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Research Article

Assessment of genetic variability within and among coffee progenies and cultivars using RAPD markers

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Abstract

The RAPD technique associated with restriction digestion of genomic DNA was used to assess the genetic variability within and among nine populations of *Coffea arabica*, including six progenies belonging to the Sarchimor germplasm, the progeny PR 77054-40-10 (Catuaí Vermelho IAC 81 x Icatu), and two commercial cultivars (IAPAR 59 and Catuaí Vermelho IAC-81). These populations were evaluated using analysis of molecular variance (AMOVA), genetic similarity among progenies, and percentage of polymorphic loci. A total of 99 RAPD markers were evaluated of which 67 (67.67%) were polymorphic. AMOVA showed that 38.5% and 61.5% of the genetic variation was distributed among and within populations, respectively. The fixation index (F_{ST}) of the genotypes was 0.385. The mean genetic variability estimated within populations ranged from 15.58 (IAPAR 59) to 8.27 (Catuaí Vermelho IAC 81). A distinct level of genetic variability was revealed for each of the coffee progenies and varieties studied. The methodology used in this investigation was useful to determine the genetic variability within and among *C. arabica* L. populations providing significant information for coffee breeding.

Key words: Coffee breeding, genetic variability, molecular variance, RAPD markers.

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Introduction

Coffea arabica L. is the most important species of the *Coffea* genus, followed by *C. canephora* P. Nowadays, three Brazilian varieties are grown worldwide, Mundo Novo (derived from Bourbon x Sumatra crossing), Caturra (a dwarf mutant of Bourbon), and Catuaí (derived from Caturra x Mundo Novo crossing). These cultivars are highly productive and exhibit an exceptional quality beverage (Anthony *et al.*, 2001). The exploitation of genetic resources from the wild *Coffea* is essential for the development of inbred lines, which can be adapted to new production systems and also to overcome possible challenges from the world market (Carvalho *et al.*, 1985). Nevertheless, breeding programs are limited due to the narrow genetic base of coffee, especially for pest and disease resistance improvement (Van der Vossen, 1985). Nowadays, *C. canephora* provides the main source for resistance genes not found in *C. arabica*, including leaf rust (*Hemileia vastatrix*), coffee berry disease (CBD) caused by *Colletotrichum kahawae*, and resistance to root-knot nematode (*Meloidogyne* sp) (Lashermes *et al.*, 2000).

Natural and artificial hybrids derived from *C. arabica* x *C. canephora* have been intensively used in breeding programs. A good example is the Timor Hybrid, which has been exploited as a bridge to transfer rust resistance genes from *C. canephora* into cultivars of *C. arabica* (Fazuoli *et al.*, 1996). The Timor Hybrid is an atypical tree, which was identified in a *C. arabica* field on the island of Timor (Bettencourt, 1973). Information about coffee germplasm introduced into Timor island suggests, on the basis of its limited fertility associated to the characteristics of disease resistance and molecular data of the original plants, that the Timor Hybrid was derived from a spontaneous interspecific cross between *C. arabica* and *C. canephora* (Lashermes *et al.*, 2000). In 1972, the Centro de Investigações das Ferrugens do Cafeeiro (CIFC, Oeiras, Portugal) sent descendants to several producer countries that were derived from the Timor Hybrid . These plants were then crossed with *C. arabica* cv Villa Sarchi giving origin to the Sarchimor germplasm.

The employment of molecular markers to access the genetic variability in accessions of *C. arabica* has shown

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some difficulties due to the lack of genetic variation as observed with isozyme (Lopes, 1993) and RFLP markers (Paillard *et al.*, 1996). Nevertheless, based on RFLP of cpDNA Lashermes *et al.* (1996a) suggested a recent speciation for the genus *Coffea*. Also, RAPD markers were an effective method for providing the genetic identification of *Coffea* accessions (Lashermes *et al.*, 1996b). Diniz (2000) showed that the association of the RAPD technique with a prior digestion of the genomic DNA with restriction enzymes improved the assessment of genetic polymorphism in 40 *C. arabica* varieties.

In this investigation, the RAPD technique associated with a prior digestion of the DNA template was employed for the identification of the genetic variability within and among coffee populations and to estimate the percentage of polymorphic loci of the assay E9601, including six progenies of Sarchimor germplasm (IAPAR 77028-11), the progeny PR 77054-40-10, and the cultivars IAPAR 59 and Catuaí Vermelho IAC 81.

