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Identification of RAPD markers for resistance to coffee berry disease, *Colletotrichum kahawae*, in arabica coffee

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Summary

Resistance to Coffee Berry Disease (CBD) in Arabica coffee is controlled by at least three genes which are present in the varieties Hibrido de Timor (*T* gene), Catimor (*T* gene), Rume Sudan (*R* and *k* genes) and K7 (*k* gene). Hibrido de Timor, Catimor and Rume Sudan are genetically distant from most of the commercial cultivars, and the utilisation of molecular markers would greatly improve the efficiency of breeding programmes concerned with CBD resistance. The objectives of the present work were therefore: (1) to identify random amplified polymorphic DNA (RAPD) markers associated with CBD resistance and (2) to identify markers which could be used to select against the genetic background of the resistance donors. Identification of RAPD markers was carried out in three steps. The first step involved the comparison of the RAPD profiles between the susceptible cultivars and the resistant donors. This was followed by comparison of the RAPD profiles between resistant and susceptible types of each donor variety. The final step involved assay of the resistance markers in the first and the second backcrosses between these donors and the recurrent parent. High genetic variability was demonstrated in Catimor, and to some extent in Rume Sudan. Three RAPD markers were shown to be closely associated to the *T* gene. Attempts to identify markers associated with the *R* and *k* genes were less rewarding. The implications of the current observations in relation to breeding for CBD resistance in Arabica coffee are discussed.

Introduction

The production of Arabica coffee (*Coffea arabica* L.) is fundamental for over 50 developing countries, for which it is the main foreign currency earner (Graaf, 1986). Its production is, however, constrained by a number of major diseases, including coffee leaf rust (*Hemileia vastatrix*), Coffee Berry Disease (CBD) caused by *Colletotrichum kahawae* and bacterial blight of coffee (*Pseudomonas syringae*). While coffee leaf rust is spread worldwide, CBD is still restricted to the continent of Africa where it is the main constraint to sustainable and economical production of Arabica coffee. Its chemical control may account for up to 45% of the annual cost of production (Nyoro & Spray, 1986). Despite such elaborate control measures, losses as high as 50% of the potential crop may still occur under unfavourable weather conditions (Agwanda & Owuor, 1989). For this reason, a number of breeding and selection programmes have been initiated in countries such as Cameroon (Bouharmont, 1995), Ethiopia (Van der Graaff, 1981), and Kenya (Van der Vossen & Walyaro, 1983).

Selection for resistance to the disease has either been based solely on the seedling inoculation method (Van der Vossen et al., 1976), or both on seedling inoculation and field expression of resistance on mature trees (Van der Graaff, 1981). Based on the seedling inoculation, three genes of resistance have been identified in the varieties Rume Sudan (*R* and *k* genes), Hibrido de Timor (*T* gene) and K7 (*k* gene) (Van der Vossen & Walyaro, 1980). Similarly, the Catimor vari-

ety has also been shown to possess the *T* gene of resistance present in Hibrido de Timor (Anon, 1978). While K7 is a Kent type commercial variety, other resistant donors (Hibrido de Timor, Rume Sudan) correspond to exotic germplasm where the valuable resistant genes are associated with undesirable traits. Hibrido de Timor resulted from a spontaneous interspecific hybridisation between *C. arabica* and *C. canephora* (Bettencourt, 1973) and shows a great deal of divergence from the commercial cultivars for most agronomic traits. Similar differences are also observable in Catimor which is a hybrid between Hibrido de Timor and the commercial variety Caturra. Rume Sudan resulted from seeds accessed from the Boma plateau in Sudan (Walyaro, 1983). It is a compact plant with small beans and poor yields. Except for its resistance to CBD and leaf rust, it is considered agronomically inferior.

The three genes of resistance have since been exploited in the Kenyan breeding programme either in pursuit of pure line varieties or for production of hybrid cultivars (Agwanda & Owuor, 1989). In either respect, the seedling inoculation method (Van der Vossen et al., 1976) has contributed significantly by shortening the time required to identify resistant progenies from crosses involving resistant and susceptible donors. However, its efficiency becomes limited when a breeder is interested in accumulating a number of resistance genes into an improved cultivar, since this would require test crossing. Given the long generation cycle (five years) characteristic of Arabica coffee, the test cross approach is highly time-consuming and thus represents a real bottleneck to rapid development of varieties resistant to CBD. In such situations, the use of molecular markers would not only facilitate the pyramiding of resistance genes through marker-facilitated selection, but would also be useful in selecting against the genetic background of the donor varieties (Melchinger, 1990; Peterson et al., 1991; Lavi et al., 1994; Michelmore, 1995). To this end, random amplified polymorphic DNA (RAPD) (Williams et al., 1990; Welsh & McClelland, 1990) has proved useful in tagging resistance genes in a number of crops, including apples (Gianfranceschi et al., 1994; Yang & Krüger, 1994), barley (Poulsen et al., 1995), *Brassica napus* (Foisset et al., 1995), rice (Nair et al., 1995), sunflower (Mouzeyar et al., 1995) and wheat (Talbert et al., 1996).

