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Partial map of *Coffea arabica* L. and recovery of the recurrent parent in backcross progenies

Antonio Carlos Baião de Oliveira[1*], Ney Sussumu Sakiyama[2], Eveline Teixeira Caixeta[3], Eunize Maciel Zambolim[2], Raphael José Nascif Rufino[4], and Laércio Zambolim[2]

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ABSTRACT - *A partial map of* Coffea arabica *L. was constructed based on a backcross population and RAPD markers. From a total of 178 markers evaluated, only 134 that segregated 1:1 (P>0.05) were used to develop the map. Seventeen markers were not linked, while 117 formed 11 linkage groups, covering a genome distance of 803.2 cM. The maximum distance between adjacent markers was 26.9 cM, and only seven intervals exceeded 20 cM. The markers were further used for assisted selection of the plants closest to the recurrent parent, to accelerate the introgression of rust resistance genes in the coffee breeding program. Three BC_1 plants resistant to coffee leaf rust and with high genetic similarity to 'Catuaí' were selected and integrated in the following backcross cycles.*

Key words: *Coffee*, molecular markers, RAPD, marker assisted selection, coffee leaf rust.

INTRODUCTION

The species *Coffea arabic*a L. (2n=4X=44), the only tetraploid and self-fertile of the genus *Coffea*, is characterized by low genetic diversity that has been attributed to its allotetraploid origin, type of reproductive biology and evolutionary process. On the other hand, the diploid species of the genus are all allogamous with considerable variability, representing an important gene pool for coffee breeding (Herrera et al. 2002). The transference of desirable traits, mainly resistance to biotic stresses, from diploid species such as *C. canephora*, *C. liberica* and *C. racemosa* to *C. arabica* cultivars, without affecting the grain quality traits of this species, has been one of the main objectives of coffee breeding programs. However, the difference in ploidy between *C. arabica* and the diploid species hampers gene introgression.

The strategy of using crosses of other species with *C. arabica* arose from the need of improving the cultivars used in Latin America and Kenya when coffee leaf rust, caused by the fungus *Hemileia vastatrix* Berk. et Br., appeared. The Arabic coffee type, cultivated until then, presented no resistance at all to this disease (Etienne et al. 2002). Researchers have concentrated their efforts on populations of natural interspecific hybrids, which seem most suitable to be integrated in backcross programs with Arabic coffee types, due to their similar genetic structure to *C. arabica*.

[1] Centro de Análise e Pesquisa Tecnológica do Agronegócio do Café "Alcides Carvalho", Instituto Agronômico (IAC/APTA), C.P. 28, 13.012-970, Campinas, SP, Brasil. *E-mail: baião@iac.sp.gov.br
[2] Laboratório de Biotecnologia do Cafeeiro, BIOAGRO, Universidade Federal de Viçosa (UFV), 36.570-000, Viçosa, MG, Brasil
[3] Embrapa-Café , UFV
[4] Sakata Seed Sudamerica LTDA, Avenida Dr. Plinio Salgado, 4320, C.P. 427, 12.906-840, Bragança Paulista, SP, Brasil

Studies focused on the natural hybrid called Híbrido de Timor (HT). This hybrid is in fact a population, probably descending from a single plant found in a *C. arabica* field on Timor Island, selected since the plant showed no symptoms of leaf rust disease. The HT was originated from a natural cross between *C. arabica* and *C. canephora* (2n=2X=22) and derived numerous factors of rust resistance from the diploid parent (Bettencourt 1973). Due to this trait and the ease of crossing with Arabic coffee varieties, HT has been widely used in genetic breeding programs aiming at improved rust-resistant coffee cultivars.

Trait introgression into species by conventional methods is frequently a time-consuming, labor-intensive process. In the case of perennial species such as coffee, the development of Arabic coffee cultivars from the HT can take up to 30 years. A feasible alternative to reduce the duration of gene introgression, by the simultaneous transference of DNA fragments from the donor (HT) to the recurrent parent (commercial cultivar), is to use marker-assisted selection (Etienne et al. 2002).

Interspecific crosses can incorporate undesirable alleles of the donor species into the cultivated, increase the time required to recover the traits of interest of the elite lines, and cause the loss of favorable alleles of other important traits (Brondani et al. 2001). Marker-assisted introgression on the other hand allows a control of the crosses, which minimizes the occurrence of these problems. The construction of a genetic linkage map based on molecular markers is therefore of utmost importance. These maps allow the identification and localization of genes and QTL (Quantitative Trait Loci) in the linkage groups and provide the base for marker-assisted selection (Lorieux et al. 2000). Besides, genetic mapping represents one of the most efficient approaches to advanced genetic studies, as it can facilitate the understanding of trait inheritance, genome structure and organization, research on evolution, isolation and cloning of desirable genes (Roose et al. 2000).