Material and Methods

Nine coffee populations were studied. They derived from the assay E9601 performed by the Instituto Agronômico do Paraná (IAPAR), located in South Brazil. The main purpose of IAPAR in developing this assay was to search for cultivars that were resistant to orange leaf rust and adapted to the environmental, economic, and technological conditions of the plantations in Paraná State. The populations included six progenies of IAPAR 77028-11, a Sarchimor germplasm in F_5 generation, two varieties represented by IAPAR 59 and Catuaí Vermelho IAC 81, and one F_5 progeny (PR 7705-40-10) derived from Icatu x Catuaí Vermelho IAC 81.

DNA extraction, amplification, and electrophoresis

Young leaves were collected from three plants of the first and second repetitions and four plants from the third repetition with a total of 10 samples for each treatment, as described by Huff *et al.* (1994). Genomic DNA was isolated following the method described by Doyle and Doyle (1987), except that CTAB was replaced by MATAB (Mixed Alyltrimethylammonium Bromide, Sigma). DNA concentration was estimated using a fluorometer (DyNA Quant 200, Höefer-Pharmacia), according to the manufacturer's instructions. DNA samples of the 90 individual plants were adjusted to 10 ng/µL and used in the amplification reactions with a final volume of 15 µL containing buffer 1x (75 mM Tris-/HCl, pH 9.0, 50 mM KCl, 2.0 mM $MgCl_2$, and 20 mM $(NH_4)_2SO_4$), 0.2 mM each of dATP, dTTP, dCTP, and dGTP; 0.4 µM of primer (Operon Technologies), 0.9 U of *Taq* DNA Polymerase (Biotools), and 20 ng template DNA. For digestion, genomic DNA was incubated just before amplification, with one of the following enzymes *Bam* HI, *Eco* RI, *Hae* III, *Hind* III, and *Rsa* I, added directly to the RAPD reaction. DNA amplification was carried out using a PTC 100 (MJ Research) thermal cycler, programmed with 60 min at 37 °C for prior digestion of the DNA template then the thermal cycler was programmed with 3 min at 94 °C for initial DNA denaturation, followed by 48 cycles of 1 min at 94 °C, 1 min 45 s at 38 °C, and 2 min at 72 °C. The last cycle was followed by a 7 min extension at 72 °C. The samples were then stored at 4 °C until electrophoresis. Amplified products were resolved in 1.4 % agarose gels in 1x TAE (40 mM Tris-acetate, 1 mM EDTA pH 8.0) buffer, at 120 V for 3 h and stained with ethidium bromide. The RAPD profiles were visualized under UV light, and photographed using a video camera (Cohu, High Performance CCD Camera).

Statistical analysis

The markers obtained were assessed for the presence (1) or absence (0) of homologous DNA bands among the individuals of each population. The analyses were performed by using only good-quality amplified fragments.

Genetic distance and genetic identity among populations were estimated according to Nei (1978). These parameters and the percentage of polymorphic loci were calculated using the 1.31 POPGENE program (Yeh *et al.*, 1997). A matrix of distance was obtained for each population using the NTSYS-pc software, version 2.1 (Rohlf, 2000). The dendrograms were constructed employing UPGMA (unweighted pair-group method with arithmetic averages) with the SAHN (Sequential Agglomerative, Hierarchical, and Nested Clustering) routine.

The analysis of molecular variance (AMOVA) was calculated to estimate the variation within and among populations using the RAPD polymorphic loci. The data were generated using the Arlequin 1.1 software (Schneider *et al.*, 1997) in which the fixation index (F_{ST}) was also estimated. The Win AMOVA software (Excoffier *et al.*, 1992; Excoffier, 1993) was used to obtain the square sum of variation within the populations. The bootstrap method was applied by using the DBOOT software version 1.1 (Coelho, 2001) to evaluate the reliability of the tree topology.

Results and Discussion

The RAPD technique combined with prior digestion of DNA samples improved the detection of polymorphism in *C. arabica* populations. Two hundred RAPD primers were tested with fifteen different restriction enzymes. Eighteen primers or primer/enzyme combinations that rendered polymorphic and reproducible fragments were then selected for DNA amplification of all samples (Table 1). The association of the selected primers with some restriction enzymes in the PCR reaction showed electrophoresis patterns different from those obtained without prior restriction as illustrated in Figure 1. This procedure allows for a straightforward analysis of the amplified products as it changes the relative intensities of the bands (Williams *et al.*, 1990; Riede *et al.*, 1994). Furthermore, digestion of genomic DNA may reveal a restriction site polymorphism

Table 1 - Primer sequences and primer/enzyme combinations used in the RAPD reaction of coffee accessions showing numbers of polymorphic and monomorphic bands.

