MAIN DNA MOLECULAR MARKERS AND THEIR USE IN BREEDING PROGRAMMES AND FOR CHROMOSOME MAPPING

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Introduction

These days, you can buy for a couple of hundreds dollars a GPS (Global Positioning System). This pocket instrument allows you to determine your co-ordinate position on land, at sea or in the air with the approximation of a few metres. But suppose you do not have access to this instrument and you are travelling on the motorway from Rome to Venice and you want, moreover, to make a stopover in Florence. Imagine also that some joker has cancelled all the signposts along the motorway. Without GPS or signposts, there would be no way of knowing where you are and when you should get off the motorway to visit Florence or Venice.

As far as Coffea arabica is concerned, we can compare the genome of this organism to a lengthy motorway along which interesting spots to visit are lined up, restaurants and hotels, i.e. "interesting" or "useful". Thus, to travel along the coffee genome and find the genes we are looking for, we need reference points and a reference chart.

The most useful reference points in a genome (signpost along the motorway) are the stretches of DNA which exhibit variability among different varieties and even between individual plants. These stretches of DNA are called polymorphic sequences and the genomes of possibly all Eukaryotes, *Coffea arabica* included, are thought to be rich in such sequences. The real problem is to find them, but once we have a reasonable number of polymorphic loci we can trace a reference map and associate polymorphisms to useful genes, as for instance the resistance to nematodes.

There are various types of DNA polymorphisms and various techniques to detect them, here we briefly mention the most important.

Restriction Fragment Length Polymorphism (RFLP)

This approach detects sequence variations (frequently single base mutations) and involves the digestion of DNA by a specific restriction enzyme, Southern blot and hybridisation with **a labelled probe. The drawback** with this technique lies in its complexity, in the fact that

you need a probe and in the low informative content (low number of alleles, usually two). Nevertheless the technique is well established and reliable and allows for the unambiguous identification of the homo and of the heterozygotes. RFLPs in the *Coffea* genus have been reported by Lashermes et al. (1996).

Random Amplified Polymorphic DNA (RAPD)

The RAPD technique allows for the identification of DNA polymorphisms in almost all organisms even when no other information is available on the DNA under study. It is based on the enzymatic amplification (PCR) of short DNA stretches (100-1,000 base pairs) lying between two inverted repeats homologous to arbitrary primers. The great advantage of this method is that there is no need for probes or for DNA sequences but the faint electrophoretic bands are difficult to reproduce and there is no way of recognising the heterozygotes. A number of publications report RAPD analysis in *C.a.* following the paper of Orozco-Castillo et al. (1994).

Amplified Fragment Length Polymorphism (AFLP)

Following the digestion of the genomic DNA by two restriction enzymes, adapters are added at both ends of the DNA fragments and some of the DNA fragments are PCR amplified by priming the adapters. This approach is very informative because many electrophoretic bands (30-90 bands) are obtained with a single PCR and the probability of finding a polymorphic band is correspondingly relatively high. Unfortunately the technique is rather complex and, as for the RAPD technique, it is impossible to identify the heterozygotes for a given band. The use of this technique in breeding programmes of *C.a.* has been reported by Lashermes et al. (2000).

Microsatellites

Microsatellites are repeated sequences in tandem and the number of repeats can vary in different organism of the same species. This type of polymorphism is normally very informative because one locus can show many alleles and because all the genotypes can be identified. Moreover the analyses are carried out by PCR and are very easy and a large number of samples can be analysed at low cost and in a short time. However, the development of these polymorphisms is difficult and expensive. The first descriptions of microsatellites in *C. arabica* have recently been reported by us (Mettulio et al., 2000; Rovelli et. al., in press).

Here we report the results of the microsatellite analysis of two crosses and describe a new type of polymorphism in *Coffea arabica*: Cleavase Fragment Length Polymorphism (CFLP).

