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Use of random amplified DNA markers to analyse genetic variability and relationships of *Coffea* species

P. Lashermes, J. Cros, P. Marmey & A. Charrier
ORSTOM, 911 Av. Agropolis, BP 5045, F-34032, Montpellier, France

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Summary

The use of random amplified DNA fragments as genetic markers in *Coffea* was investigated. Arbitrary oligonucleotides were used as primers to amplify genomic DNA of different coffee accessions representing major *Coffea* species by polymerase chain reaction. Intraspecific variation was easily detected in *C. canephora* and *C. liberica* whereas the primers assayed failed to reveal polymorphism between *C. arabica* accessions. Extensive interspecific variation was observed. Genetic relationships between *Coffea* species are deduced from the degrees of similarity in amplified product profiles. Random amplified DNA markers appeared to be of high value for characterization, analysis and utilization of coffee genetic resources.

Introduction

Coffees belong to the family Rubiaceae. The genus *Coffea* L. has recently been reorganized into two subgenera: *Coffea* and *Paracoffea* (Bridson, 1987). The subgenus *Coffea* consists of approximately 100 taxa so far identified, including all the agronomically important species. *C. arabica* and *C. canephora* are the only cultivated species of economic importance but many *Coffea* species form a valuable gene reservoir for different breeding purposes (Berthaud & Charrier, 1988). *C. arabica* is a natural allotetraploid (2n = 4x = 44), and is self-fertile. Other species are diploids (2n = 22) and generally self-incompatible. Phylogenetic relationships among *Coffea* species have been established based on cytological analyses (Charrier, 1977; Louarn, 1982) and taxonomic data including morphology, geographical distribution, ecological adaptation as well as biochemical markers (Berthou et al., 1980; Berthaud, 1986; Anthony et al., 1989; Clifford et al., 1989) and variation of the cytoplasmic DNAs (Berthou et al., 1983). However, many uncertainties remain and questions such as the origin of *C. arabica* or extent of genomic variation within the diploid species are still being discussed.

Recently developed techniques based on the polymerase chain reaction (PCR) such as the random amplified polymorphic DNA (RAPD) system (Williams et al., 1990) offer a new class of DNA markers which present particular interests. Number and size of fragments generated by the RAPD system strictly depend on the nucleotide sequence of the primer used and on the source of the template DNA, resulting in a genome-specific "fingerprint" of random DNA fragments. Detection of polymorphisms by using RAPD technology is faster and less laborious than by using RFLP technology, and do not require any specific sequence information on the target genome (Welsh et al., 1991; Carlson et al, 1991).

In the present study, we examined intraspecific variation in *C. arabica* and *C. canephora*,

and genetic relationships among a set of coffee accessions representing major *Coffea* species using RAPD markers. In addition, we analysed Hibrido de Timor, an agronomically important spontaneous interspecific hybrid. A more general objective was to assess the potential of RAPD technique for analysis, management and utilization of coffee genetic resource collections.

Materials and methods

Plant material and DNA extraction

The accessions used and their origin are shown in Table 1. Accessions of *C. arabica* and *C. canephora* were chosen to represent a wide genetic variability. Hibrido de Timor is a tetraploid genotype which could derive from a spontaneous interspecific cross between *C. arabica* and *C. canephora* (Gonclaves & Rodrigues, 1976). *C. sp.* A801 is an unidentified accession which origin is either East Africa or Madagascar (Rakotomalala, pers. com.). DNA samples were extracted from young leaves. Freeze-dried tissue (0.4 g dry weight) was ground under liquid nitrogen, dispersed in 3 ml of CTAB isolation buffer (Saghai-Maroof et al., 1984) and incubated at 60°C for 45 min. Chloroform : isoamyl alcohol (24:1; 3 ml) was added and the solution was mixed by inversion to form an emulsion. After separation of the phases by centrifugation, the aqueous phase was removed. DNA was precipitated with isopropanol, rinsed in 70% ethanol, dried and resuspended in TRIS-EDTA buffer. The amount of DNA in the solution was estimated by both UV spectrometer and agarose gel electrophoresis followed by ethidium staining.