		Number of bands		
Primer/Restriction enzyme	sequence (5' to 3')	Polymorphic	Monomorphic	Total
OPAD-05	CAATCGGGTC	1	2	3
OPP-20	GACCCTAGTC	3	1	4
OPN-20	GGTGCTCCGT	3	3	6
OPN-20 *Hae* III	GGTGCTCCGT	11	0	11
OPP-18	GGCTTGGCCT	0	4	4
OPP-18 *Hae* III	GGCTTGGCCT	11	0	11
OPZ-14	TCGGAGGTTC	2	2	4
OPZ-14 *Hind* III	TCGGAGGTTC	8	1	9
OPAA-09	AGATGGGCAG	1	1	2
OPAA-09 *Hae* III	AGATGGGCAG	2	1	3
OPAD-02	CTGAACCGCT	3	2	5
OPAD-02 *Hae* III	CAAAGGGCGG	3	1	4
OPS-15 *Hae* III	CAGTTCACGG	5	1	6
OPY-16 *Rsa* I	GGGCCAATGT	3	3	6
OPY-20 *Bam* H I	AGCCGTGGAA	1	3	4
OPAX-10 *Rsa* I	CCAGGCTGAC	3	2	5
OPZ-07 *Eco* R I	CCAGGAGGAC	4	2	6
OPZ-07 *Hind* III	CCAGGAGGAC	3	3	6
Total		67	32	99

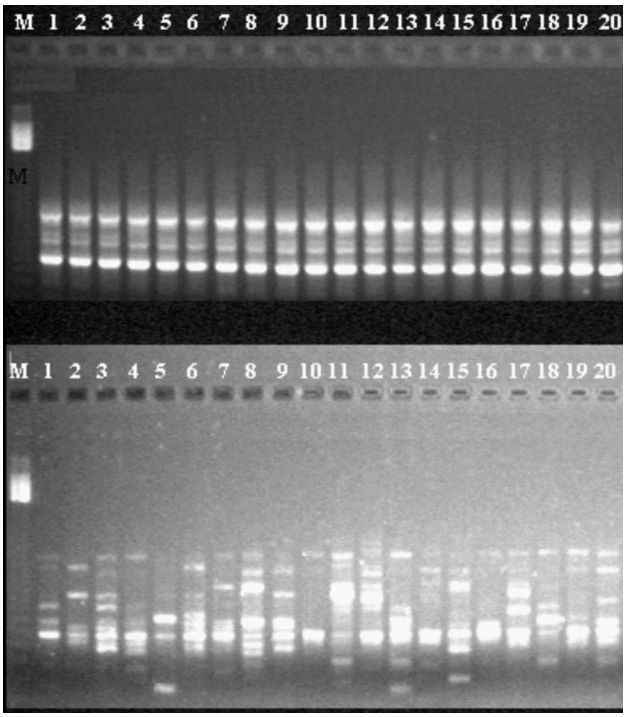

Figure 1 - Amplification profile of partial DNA coffee samples obtained with OPP-18 RAPD primer used alone (top) and in combination with restriction enzyme *Hae* III (bottom).

located between the primer-annealing sites in an otherwise monomorphic band. In wheat, this method improved the level of polymorphism (Kobner, 1995) and the results were attributed to a greater efficiency of primer annealing along shorter DNA fragments, where a simplified secondary structure is unlikely to interface with the process. Kobner (1995) suggested that these differences occur because distinct annealing sites became accessible, depending on the restriction enzyme employed. Whereas Orozco-Castilho *et al.* (1994) and Lashermes *et al.* (1996b) revealed that few RAPD primers were efficient in detecting polymorphism and in generating reproducible DNA fragments in *C. arabica*, the use of restriction digestion of genomic DNA before amplification with RAPD primers changed the electrophoretic profiles in 40 varieties of *C. arabica* (Diniz, 2000). In this study, the association of restriction digestion of DNA with RAPD reaction allowed for a more efficient assessment of the existing genetic variability (Figure 1). Thus, the technique is especially suitable for the analysis of genetic differentiation and to detect loss of genetic variation in coffee populations.