In coffee, the RAPD technique has been used to study the genetic diversity and relationships among *Coffea* species (Lashermes et al., 1993, 1996b, 1996c; Orozco-Castillo et al., 1994). The narrow genetic base characteristic of the cultivated varieties within *C. arabica* species was showed. However, some degree of polymorphism was demonstrated in varieties with more or less wild characteristics, such as Rume Sudan and Catimor (Lashermes et al., 1993; Orozco-Castillo et al., 1994). Likewise, interspecific gene introgression between *C. arabica* and *C. canephora* was revealed in these two studies. The objectives of the present study were: (1) to identify random amplified polymorphic DNA (RAPD) markers associated with CBD resistance and (2) to identify markers which could be used to select against the genetic background of CBD resistance donors.

Materials and methods

Plant materials

Identification of RAPD markers for CBD resistance first involved comparison of the RAPD profiles of susceptible cultivars and resistance donors. SL28 and Caturra were used as the susceptible cultivars and Rume Sudan, K7 and Catimor were used as the resistance donors. The choice of SL28 was influenced by the fact that it is used as recurrent parent in the selection programme for CBD resistance in Kenya. Caturra was used because it is the susceptible parent in the cross that was used to develop Catimor (Moreno 1989). Primers which generated polymorphic bands specific to or shared amongst any of the resistant varieties were selected for further screening to verify whether such markers were specific to the resistant progenitors. For this purpose, RAPD profiles of resistant and susceptible types of each resistance donor were compared. Five resistant and two susceptible Rume Sudan, seven resistant and two susceptible Catimor and two resistant and two susceptible K7 individuals were used. Susceptible Catimor and Rume Sudan were obtained from Centro Nacional de Investigaciones de Café (CENICAFE), Colombia. The final step involved assay of the resistance markers in the first and the second backcrosses (BC_1 & BC_2, respectively) between these donors and SL28. Eleven BC_1 and four BC_2 were used to confirm markers for the *R* gene, whereas five BC_1 and two BC_2 were used for the *T* gene. All selected BC plants were considered as CBD-resistant following seedling inoculation tests and field evaluation on mature trees. Further description of the materials is given in Table 1.

Table 1. Description of coffee genotypes used in identification of RAPD markers for CBD resistance

Name	Type	Pedigree[1]	Resistance phenotype	No. of tree/genotypes analysed
SL28	Cultivar		Highly susceptible	1
Caturra (Ct)	Cultivar		Highly susceptible	1
K7	Cultivar		Medium resistant	2
K7	Cultivar		Susceptible	2
Rume Sudan (RS)	Semi-wild		Resistant	5
Rume Sudan	Semi-wild		Susceptible	2
Catimor	F_3 selections	Ct X HT	Resistant	7
Catimor	F_4 selections	Ct X HT	Susceptible	2
Hibrido de Timor (HT)	Wild	Interspecific hybrid	Resistant	1
BC_1 selections	Breeding lines	SL28 X [(SL28 X RS) X HT]	Highly resistant	5
BC_2 selections	Breeding lines	SL28 X [SL28 X ((SL28 X RS) X HT)]	Highly resistant	2
BC_1 selections	Breeding lines	SL28 X [SL28 X ((SL28 X LY7) (SL28 X RS))]	Highly resistant	11
BC_2 selections	Breeding lines	SL28 X [SL28 X ((SL28 X LY7) (SL28 X RS))]	Highly resistant	4