PCR technique

Oligonucleotides were used singly as primer for the amplification of random DNA sequences from genomic DNA. The sequence of each primer was chosen arbitrarily and is as follows (5′-3′):
(1) GGTCGGAGAA, (2) TCGGACGTGA,
(3) AGACGTCCAC, (4) GGAAGTCGCC,
(5) AGTCGTCCCC, (6) ACGCATCGCA,
(7) CTGCATCGTG, (8) GAAACACCCC,
(9) TGTAGCTGGG, (10) CCTACGTCAG,
(11) CTTCCGCAGT, (12) ACGCGCATGT,
(13) GACGCCACAC, (14) ACCAGGTTGG,
(15) AATGGCGCAG, (16) TCTCAGCTGG,

Table 1. List of accessions surveyed for RAPD markers

Code No.	Species	Code accession/ Variety	Spontaneous/ Cultivated	Origin/ Cultivation	Source
ar 1	*C. arabica* Linné	PDRY 005	cult.	Yemen	IRCC
ar 2	*C. arabica* Linné	caturra amarillo	cult.	Brazil	IRCC
ar 3	*C. arabica* Linné	ET1-1	subspont.	Ethiopia	ORSTOM
ar 4	*C. arabica* Linné	ET 20-4	subspont.	Ethiopia	ORSTOM
ar 5	*C. arabica* Linné	java	cult.	Cameroon	ORSTOM
ar 6	*C. arabica* Linné	sarchimor	cult.	Brazil	IRCC
hT	(*C. arabica*?)	hibrido de Timor/832-1	spont.	Timor	CIFC
ca 1	*C. canephora* Pierre	D4	cult.	Central Africa	IRCC
ca 2	*C. canephora* Pierre	IF182	cult.	Ivory Coast	IRCC
ca 3	*C. canephora* Pierre	2GA	spont.	Ivory Coast	ORSTOM
ca 4	*C. canephora* Pierre	caféier de la Nana	spont.	Central Africa	ORSTOM
co	*C. congensis* Froehner	D7	spont.	Central Africa	IRCC
eu	*C. eugenioides* Moore	eug	spont.	Kenya	IRCC
li 1	*C. liberica* Hiern	5LA	spont.	Ivory Coast	ORSTOM
li 2	*C. liberica* Hiern	5CA	spont.	Central Africa	ORSTOM
ps	*C. pseudozanguebariae* Brids.	pop. Diani	spont.	Kenya	ORSTOM
re	*C. resinosa* (Hook. f.) Radlk.	res	spont.	Madagascar	IRCC
sp	*C. sp*	A801	spont.	?	IRCC
st	*C. stenophylla* G. Don	D8A	spont.	Ivory Coast	IRCC

CIFC: Centro de Investigaçao des Ferrugens do Cafeeiro, Oeiras, Portugal.
IRCC/CIRAD: Centre de Coopération Internationale de Recherche Agronomique pour le Développement, Montpellier, France.
ORSTOM: Institut Français de Recherche Scientifique pour le Développement en Coopération, Montpellier, France.

(17) CACTCTCCTC, (18) GAATCGGCCA, (19) CTGACCAGCC, (20) GGGAGACATC, (21) GAAGTGGGGAAGTGGG, (22) ACCTCGAGCACTGTCT, (23) CTGTTGCTAC. The primers 1 to 20 were purchased from Operon Technologies, Inc (Alameda/CA, USA) and the others obtained from investigators at ORSTOM, Montpellier. All 23 primers were used in amplification reactions with template DNA from the 6 accessions of *C. arabica* (Table 1) and Hibrido de Timor. In a separate set of reactions, the primers 2, 3, 4, 5, 6, 7, 8, 11, 15, 21, 22, 23 were used to amplify genomic DNA from all coffee accessions listed in Table 1.