The DNA amplification of 90 individual DNA samples, belonging to six Sarchimor progenies, two cultivars (IAPAR 59, Catuaí Vermelho IAC 81), and the progeny PR 77054-40-10, rendered 99 reproducible bands of which

67.67% were polymorphic. The data were used to estimate the percentage of polymorphic loci for each population (Table 2), the differences within populations and among pairs of populations (Table 3) and for the analysis of molecular variance among populations (Table 4). The square sum of the variations within each population is illustrated in Figure 2. The dendrograms showing the associations among individuals within populations and among all individuals are displayed in Figures 3 and 4, respectively. Bootstrap analysis was used to estimate the number of bands necessary to obtain a stable association of all accessions. It was observed that approximately 70 markers were sufficient for dendrogram stability (coefficient of variation = 4.2%) and the rate of decrease was comparatively minimal beyond that (Figure 5), suggesting that 99 markers were sufficient for the analysis of coffee cultivars and progenies.

Analysis of molecular variation

The RAPD showed that 38.5% and 61.5% of the genetic variation were found among and within the populations, respectively (Table 4). These data were supported by a F_{ST} = 0.385 which measures the magnitude of genetic differentiation among populations. It has been demonstrated

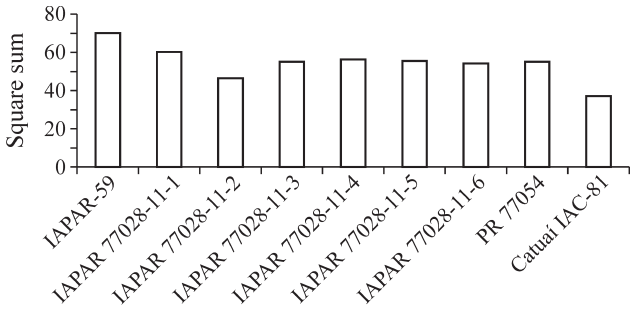

Figure 2 - Square sum diagram of variations (Excoffier, 1993) within Sarchimor progenies and cultivars of *C. arabica.*

that the range of F_{ST} variation for autogamous species is large, varying from 0.026 to 0.78 as shown in wild bean (Cattan-Toupance *et al.*, 1998). Anthony *et al.* (2001) determined a value of $G_{ST} > 0.50$ for 88 *C. arabica* accessions, collected by FAO in 1968 from forests and farms in Ethiopia, and two accessions derived from genetic populations of *C. arabica* var arabica and *C. arabica* var bourbon. According to Nei (1977), the G_{ST} and the F_{ST} of Wright (1965) are equivalent values. The genotypes studied in this research were submitted to several selection cycles, a fact that should have contributed to the reduction of F_{ST} value (F_{ST} = 0.385). As demonstrated by the FST value obtained in this investigation, the coffee genotypes still display high levels of genetic polymorphism. The overall genetic variation probably derived from DNA markers introgressed from the *C. canephora* gene pool, which are present in the

Table 2 - Percentage of polymorphic loci for *C. arabica* progenies and cultivars estimated according to Yeh *et al.* (1997).

Progenies	Number of polymorphic loci	Percentage of polymorphic loci
1- IAPAR-59	42	42.42%
2- IAPAR 77028-11-1	37	37.37%
3- IAPAR 77028-11-2	31	31.31%
4- IAPAR 77028-11-3	33	33.33%
5- IAPAR 77028-11-4	34	34.34%
6- IAPAR 77028-11-5	40	40.40%
7- IAPAR 77028-11-6	34	34.34%
8- PR 77054-40-10	34	34.34%
9- Catuaí Vermelho IAC 81	26	26.26%

Table 4 - Analysis of molecular variance (AMOVA) among and between the Sarchimor progenies and *C. arabica* cultivars.

Source of variation	DF	Square sum	Variance component	Percentage variation (%)
Between populations		351.4	3.79	38.5***
Within populations	81	490.1	6.05	61.5
Total	89	841.5	9.84	

(F_{ST}) = 0,385. ***p < 0.001.

Table 3 - Mean differences of the genetic differentiation among individuals within populations and between pairs of *C. arabica* populations estimated according to Yeh *et al.* (1997).