[1] LY7 = Lyamungu 7

DNA extraction and PCR amplification of fragments

Genomic DNA of each genotype was extracted from 4 g of lyophilised leaves. The leaves were first ground into fine powder. DNA was then extracted in 200 ml of extraction buffer (350 mM sorbitol, 100 mM Tris-HCl pH 8, 5 mM EDTA pH 8, 0.5% sodium bisulphate) and the solution was filtered through a muslin cloth. The extract was centrifuged at 3000g for 20 minutes, and the supernatant was discarded. The precipitate was incubated in 30 ml of lysis buffer (1.5M NaCl, 100mM Tris-HCl pH 8, 20 mM EDTA pH 8, 4% mixed alkyl-trimethylammonium bromide) for 4 hours at 65degC with occasional mixing. After cooling for about 5 minutes at room temperature, the extract was adjusted to 50 ml by adding chloroform/isoamyl alcohol (24/1 vv). The mixture was then homogenised by gentle inversion before being centrifuged at 3000g for 10 minutes. The aqueous supernatant was recovered and the chloroform/isoamyl alcohol extraction procedure was repeated. The resulting aqueous fraction was incubated with 100 μl of RNase 10 mg/ml (Boehringer Mannheim) for 30 minutes at 37 degC before precipitating the DNA with an equal volume of isopropanol. The precipitated DNA was recovered in 1 ml of 70% (v/v) ethanol. Where no precipitate was directly obtained, the mixture was centrifuged at 4000 rpm for 10 minutes and the sedimented DNA was recovered in 1 ml of 70% (v/v) ethanol. The extract was then dried and dissolved in 300 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8).

A total of 286 random decamer primers (Operon Technologies, CA, USA) were used for PCR in reaction conditions similar to those described by Lashermes et al. (1996c). The RAPD products were then fractionated according to size on agarose gel (1.8% w/w) subjected to electrophoresis (10V/cm for $4^1/_2$ hours) in 1X TBE buffer. The DNA fragments were then uniformly stained in a solution of ethidium bromide (10 μg/ml) for 15 minutes. DNA was then visualised on a UV transilluminator and photographed using Polaroid film. Data were recorded as the presence (1) or absence (0) of the amplified products. Genetic distances (GD) between genotypes were estimated as follows: GDxy = (Nx +Ny)/(Nx +Ny +Nxy), where Nx is the number of bands in line x and not in line y, Ny is the number of bands in line y and not in line x, and Nxy is the number of bands in lines x and y. Cluster analysis by the unweighted pair group method using arithmetic averages (UPGMA) was performed with the TREECON (version 1.1) package (Van der Peer & De Wachter, 1994). The probability of a RAPD marker being associated to CBD resistance in the nine Catimor genotypes, the five BC_1s and the two BC_2s due to chance was calculated based on binomial distribution.

Table 2. Matrix of genetic distance between 7 genotypes representing 5 Arabica varieties based on 120 polymorphic RAPD markers

	SL28	Caturra	RS-1*	RS-2*	K7	Catimor-1	Catimor-2
Caturra	0.39	0.0					
RS-1*	0.69	0.68	0.0				
RS-2*	0.66	0.62	0.39	0.0			
K7	0.39	0.40	0.63	0.54	0.0		
Catimor-1	0.67	0.70	0.88	0.84	0.74	0.0	
Catimor-2	0.65	0.70	0.84	0.85	0.71	0.48	0.0

* RS-1 and RS-2 represent first and second Rume Sudan individuals, respectively.

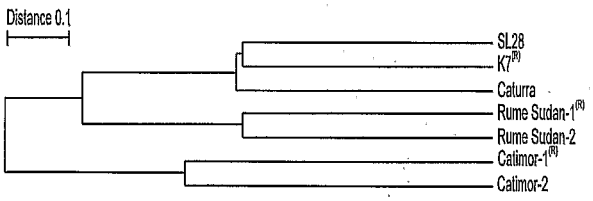

Figure 1. Dendrogram showing relationships between 7 Arabica genotypes generated by group average clustering analysis (UPGMA) using RAPD-based genetic distance. The genotypes resistant to CBD are indicated by ®.