Polymerase chain reaction was set up as described by Williams et al. (1990) in volume of 50 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.1 mM of each dATP, dCTP, dGTP and dTTP (Boehringer), 80 ng of genomic DNA, 0.8 µM of primer and 1 unit of Taq polymerase (Perkin Elmer Cetus). The reaction mixture was overlain with approximately 25 µl of light mineral oil. PCR amplification profile consisted of initial denaturation (7 min at 95°C) followed by 45 cycles (1 min at 94°C, 1 min at 35°C and 2 min at 72°C). The amplifications finished with an incubation at 72°C for 7 min.

Reaction products were analysed by electrophoresis in 2.0% agarose horizontal gels at 4 volt/cm for four hours (TAE buffer: 1 mM EDTA, 40 mM Tris, 20 mM sodium acetate) and detected by staining with ethidium bromide. Molecular standards were the lambda DNA digested with Eco RI and Hind III.

Data analysis

The amplified products detected were named by primer code followed by the size of amplified fragment in base pairs. Once the proper conditions were established, reaction products were highly reproducible. However, the quantitative reproducibility was not sufficient for identifying heterozygotes and homozygotes based on relative intensities of DNA bands. In consequence, RAPD were scored as dominant markers (presence versus absence of specific product) and transformed into a 1 (present) and 0 (absent) matrix over all accessions and all RAPD identified. A similarity index D, expressing the probability that a RAPD in one accession is also found in another was calculated according to Wetton et al. (1987) for all possible pairwise comparisons between accessions: $D_{AB} = 2 \times$ [no. shared amplified product/(no. fragmentsA + no. fragmentsB)]. In addition, a hierarchical clustering analysis (Benzecri, 1973) based on the similarity index was performed using TAXO program (Serres & Rioux, 1986). The weighted average linkage option was used to generate a dendrogram.

Southern analysis

A southern analysis of PCR products obtained with the primer 11 was performed. After electrophoresis, PCR products for all accessions were transferred to a nylon membrane (Hybond N+, Amersham, UK) using southern blotting. The amplified fragment of 1500 bp produced from Hibrido de Timor using the primer 11 was purified from agarose after electrophoresis using the freeze/squeeze method (Sambrook et al., 1989) and amplified one more time by PCR using the standard conditions. Thirty ng of amplified DNA was labelled and used as probe for southern analysis. Reaction of labelling, hybridization and detection were based on the enhanced chemiluminescence system (ECL, Amersham, UK). The blot was washed with high stringency (6 M urea, 0.1 × SSC, 0.4% SDS) and the film was exposed for one minute.

Results

Analysis of C. arabica *accessions and Hibrido de Timor*

Depending the primer, the number of amplified DNA fragment varied from 1 to 8, and ranged in length from 200 to 2100 bp (Fig. 1). Using 23 primers, a total of 112 amplified DNA fragments were scored. No polymorphism was detected among the 6 accessions of *C. arabica*. Hibrido de Timor diverged only from the *arabica* accessions by the absence of two amplified products and the presence of one additional band. This RAPD of 1500 bp in length was produced with the primer 11.