Genotypes	1	2	3	4	5	6	7	8	9
1- IAPAR-59	15.58								
2- IAPAR 77028-11-1	4.92	13.38							
3- IAPAR 77028-11-2	5.98	1.58	10.31						
4- IAPAR 77028-11-3	5.59	2.75	4.72	12.24					
5- IAPAR 77028-11-4	5.52	8.34	6.81	7.04	12.51				
6- IAPAR 77028-11-5	6.48	5.48	7.35	4.02	6.25	12.31			
7- IAPAR 77028-11-6	8.24	6.44	8.31	4.50	8.93	7.19	12.07		
8- PR 77054-40-10	9.13	12.41	13.72	9.52	6.32	9.56	5.84	12.24	
9- Catuaí Vermelho IAC 81	11.62	12.28	13.73	12.58	8.81	11.33	6.87	2.54	8.27

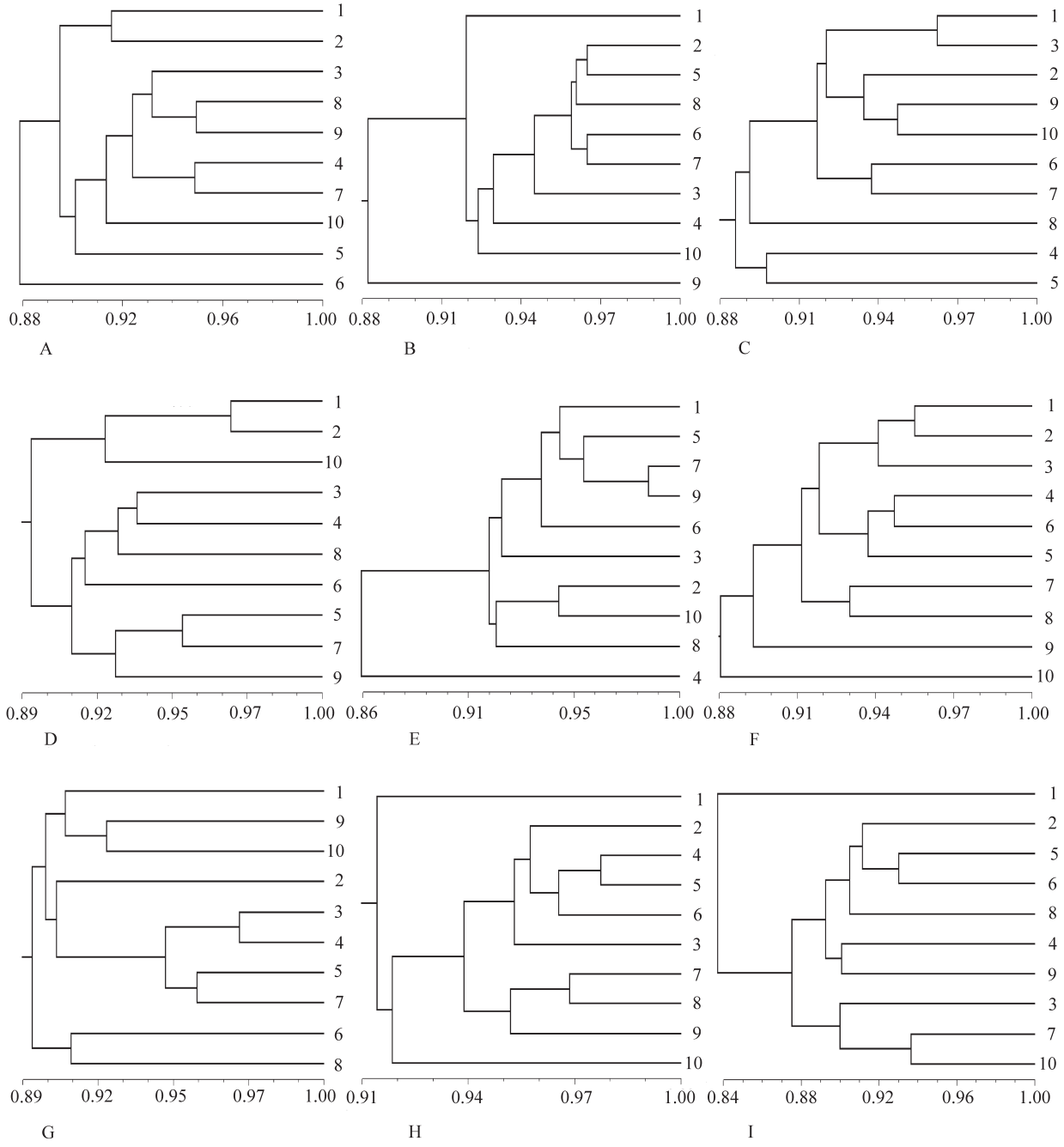

Figure 3 - Cluster diagrams based on Dice's genetic similarity among individual plants of coffee: A. progeny IAPAR 77028-11-1; B. progeny IAPAR 77028-11-2; C. progeny IAPAR 77028-11-3; D. progeny PR 77054-40-10; E. progeny IAPAR 77028-11-4; F. progeny IAPAR 77028-11-5; G. progeny IAPAR 77028-11-6; H. cultivar Catuai Vermelho IAC 81; and I. cultivar IAPAR 59.