Table 3. Nucleotide sequence of primers generating amplification products specific to the different CBD resisance donors

Primer code	Base sequence (5′-3′)	Resistant variety
B-06	TGCTCTGCCC	Catimor
B-11	GTAGACCCGT	Rume Sudan
C-08	TGGACCGGTG	Rume Sudan
C-18	TGAGTGGGTG	Catimor
E-06	AAGACCCCTC	Catimor
J-11	ACTCCTGCGA	Catimor
J-17	ACGCCAGTTC	Rume Sudan
K-09	CCCTACCGAC	Rume Sudan
L-19	GAGTGGTGAC	Rume Sudan
M-06	CTGGGCAACT	Catimor
M-15	GACCTACCAC	Rume Sudan
M-20	AGGTCTTGGG	Catimor
N-06	GAGACGCACA	Catimor
N-12	CACAGACACC	Catimor
N-18	GGTGAGGTCA	Rume Sudan, Catimor
P-09	GTGGTCCGCA	Rume Sudan, Catimor
S-15	CAGTTCACGG	Rume Sudan
X-11	GGAGCCTCAG	Catimor
X-15	CAGACAAGCC	Catimor
Z-01	TCTGTGCCAC	Catimor
Z-07	CCAGGAGGAC	Rume Sudan
Z-14	TCGGAGGTTC	Catimor

Results

The initial evaluation involved 7 genotypes representing 5 varieties either resistant or susceptible to CBD. Of the 286 primers assayed, 272 (95%) produced discernible amplification products, whereas the remaining 14 (5%) did not generate any amplification. The mean number of amplification bands per primer varied between 1 and 15, with a mean of 8 bands per primer. The amplified fragments varied in size between 250 bp to 3000 bp. One hundred and twenty of the amplified bands (5.2%) were polymorphic amongst the varieties. Five of the polymorphic bands were specific to SL28, five to Caturra, 16 to Rume Sudan and 25 to Catimor. The remaining polymorphic bands were shared among the varieties in various combinations. No band specific to K7 was observed. Among the polymorphic marker loci, the frequency of band presence was close to that of band absence in SL28, Caturra and Catimor. In Rume Sudan and K7, the frequency of absence of bands was nearly double that of presence of bands.

Genetic similarity analysis of the seven genotypes (Table 2) revealed close proximity between the three cultivated varieties, namely SL28, K7, and Caturra (Figure 1). Catimor progenies were shown to be genetically divergent from the rest of the varieties. Appreciable differences were observed between the two individuals of Rume Sudan and those of Catimor. The largest difference was registered between the individuals of Catimor and Rume Sudan.

The polymorphic marker-bands specific to the resistant varieties, either by presence or absence, were further considered as potential markers for CBD resistance in Catimor (51 markers), in Rume Sudan (30 markers) and in K7 (9 markers). Comparisons between resistant and susceptible types for each variety revealed that only 14 markers were specific to resistant Catimor,