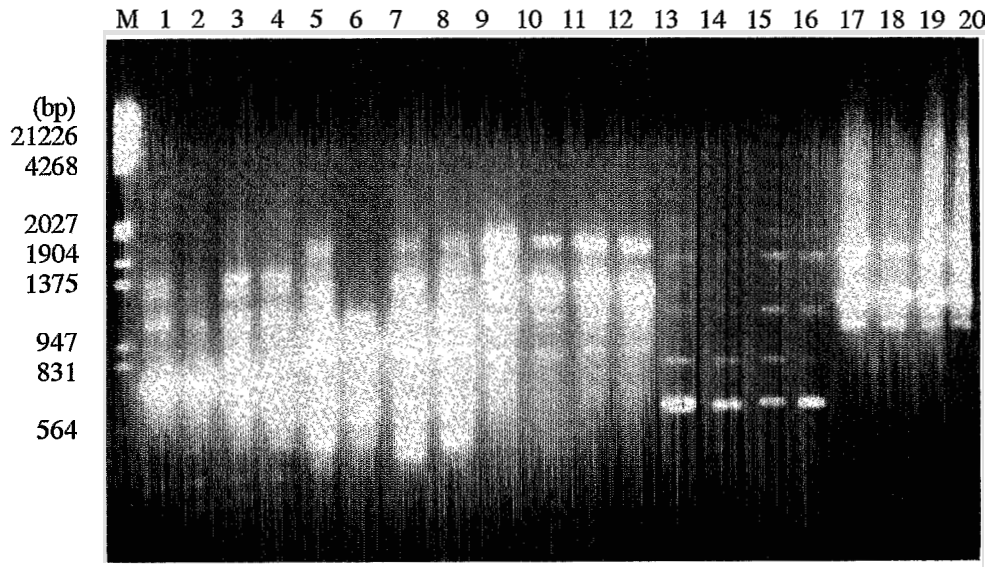

Fig. 1. Examples of amplification fragments produced from *C. arabica* accessions using different arbitrary decamer primers.