Sarchimor-derived germplasm and in the PR 77054-40-10 progeny.

Analysis of genetic variability within and among the cultivars IAPAR-59 and Catuaí Vermelho IAC 81

The IAPAR 59 cultivar showed a higher percentage of polymorphic loci (42.42%) than the Catuaí Vermelho IAC 81 cultivar (26.26%) (Table 2). The genetic diversity was also higher within the cultivar IAPAR 59 cultivar than within the Catuaí cultivar (Table 3, Figures 2, 3, 4). The origin of these cultivars gives support to these observations. As outlined in Figure 6, IAPAR 59 derived from an artificial hybridization between Villa Sarchi (CIFC 971/10) and the Timor Hybrid (CIFC 832/2) accomplished in Centro de Investigações das Ferrugens do Cafeeiro (CIFC) located in Portugal. The F_2 progeny, denoted as LC 1669, was brought

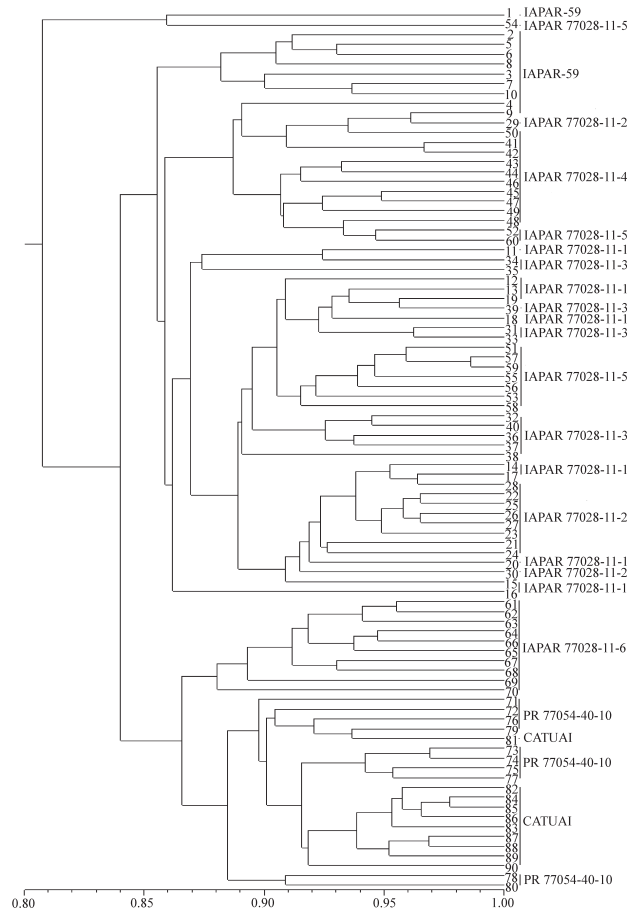

Figure 4 - Genetic relationships obtained among plants of progenies and cultivars of *C. arabica* on the basis of Dice's similarity coefficient.

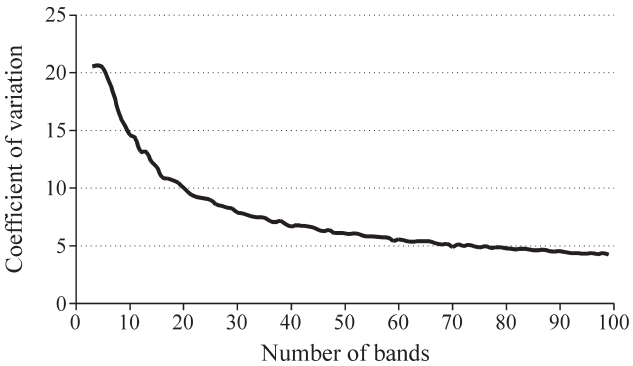

Figure 5 - Sample variance for the Sarchimor progenies and cultivars of *C. arabica*, derived from a bootstrap procedure after 1000 sampling, illustrating the relationship between the mean coefficient of variation (%) and the number of bands.