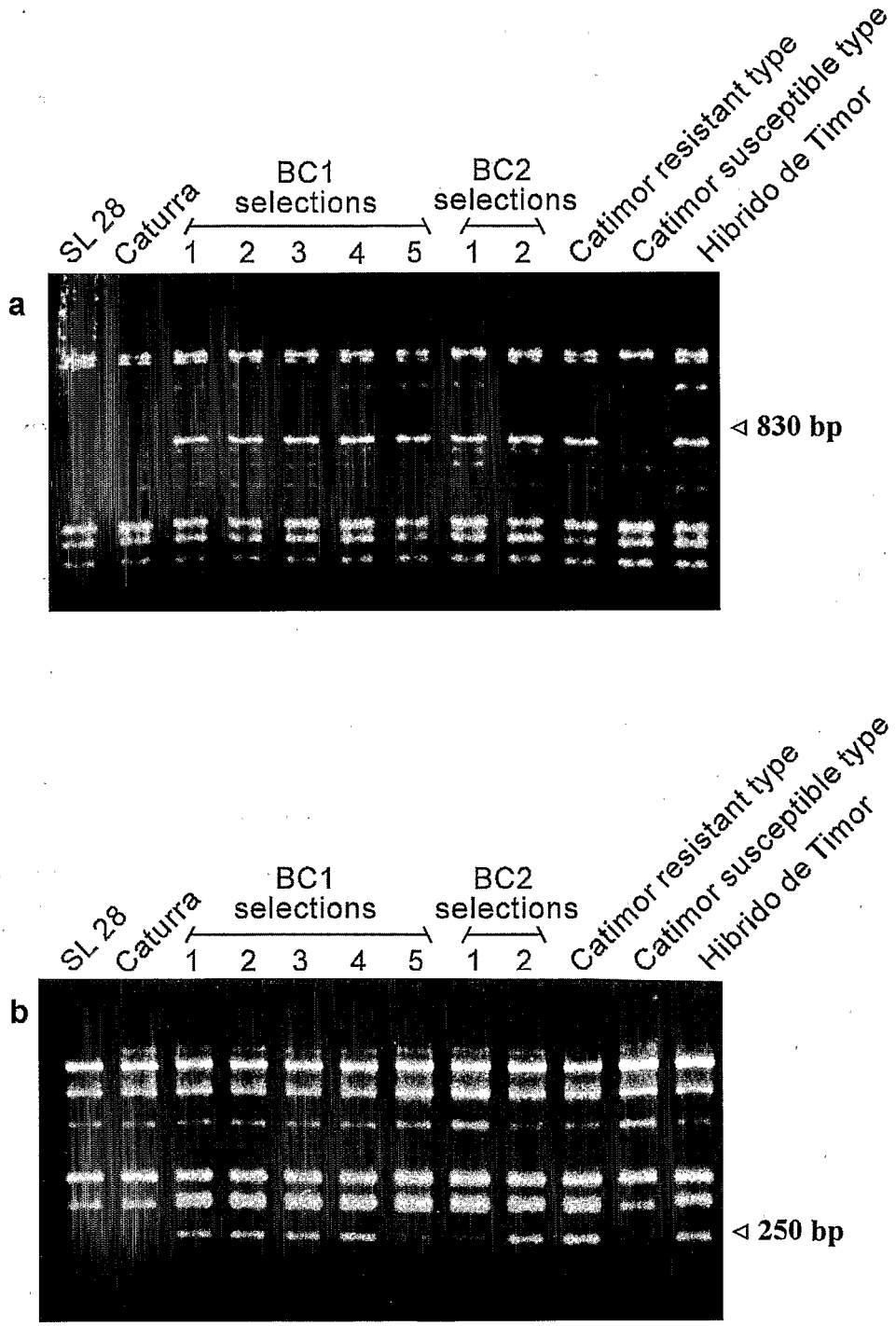

Figure 2. RAPD amplification pattern using primers M20 (a) and N18 (b) in CBD-susceptible cultivars (SL 28, Caturra), resistance donors (Catimor, Hibrido de Timor), and in some backcrosses selected for CBD resistance. The polymorphisms associated with CBD-resistance are marked by arrows.

Table 4. Segregation of RAPD markers among Arabica coffee genotypes showing contrasting levels of CBD resistance.

Genotype	Pedigree[1]	Resistance Class[2]	Presence (1) or absence (0) of marker		
			$M6_{2027}$	$M20_{830}$	$N18_{250}$
SL28		12	0	0	0
Caturra (Ct)		12	0	0	0
Catimor (F_3)accession 1	Ct X HT	2–3	1	1	1
Catimor (F_3)accession 2	Ct X HT	2–3	1	1	1
Catimor (F_3)accession 3	Ct X HT	2–3	1	1	1
Catimor (F_3)accession 4	Ct X HT	2–3	1	1	1
Catimor (F_3)accession 5	Ct X HT	2–3	1	1	1
Catimor (F_3)accession 6	Ct X HT	2–3	1	1	1
Catimor (F_3)accession 7	Ct X HT	2–3	1	1	1
Catimor (F_4)accession 8	Ct X HT	12	0	0	0
Catimor (F_4)accession 9	Ct X HT	12	0	0	0
Hibrido de Timor (HT)	Wild	2–3	1	1	1
BC_1selections	SL28 X [(SL28 X RS) X HT]	1–2	1	1	1
BC_1selections	SL28 X [(SL28 X RS) X HT]	1–2	1	1	1
BC_1selections	SL28 X [(SL28 X RS) X HT]	1–2	1	1	1
BC_1selections	SL28 X [(SL28 X RS) X HT]	1–2	1	1	1
BC_1selections	SL28 X [(SL28 X RS) X HT]	1–2	1	1	1
BC_2selections	SL28 X [SL28 X ((SL28 X RS) X HT)]	1–2	1	1	1
BC_2selections	SL28 X [SL28 X ((SL28 X RS) X HT)]	1–2	0	1	1

[2] Resistance class according to Van der Vossen et al. (1976), 1 = highly resistant, 12 = highly susceptible

10 to resistant Rume Sudan and none to resistant K7 types (Table 3).

Three of the markers specific to resistant Catimor type were assumed to be tightly associated with the *T* gene based on their co-transmission with CBD resistance in the BC_1 and BC_2 generations (Table 4 & Figure 2). The three marker-bands designated $M6_{2027}$,$M20_{830}$ and $N18_{250}$ generated by primers M6, M20 and N18, respectively, were observed in all the backcrosses studied, with the exception of one backcross for marker $M6_{2027}$. The probability of having at least one of the 51 markers in Catimor being associated with CBD resistance in the nine Catimor genotypes and the seven backcrosses was 0.0008. Thus, the observed associations between these markers and resistance to CBD were most probably due to genetic linkage.