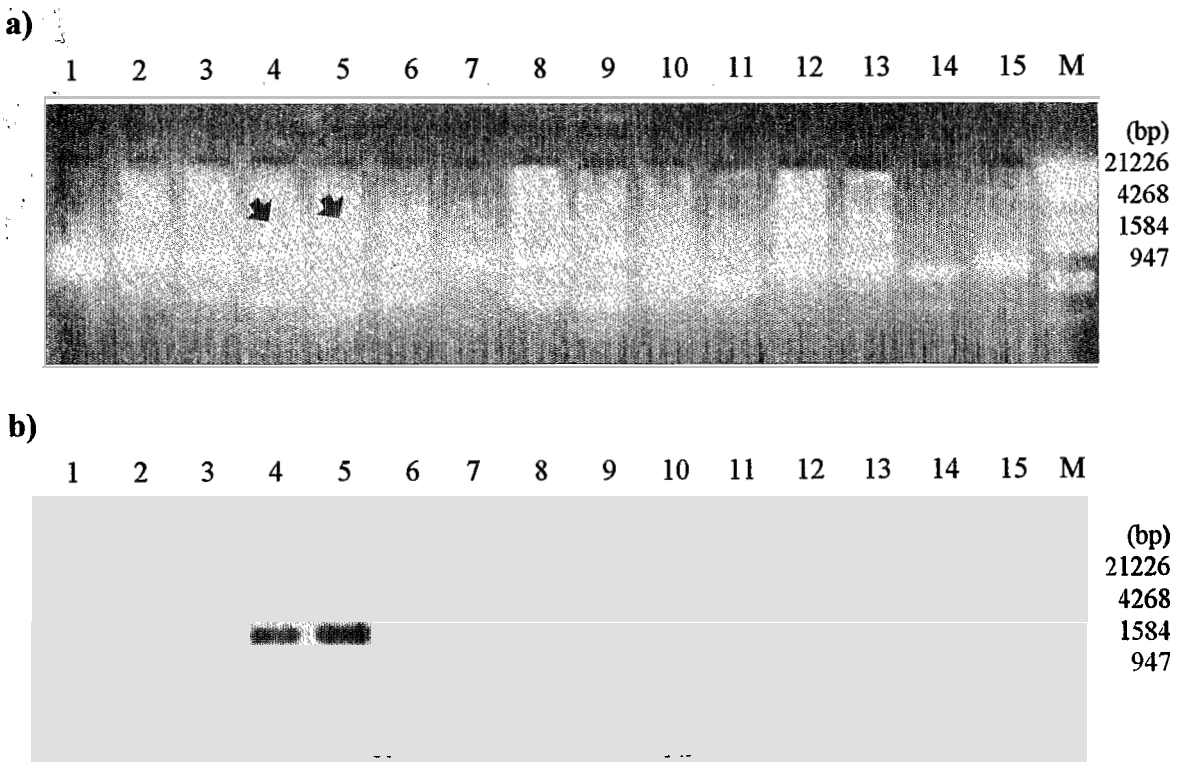

Fig. 2. Polymorphism between Hibrido de Timor and *C. arabica* revealed with the primer 11. a) A 1500 bp RAPD marker absent in *C. arabica* accessions and present in Hibrido de Timor (lane 4) and *C. canephora* acc. ca3 (lane 5) is indicated by arrows. b) Southern blot of the amplified fragments produced with the primer 11 was hybridized with the 1500 bp fragment from hibrido de Timor used as probe.

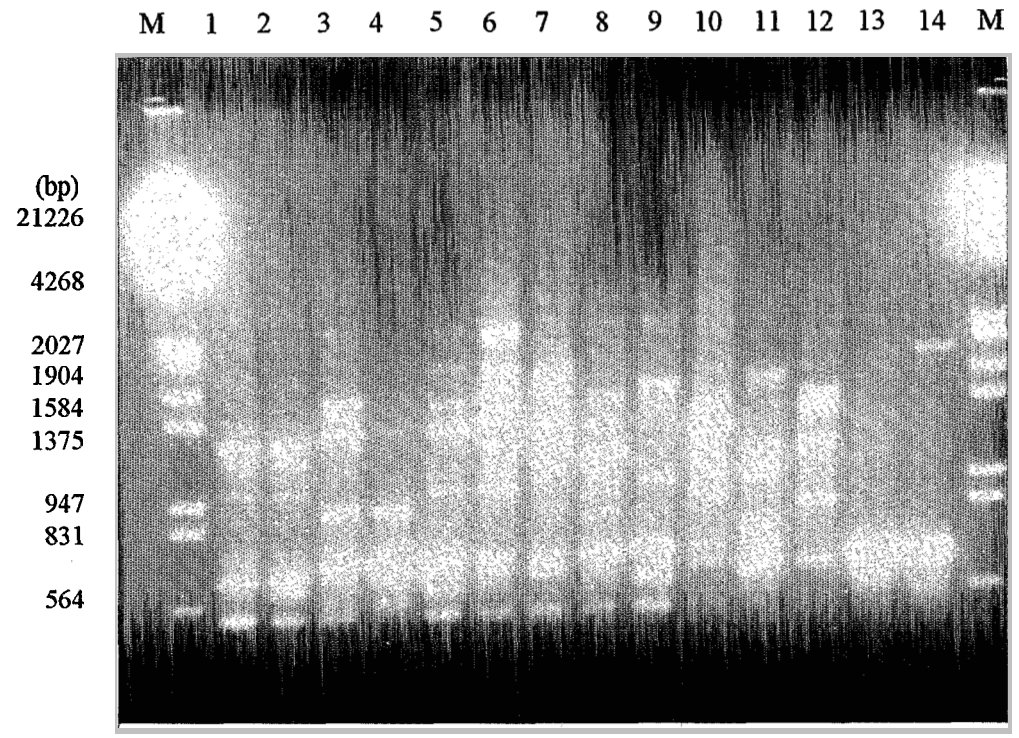

Fig. 3. Examples of genome-specific amplification fragments produced with an arbitrary decamer primer (primer 5).

Attempt was done to investigate if this additional band was whether or not specific to Hibrido de Timor. The accessions listed in Table 1 were surveyed, only the *C. canephora* acc. ca3 produced a similar amplified DNA fragment (Fig. 2a). Sequence homology between the fragments produced from Hibrido de Timor and *C. canephora* acc. ca3 was confirmed by southern hybridization using the 1500 bp fragment from Hibrido de Timor as probe (Fig. 2b).

