		
	C.a. Caturra	C.a. Et-30	F 2
I9-3CTG	200-214	198-200-214	36 samples: 200-214 48 samples: 198-200-214 12 samples: 198-214
17-2CTG	204-215	202-215	39 samples: 204-215 32 samples: 202-204-215 25 samples: 202-215
32-2CTG	121-128	119-126	40 samples: 119-121 56:121-126
E10-3CTG	135	137	23 samples: 135 43 samples: 135-137 30 samples: 137
14-2CTG	130	128-130	96 samples: 130

Table 2.

Microsatellite	Alleles (expressed in bp)		
	Catimor	C.a. Icatuai	F1
20-6CTG	105-109	105-107-109	2 samples: 105-109 4 samples: 105-107-109
37-6CTG	119	121	6 samples: 119-121
24-4CTG	112	108	6 samples: 108-112

Table 3.

Microsatellite	Alleles (expressed in bp)		
	Sarchimor	C.a. Et-6	F1
I9-3CTG	200-214	198-200-214	10 samples: 200-214 7 samples: 198-200-214
14-2CTG	204-217	202-217	17 samples: 202-204-217
E10-3CTG	135	137	17 samples: 135-137
20-6CTG	104-106-108	104-106	17 samples: 104-106-108

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Varieties of *C. arabica* (Ethiopica, Caturra, Mundo Novo, Laurina and a wild variety) and *C. canephora* were examined.

Nonetheless, we could identify 5 different patterns between the two species of *Coffea arabica* and *Coffea canephora*. Table 5 shows the size of the different bands of the two species.

These traits of genomic DNA were analysed with 8-11 restriction enzymes (AciI, AluI, BamHI, EcoRI, FokI, HindIII, HinfI, HphI, MboI, MnlI, MspI, Tsp45I, Tsp509I). The analysis of the restriction patterns to date have not uncovered differences between the varieties of *C. arabica* in the size and number of bands.

Table 4.

Clone	Homology	Clone	Homology
RM-0-L19	unknown	RM D04	thioredoxin h
RM B11	translationally controlled tumor protein (TCTP)	RM-0-I05	antimicrobial peptides precursor
RM A11	A.thaliana hypothetical protein	RM-0-E12	germin-like protein
RM C05	chlorophyll a/b-binding protein	RM B08	unknown
RMi-5-B10	60S ribosomal protein L22	RM B10	40S ribosomal protein S23
RMi-1-E03	cysteine proteinase	RM A01	60S ribosomal protein L34
RM C11	A.thaliana hypothetical protein		

DISCUSSION

In total we identified 8 polymorphic microsatellites in varieties of *C. arabica* and 5 polymorphic restriction sites amongst *C. arabica* and *C. canephora*.

Table 5.

EST	ENZYME	*C. canephora* (in bp)	*C. arabica* (in bp)
RM A11	MnlI	600	400 + 200
Rmi-5-B10	MspI	not cut	900 + 800
RM C11	AluI	600	500 + 100
RM C11	MboI	not cut	700 + 300
RM D04	MspI	800 + 100	not cut

With regard to the microsatellites, we found between one and three alleles in the parental samples. Where there were two or three alleles, one of these, such as the microsatellites I9-3CTG or 17-2CTG, was always present both in the parents and the progeny. We therefore hypothesised that another locus was involved which became coamplified. Only the microsatellite 20-6CTG in the *C. a.* Sarchimor x *C. a.* Et-6 cross presents three alleles in one parent and the progeny, but two alleles in the other parent. In this case there could be three alleles for the same locus; however, double haploid plants would be necessary to verify this.

The analysis of the microsatellites such as E10-3CTG, 37-6CTG or 24-4CTG suggests that even in this allotetraploid species the microsatellites are inherited in accordance with Medel's

laws: when the parental samples displayed one allele in homozygosis but the parents had different alleles, then the F_2 displays the classic Mendelian distribution of 1:2:1.

The microsatellites can be used to follow the traces of certain genes within specific crosses. Indeed, the *C. a.* Sarchimor x *C. a.* Et-6 cross was produced with the intention of selecting plants resistant to nematodes. With the identification of new polymorphic microsatellites we should be able to verify the distribution of the progeny of genes linked to resistance.

In general it is worth noting that the parental samples are heterozygotes only in 7 out of 24 loci: this demonstrates the high level of homozygosity in which the species *C. arabica* is found and explains the high genetic uniformity of the species.

The RFLP polymorphisms to date have not provided positive results within the species *C. arabica*, but even polymorphisms between the two species are highly useful. Moreover, each polymorphism found in this manner is related to differences in coding regions which could account for the phenotypic differences between the two species, as well as identify the genes originating from *C. canephora* in inter-specific crosses, in which attempts are made to bring the positive qualities of resistance of *C. canephora* to the cultivated varieties of *C. arabica*.

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