With respect to Rume Sudan, all the 10 markers showed varying degrees of recombination in backcrossing. Their linkage to CBD resistance was thus assumed to be weak.

Discussion and conclusions

The narrow genetic base of cultivated *C. arabica* varieties is apparent from the low level of polymorphism we observed between cultivars K7, SL28 and Caturra. Similar observations were made by Moreno (1989) who studied isozyme polymorphism in 14 accessions of Arabica coffee, and by Lashermes et al. (1996c) in a study of RAPD polymorphism in 20 *C. arabica* accessions. The low molecular polymorphism is attributable to the allotetraploid origin and mode of speciation of *C. arabica* (Lashermes et al., 1996a), and the restricted genetic base of the original population from which the varieties evolved (Van der Vossen, 1985). The single tree selection procedures used to develop most of these varieties could have further accentuated the high level of uniformity observed.

The difference observed in the present study between Rume Sudan and the cultivated varieties may reflect the genetic diversity present in the primary centre of genetic diversity of Arabica coffee situated in the highlands of South West Ethiopia, and the Boma Plateau of Sudan (Lashermes et al., 1996c). However, other hypothesis, such as gene introgression from a gene pool alien to *C. arabica*, cannot be excluded. Catimor's divergence from the other varieties studied is attributable to the interspecific origin of one of its progenitor, Hibrido de Timor (Bettencourt, 1973). The numerous segregating marker loci observed between the two Catimor progenies are most likely due to the

residual heterozygosity within the Hibrido de Timor parent and /or the heterozygosity still present within the Catimor hybrid.

The large number of marker-bands specific to the resistant genotypes but not concerned with CBD resistance may be used as basis for selecting against the genetic background of the donor parents resulting from linkage drag. The use of semi-wild varieties such as Rume Sudan, or interspecific crosses such as Hibrido de Timor, as resistance donors results into the concomitant introgression of other un-targeted and agronomically undesirable genes. This would necessitate a number of backcrosses to restore the genetic constitution of the recurrent parent. A low turnover of improved varieties would thus result from the conventional breeding procedures. However, the combined use of such donor-specific markers with the resistance markers should enable breeders to select for both disease resistance and good agronomic attributes in one step, and thus reduce the time required to develop CBD-resistant varieties with superior agronomic characteristics (Melchinger, 1990).

The three RAPD markers, $M6_{2027}$, $M20_{830}$ and $N18_{250}$, shown to be closely related to the *T* gene of resistance in Catimor and Hibrido de Timor, should provide a more efficient way to select for the *T* gene in crosses involving Catimor and Hibrido de Timor. Furthermore, their use as selection criteria should enable pre-emptive breeding against CBD in countries where quarantine barriers are still effective against the disease. So far, such methods are not available and countries wishing to commence breeding programmes in the absence of the CBD pathogen have had to rely on the services of laboratories situated in countries where the disease is already endemic or on those situated in non-coffee-producing countries.

As concerns the *R* and *k* genes, it was not possible to identify appropriate markers, mainly due to the low polymorphism detected, especially in K7. Further investigations involving well-characterised F_2 genotypes segregating for the *R* gene and showing a large polymorphism would be particularly relevant. To enhance the chance of success, only individuals showing extreme reaction to the disease (highly resistant and highly susceptible) could be involved in a bulked segregant analysis (Michelmore et al., 1991).

The availability of *T*-linked RAPD markers represents a starting point in the use of markers to enhance backcross programmes in Arabica coffee. After further investigation and characterisation on a large F_2 population, the identified RAPD markers could be converted into co-dominant PCR-based sequence-tagged site (STS) markers, in order to be used for marker-assisted selection (Paran & Michelmore, 1993). Furthermore, in regard to the present results, it seems readily feasible to identify RAPD markers associated with different introgressed fragments present in Hibrido de Timor which are assumed to confer resistance to a number of pests and diseases. Potential targeted genes include important traits such as resistance to root-knot nematodes, leaf rust and bacterial blight of coffee (Moreno, 1989). Since Hibrido de Timor is being used worldwide as a major source of resistance, such a possibility is likely to have a considerable impact on the different coffee breeding programmes.

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