Diversity among Coffea *accessions, estimation of genetic relatedness*

Considerable variation was detected with all twelve primers within the range of *Coffea* accessions (Fig. 3). An overall mean of 4.3 amplified fragments per primer was produced. All accessions generated comparable number of amplified products with the exception of *C. pseudozanguebariae* and *C. sp* A801 which produced consistently less

Table 2. D-Values for pairwise comparisons of amplified product profiles

ca1	0.46											
ca2	0.50	0.68										
ca3	0.49	0.70	0.63									
ca4	0.52	0.65	0.62	0.73								
co	0.49	0.48	0.51	0.55	0.49							
eu	0.51	0.44	0.45	0.43	0.45	0.41						
li1	0.48	0.46	0.48	0.47	0.50	0.45	0.45					
li2	0.50	0.48	0.49	0.50	0.53	0.42	0.46	0.66				
ps	0.27	0.25	0.28	0.31	0.29	0.25	0.24	0.30	0.28			
re	0.29	0.28	0.30	0.35	0.28	0.39	0.33	0.32	0.34	0.24		
sp	0.23	0.26	0.23	0.25	0.30	0.23	0.26	0.23	0.21	0.70	0.22	
st	0.40	0.42	0.42	0.50	0.44	0.46	0.39	0.45	0.50	0.27	0.32	0.25
	ar	ca1	ca2	ca3	ca4	co	eu	li1	li2	ps	re	sp

fragments (mean of 3.3 fragments per primer). D-values (similarity index) for all possible pairwise comparison are given in Table 2. Since no polymorphism was found between *arabica* accessions, only one arbitrary-selected accession of *arabica* was used. The highest D-values were obtained from comparisons between accessions belonging to the same species. Comparisons with *canephora* group yielded D-values of 0.62 to 0.73. The accession of *C. liberica* from Ivory Coast yielded D = 0.66 when compared with the *liberica* genotype issuing from Central Africa. A high value is obtained also for the comparison of *C. pseudozanguebariae* and *C. sp.* A801. Comparisons between *Coffea* species originated from West Africa (*C. canephora*, *C. congensis*, *C. liberica*, *C. stenophylla*) yielded intermediate D-values ranging from 0.42 to 0.55. Comparable results were obtained for *C. arabica* compared to West Africa species (average D = 0.47) or to *C. eugenioides* (D = 0.51). *C. resinosa* showed consistently low D-value (average D = 0.30) when compared to all other species. Similarly, comparisons of *C. pseudozanguebariae* and *C. sp.* A801 with other species yielded an overall average D as low as 0.26.

The hierarchical clustering analysis presented in Figure 4 showed different groups. A large group encompassed *C. arabica*, *C. eugeneioides* and all West African species (*C. canephora*, *C. congensis*, *C. liberica*, *C. stenophylla*). *C. canephora* accessions as well as the two accessions of *C. liberica* were clustered before they joined the clusters of other species. A second group involved *C. resinosa* which appeared distantly related to the main cluster. *C. pseudozanguebariae* and *C. sp.* A801 were closely related from each other and formed a third group.

Discussion

Intraspecific variations were easily detected in *C. canephora* and *C. liberica* using RAPD technique. On the other hand, the set of primers assayed failed to disclose variation between the