Material and Methods

The DNA was extracted from leaves of two families and from seven different varieties coming from Brazil. The two crosses were the following: 1) MRFPB H-25-1 (Icatuai) x

Catimor II III-2-6 (Iapar 59) plus 6 F_1 plants, performed at IAPAR; 2) Sarcimor T5296 x ET-6 plus 17 F_1 plants, performed at CATIE.

DNA extraction

2 ml of extraction buffer (100 mM Tris-HCl, pH 8; 4% CTAB; 1,4 M NaCl; 20 mM EDTA; 0,07% freshly added β-mercaptoethanol) were added to 0,08 g of finely ground leaves and incubated for 1h at 65°C; the samples were extracted with 0,75 volumes of 24:1 $CHCl_3$/IAA and the aqueous phase was precipitated twice with 0,6 volumes of propan-2-ol, resuspended in 350ul of CTAB 2% buffer (100 mM Tris-HCl, pH 8; CTAB 2%; 1,4 M NaCl; 20 mM EDTA), extracted again with $CHCl_3$/IAA and precipitated once in 50 mM Tris-HCl, pH 8 containing 1% CTAB. The pellet was resuspended, precipitated twice with ethanol, and kept in 50 ul TE for subsequent analysis.

Microsatellite analysis

The microsatellite analysis was performed as reported by Rovelli et al. (2000).

CFLP analysis

The PCR products were concentrated by freeze-drying and then resolved in 2% low-melting agarose using 1xTAE as the electrophoresis buffer. Recovery of the bands of interest from agarose gel and subsequent purification were performed using Boehringer-Mannheim Agarase®, following the manufacturer's protocol. The CFLP analysis was performed following the protocol in the Boehringer-Mannheim operating manual, with minor modifications: 2 ul of water per sample were added to the reaction mix, and the stop solution was replaced by a solution lacking the xylene-cyanol. All other technical details were as reported by Brow and Fors (1997). The extranuclear DNA was amplified using the universal primers reported by Demesure et al. (1995).

Results and Discussion

Microsatellite analysis of the two families

The CATIE family was analysed for 51 microsatellite and the parents had different alleles for 4 systems. All the progeny had the same alleles as expected by Mendelian inheritance. Below (Fig.1) we report the results for the E10-3CTG microsatellite: the mother was homozygote for the 135bp allele and the father was homozygote for the 137bp allele, all the progeny were heterozygotes 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 IAPAR family was analysed for 30 microsatellites, two of which were homozygote in the parents while all the progeny were heterozygotes 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	108/112	108/112	108/112	108/112	108/112	108/112
37/6CTG	121/121	119	119/121	119/121	119/121	119/121	119/121	119/121

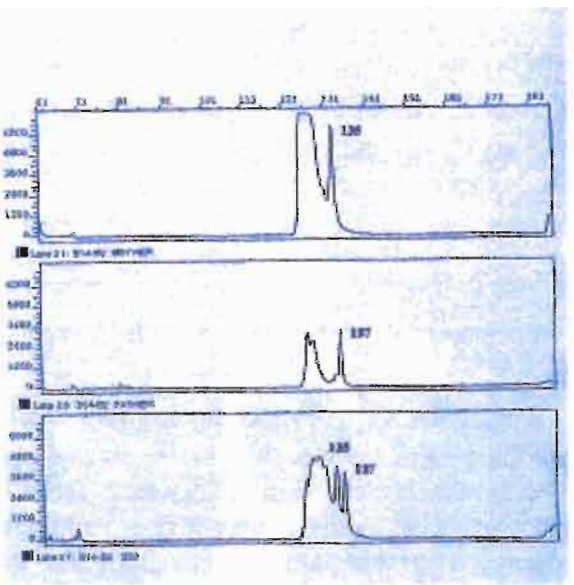

Fig. 1. GENESCAN analysis of the cross T5296 x ET6. Upper panel: mother homozygote for the 135bp allele. Middle panel: father homozygote for the 137bp allele. Bottom panel: F_1 progeny heterozygotes 135/137.