to the Instituto Agronômico de Campinas (IAC). The F_3 generation (IAC 1669 EP127 C506) was then sent to the Instituto Agronômico do Paraná (IAPAR) and designated as IAPAR 75163. The selection and adaptation of IAPAR 75163 plants gave rise to the IAPAR 75163-22 progeny, which expressed several important agronomic traits, such

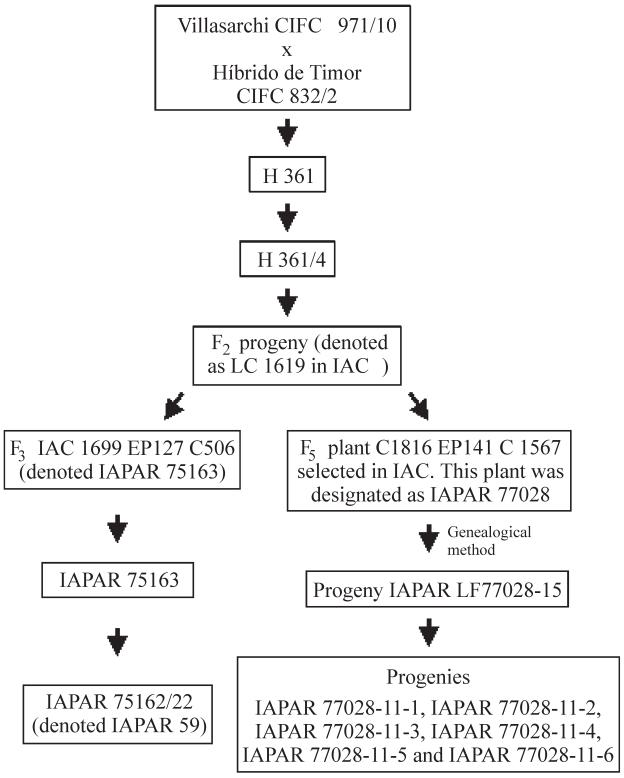

Figure 6 - Origin of IAPAR 59 and progenies of the Sarchimor germplasm.

as leaf rust resistance. The IAPAR 75163-22 progeny was then nominated IAPAR 59 and released as commercial cultivar (Sera *et al.*, 1996). Catuaí Vermelho IAC 81, on the other hand, is a cultivar that resulted from crossing between the arabica genotypes Mundo Novo and Caturra. Genotypes derived from Timor Hybrid have shown notable genetic diversity, which appears to be approximately twice that detected in the group of cultivated and wild *C. arabica* accessions (Lashermes *et al.*, 2000). Anthony *et al.* (2001) applied RAPD markers to the analysis of 88 wild *C. arabica* accessions from Ethiopia and two accessions derived from genetic populations of *C. arabica* var *arabica* and *C. arabica* var *bourbon.* According to the authors, the absence of within genetic variation strengthened the evidence of the narrow genetic base of Arabic coffee. Our results denote that much more polymorphism is present in the Sarchimor-derived germplasm than in the Arabic coffee (Catuaí Vermelho IAC 81). The analysis of genetic similarity estimated for the 10 plants of each population ranged from 0.78 to 0.94 and from 0.88 to 0.98 within the IAPAR 59 and Catuaí Vermelho IAC 81 populations, respectively. The lower genetic similarity (Table 3, Figure 3) observed among Catuaí Vermelho IAC 81 plants (Arabic germplasm), when compared to IAPAR 59 plants (Sarchimor germplasm), is consequent of the narrow genetic base of the Arabic gene pool. Nevertheless, it was evident that the Catuaí genotypes studied still display enough

genetic variation, which could be useful for breeding programs.

Progenies of Sarchimor germplasm

Besides IAPAR 59, the other progenies of the Sarchimor germplasm studied were also derived from the hybridization between Villa Sarchi (CIFC 971/10) and the Timor Hybrid (CIFC 832/2). The plant C1816 EP141 C1567, selected at IAC, was sent to IAPAR and the F_3 generation was designated as IAPAR 77028. The IAPAR LF 77028-15 progeny was then selected using the genealogical method and named, in the F_5 generation, as IPR 99. The Sarchimor progenies analyzed in this research are sister progenies of IPR 99.