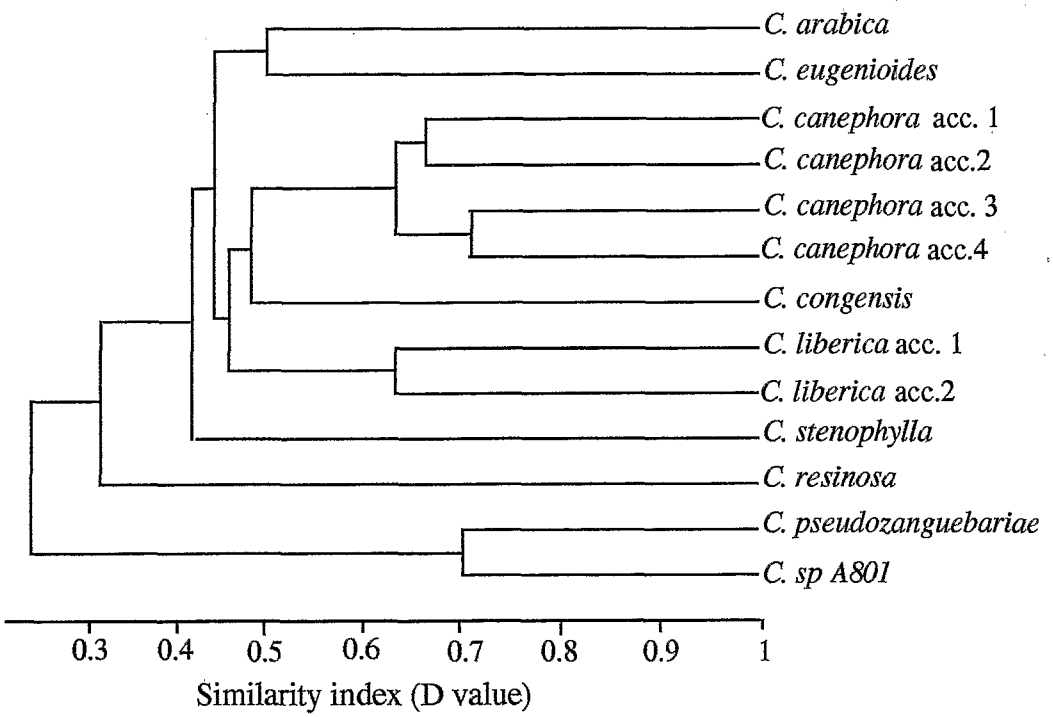

Fig. 4. Dendrogram showing genetic relationships between *Coffea* accessions constructed by hierarchical clustering analysis using RAPD markers.

C. arabica accessions although they are of various origins. Similarly, Moreno (1989) reported very low polymorphism in a survey of 14 *arabica* accessions for 18 isozyme markers. The low molecular diversity detected in *C. arabica* could have different origins. First, *C. arabica* as a self-pollinated crop is highly homozygous and all deleterious mutations that would contribute to molecular diversity are eliminated by selection. Second, *C. arabica* may present a very narrow genetic base in relation with its genesis. The formation of the allotetraploid species, *C. arabica*, could be a relatively recent event involving a limited number of plants. Another possibility could be a drastic loss of genetic diversity during glaciation phases of the quaternary period (Hamilton, 1976).

Hibrido de Timor was found in a *C. arabica* field on the island of Timor in 1927 (Goncalves & Rodrigues, 1976). It is a tetraploid genotype which presents a phenotype alike *C. arabica* and combines important resistances to coffee berry disease (CBD) and to most rust races (Moreno, 1989). This material has been intensively used as source of resistance in coffee breeding programmes all over the world. Based on information relating to the coffee germplasm introduced in the Timor island at the beginning of the century, the limited fertility of the original plant and characteristics of disease resistances, it is generally believed that Hibrido de Timor originated from a spontaneous interspecific cross between *C. arabica* and *C. canephora* (Bettencourt, 1973; Goncalves & Rodrigues, 1976; Moreno, 1989). This presumption is notably strengthened by our results showing that Hibrido de Timor share a common amplified product with one accession of *C. canephora*. In addition, the high degree of similarity (D-value = 0.99) in the amplified product patterns observed between *C. arabica* and Hibrido de Timor suggest that an initial interspecific hybridization should have been followed by several spontaneous backcrosses with *C. arabica* to generate Hibrido de Timor.

Extensive interspecific genetic variation was revealed between *Coffea* species. On average, more than 50% of the amplified DNA fragments were different between two species.