CFLP polymorphisms

When double-stranded DNA is denatured and quickly cooled in a low-salt buffer, "hairpin" structures are formed. Cleavase I cuts the DNA at the 5' region of the hairpin producing a set of fragments which can be analyzed on denaturing polyacrilamide gels. The banding pattern reflects single nucleotide substitution or deletion. This technique proved to be very sensitive; it can, in fact, detect single base mutations even in fragments of large size (up to 2.7 Kb in length. Brow & Fors, 1997).

We analysed about 20 microsatellites and 5 extranuclear systems. We conducted nearly 200 analyses but none of the microsatellites displayed a CFLP polymorphism. We do not know whether this negative result was due to the small dimension of the amplified microsatellites (200-400 bp) or to the particular nature of these sequences.

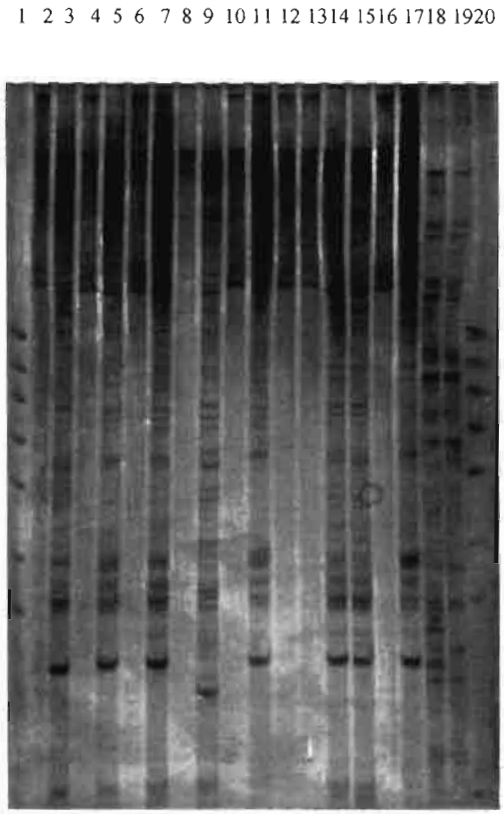

Fig. 2. CFLP Polymorphism for the intergenic spacer between the *trnH* and the *trnK1* tRNA genes (fragment of 1,300 bp in *C.a.*) of the chloroplast. About 150 ng of ethanol-precipitated PCR product were digested at 52,5°C for 5 minutes and then run on 6% denaturing polyacrylamide gel at 200V overnight. Lanes: 1 and 20, molecular weight markers; 2 and 3, Aramosa, undigested and digested; 4 and 5, Bourbon undigested and digested; 6 and 7, ET-6, undigested and digested; 8 and 9, *C. canephora*, undigested and digested; 10 and 11, Rume Sudan, undigested and digested; 12 and 14, Amphilo, undigested and digested; 13 and 15, Catimor, undigested and digested; 16 and 17, Sarcimor, undigested and digested; 18, CFLP® kit 1059 bp Wild type Control DNA; 19, CFLP® kit 1059 bp Mutant Control DNA (differs from the wild type for a single nucleotide).

On the contrary the extranuclear DNA (chloroplastic and mitochondrial) showed relatively high polymorphism: 2 out of 5 systems proved to be polymorphic. In fig. 2 we report the analysis of the intergenic spacer between the *trnH* and the *trnK1* tRNA genes (fragment of 1,300 bp in *C.a.*) of the chloroplast. Almost all the varieties analyzed showed specific bands while *C. canephora* had a very different pattern. Clearly these polymorphisms cannot be used for mapping the genome but they could be very useful in assessing the maternal contribution in inter- and intra-specific crosses when there are doubts about the origin of the gametes.

In our opinion, the absence of CFLP in the microsatellite amplicons does not imply absence of this type of polymorphism in the nuclear DNA. Most probably the amplification of longer stretches of nuclear DNA could give results as good as the extranuclear DNA. Moreover, nuclear CFLPs could be a new source of polymorphisms for mapping the *Coffea arabica* genome and for molecular assisted breeding programs.

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