The values of the genetic similarities observed within the Sarchimor-derived progenies ranged from 0.78 (within IAPAR 59) to 0.99 (within IAPAR 77028-11-4). The percentage of polymorphic loci and the level of within genetic variation showed that IAPAR 77028-11-1 and IAPAR 59 display similar levels of polymorphism, whereas IAPAR 77028-11-2 is more uniform (Tables 2, 3; Figure 3). The differences in genetic variability observed within the Sarchimor progenies are probably the result of both genetic segregation and selection that occurred in the origin of these progenies. The differences among the Sarchimor progenies revealed a smaller genetic difference (4.92) between IAPAR 59 and the IAPAR 77028-11-1 progeny and higher divergence (8.24) between the IAPAR 59 and the IAPAR 77028-11-6 progeny (Table 3, Figure 4). The genetic divergence between IAPAR 59 and IAPAR 77028-11-6 was accompanied by the loss of resistance that resulted in the appearance of new physiological races for the leaf rust disease. Hence, IAPAR 77028-11-6 probably carries fewer genes for leaf rust resistance in relation to the other genotypes analyzed. On the other hand, progenies that maintain genetic resistance are more related to the IAPAR 59 variety (Table 2) as for example, the progeny IAPAR 77028-11-1. The mean genetic differentiation was higher within the Sarchimor-derived progenies than within the Catuaí cultivar, probably because of the narrow genetic base of Catuai coffee.

PR 77054-40-10 progeny

The analysis of the PR 77054-40-10 progeny revealed that the values of within genetic similarity and the percentage of polymorphic loci are similar to those of the Sarchimor progenies (Tables 2, 3; Figures 2, 3). As expected, the PR 77054-40-10 progeny was more related to the cultivar Catuaí Vermelho IAC-81 than any other progeny evaluated (Figure 3). The PR 77054-40-10 progeny was obtained by selection from progenies derived by crossing between plants of the Catuaí and Icatu gene pools. The Icatu germplasm derived from interspecific hybridization between *C. canephora* var robusta and *C. arabica* var bourbon (Arabic cultivar) followed by crossing of the F_1 progeny with plants of the Mundo Novo (Bourbon x Sumatra) germplasm. Therefore, the genetic diversity conserved within the PR 77054-40-10 progeny (Table 3) is derived from the combination of genes from the *C. arabica* and the *C. canephora* gene pools.

Exploitation hybrids in coffee

It is acknowledged that there is a strong association between heterozigosity and heterosis and this phenomenon has led to the success of the improvement in crop yields (Duvick, 1984). Hybrid varieties have revolutionized crop production, including cross- and self-pollinated species. The usefulness of RAPD markers in the selection of hybrids with high productivity was demonstrated in rice (Xiao *et al.*, 1996). Lanza *et al.* (1997) described a positive correlation between RAPD based genetic distance and single-cross hybrid grain yield of tropical maize inbred lines. In coffee, we demonstrate that the IAPAR 77028-11-4 and IAPAR 77028-11-6 progenies are the most divergent among the Sarchimor progenies (Table 2, Figure 3). Therefore, plants of these progenies may be exploited to potentialize the hybrid performance of the Sarchimor germplasm. The same approach may be used for individual plants of the six Sarchimor progenies, PR 77054-40-10 progeny, and also the IAPAR 59 and Catuaí Vermelho IAC 81 cultivars (Table 3, Figures 2, 3). Diniz (2000) demonstrated that IAPAR 59 and Mundo Novo exhibit low genetic similarity (75%). The association between genetic divergence and heterosis was demonstrated in coffee by combining the IAPAR 59 and Mundo Novo cultivars. Hybridization between these cultivars produced a heterosis of 25% (Sera, personal communication) that was possibly the result of different allele combination in the hybrid. Thus the RAPD-derived markers could be used to obtain hybrids between plants of the same progeny and between progenies or varieties of the germplasms studied.

The RAPD markers provided useful information to study genetic variability within and among *C. arabica* populations. The IAPAR 59 cultivar presented more genetic variation than Catuaí Vermelho IAC 81. This difference is probably the result of the hybrid origin of IAPAR 59. Furthermore, it was verified that genotypes which are more susceptible to leaf rust disease also exhibit higher genetic distance from the genotypes that maintain a high number of genes for resistance such as the IAPAR 59 cultivar. It was also evident that the level of genetic variability is not the same for the six Sarchimor progenies. The overall results of this investigation demonstrated that all genotypes studied could provide valuable material for coffee breeding.

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