Because of its allotetraploid structure, it would be expected that *C. arabica* produced significantly higher numbers of amplified fragments than the diploid species. The lack of variation in number of amplified fragments between *C. arabica* and most diploid species observed in this study could be related to differences in level of heterozygosity. *C. arabica* material has been reported to show a very high level of homozygosity while the diploid species are allogamous and tend to present more heterozygosity (Berthou et al., 1980; Berthaud, 1986). In the same way, the lower number of amplified fragment generated from both accessions of *C. pseudozanguebariae* and *C. sp.* A801 when compared to other diploid accessions may be due to a high level of homozygosity. However, other possibilities such as important reduction in size of genome during species divergence (Flavell, 1982) or technical artefact cannot be discarded.

The coffee species from West Africa (*C. canephora*, *C. liberica*, *C. congensis* and *C. stenophylla*), and from the highland forest of Kenya and Ethiopia (*C. arabica* and *C. eugenioides*) present a high similarity. *C. resinosa* originated in Madagascar was very differentiated from all species surveyed. *C. pseudozanguebariae* indigenous to the coastal region of East Africa present also a low similarity with all species analysed. Only *C. sp.* A801, accession unidentified, appeared closely related to *C. pseudozanguebariae*. These results are consistent with the classification in three sections proposed by Berthaud (1986) based on morphological and cytological studies: *Erythrocoffea*, *Mozambicoffea* and *Mascarocoffea* including respectively *Coffea* species from West and Central Africa, Indian Ocean coast of East Africa and Madagascar.

Meiotic chromosome pairing in interspecific hybrids suggested that *C. arabica* share a common genome with *Coffea* diploid species (Charrier, 1977; Berthaud & Charrier, 1988). Based on restriction enzyme analysis of chloroplast and mitochondrial DNAs, Berthou et al. (1983) proposed that *C. arabica* diverged from an ancestor similar to *C. eugenioides*. Results presented here indicate that *C. arabica* is related to the diploid species of West and Central Africa but *C. eugenioides* did not appear much more closely related than other species such as *C. canephora*, *C. liberica* or *C. congensis*.

Caution needs to be exercised in application of DNA fingerprinting to ascertain genetic distance (Linch, 1988; Lander, 1989). A number of problems arise in interpreting "RAPD fingerprint".

First, it is unknown which markers belong to which loci. Second, it cannot be ascertained whether individuals are homozygous or heterozygous. Consequently, the only feasible measure of similarity between accessions, i.e. the fraction of shared marker bands, will not be equivalent to the fraction of shared genes (Linch, 1988). Nevertheless, the degrees of similarity in amplified product pattern between *Coffea* species observed in this study conform to the phylogenetic relationship deduced for the species by conventional methods. However, this study did not provide substantial information on the genetic relationships between species of West and Central Africa. Utilization of a larger number of accessions and selection for analysis of only the most conserved RAPD markers might be necessary when studying closely related species.

RAPD assay provides a highly effective and convenient means to "fingerprint" coffee accessions. This method should therefore be of high value for germplasm characterization and genetic resource maintenance in *Coffea*. Applications could include fingerprinting of genotype, identification of duplicate accessions, analysis of genetic diversity in a collection and establishment of a core collection. The usefulness of RAPD markers for genetic mapping has been largely reported (Carlson et al., 1991; Klein-Lankhorst et al., 1991; Martin et al., 1991). The difficulty in detecting polymorphism in *C. arabica* could slow applications in genetic resources/breeding programmes for *arabica* coffee unless more informative markers are found. However, in connection with assisted backcross-breeding (Tanksley et al., 1989; Hillel et al., 1990; Melchinger, 1990), RAPD technology is obviously a very powerful tool to increase the effectiveness of introgression of desirable traits (e.g. rust resistance) from wild coffee material or spontaneous hybrid such as Hibrido de Timor.

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