Two maps have been constructed for coffee: a complete linkage map for *C. canephora* (Lashermes et al. 2001) and another partial linkage map, based on an interspecific cross between the species *C. pseudozanguebarie* x *C. liberica* (Ky et al. 2000). In *C. arabica*, the establishment of saturated linkage maps and, therefore, with wide genome cover, is hampered by the low polymorphism level in cultivars (Orozco-Castillo et al. 1994, Lashermes et al. 1999) and by the presence of polyploidy in this species (Paillard et al. 1996). The construction of partial maps, using different genetic backgrounds and followed by the integration of these partial maps, is the strategy that has been used to achieve complete maps of the Arabic coffee genome. Two partial maps of *C. arabica* have been constructed recently by this strategy (Pearl et al. 2004, Teixeira-Cabral et al. 2004).

In our study, RAPD markers were used to construct a partial gene linkage map of *C. arabica*. In the segregating population under study, these markers were also used to identify plants with the highest proportion of the recurrent parent. These were selected to integrate new backcross cycles, aiming at the incorporation of rust resistance genes into the commercial cultivar Catuaí.

MATERIAL AND METHODS

Mapping population

The commercial cultivar Catuaí Amarelo IAC 30, susceptible to coffee leaf rust, was crossed with a resistant Híbrido de Timor UFV 445-46 line. The cross originated the resistant F_1 hybrid, designated H 419-1. Pollen of this F_1 hybrid was used in a backcross with the parent Catuaí to generate the BC_1 population with 59 plants used in the linkage map construction.

DNA extraction

The DNA of 59 BC_1, parents and F_1 plants was extracted from young and completely developed leaves following the slightly modified method of Paillard et al. (1996). The leaf samples were ground in liquid nitrogen and 500 mg of powder was transferred to 15 ml Falcon tubes containing 3 mL of extraction buffer A (0.35 M sorbitol, 0.1 M Tris-HCl pH 8.0, 0.005 M EDTA) and B (0.05 M EDTA, 2.0 M NaCl, 0.2 M Tris-HCl pH 8.0, 2% CTAB), plus 0.8% sarcosyl, 1% sodium bisulfite, 2% PVP-40 and 0.1% activated carbon. The mixture was incubated at 65 ºC for 60 min. The DNA was extracted with phenol-chloroform/isoamilic alcohol (24:1) and precipitated with 2/3 volume of isopropanol. The DNA pellet was washed with 70 and 95% ethanol, air-dried and resuspended in 200 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), containing 80 mg mL^{-1} of RNase. The solution was incubated at 37 ºC for 30 min, and the DNA reprecipitated with frozen isopropanol and 30ml 5M NaCl or 3M NaOAc. The resultant DNA pellet was washed with ethanol, dried and resuspended in 200 mL TE. The quality of the DNA

was determined by 1% agarose gel electrophoresis and quantified by a fluorometer (DyNA Quant™ 200, Hoefer Pharmacia Biotech Inc.).

PCR amplification, electrophoresis and gel staining

DNA was amplified by RAPD in a thermocycler (*Perkin-Elmer* 9600) programmed for an initial DNA denaturation at 95 °C for 1 min, 40 cycles of 15 sec at 94 °C, 30 sec at 35 °C and 1 min at 72 °C, followed by a final extension of 7 min at 72 °C. The PCR was performed in a 25 mL reaction mixture containing 25 ng of genomic DNA, 1 U of *Taq* DNA polymerase, 0.1 mM of each dNTP, 0.2 mM primer, 50 mM KCl, 10 mM Tris-HCl pH 8.3, and 2 mM $MgCl_2$. The amplified fragments were separated in 1.2% agarose gel, stained with ethidium bromide (10 ng mL^{-1}) and visualized under UV light for photo documentation.

Screening of RAPD primers

A total of 1200 primers (*Operon Technologies* kits OPA-OPZ, OPAA-OPAZ and OPBA-OPBH) and 698 arbitrary combinations of 99 primers were evaluated in both parents and F_1 DNAs. The choice of these 99 primers was based on consistent amplifications and absence of polymorphisms when used separately in previous reactions.

Polymorphic and consistent markers were selected based on these analyses. Only markers present in the non-recurrent parent (Híbrido de Timor) and F_1 and absent in the recurrent parent (Catuaí) were used in the analysis of the BC_1 population. To confirm the repeatability of the polymorphic bands, 12-tree-samples of the BC_1, parents and F_1 were evaluated. After that, all BC_1 plants were analyzed.

Rust inoculation and evaluation

BC_1 plants were inoculated with *Hemileia vastatrix* race II to select coffee leaf rust resistant plants. For the inoculation, the leaf disc technique was used and the symptoms evaluated after 49 days, according to a score scale proposed by Tamayo et al. (1995).

Data analysis and map construction

A matrix was created based on the RAPD markers, using 1 for the presence of band (heterozygous genotype), 0 for absence (homozygous recessive genotype) and 9 for lost data. The segregation of RAPD markers was analyzed by the chi-square (χ^2) test. Each marker was designated based on the primer code it was derived from and its base-pair size.

Data were analyzed with the software Genes (Cruz 2001) and GQMOL (http://www.ufv.br/dbg/gqmol/gqmol.htm). Only markers segregating 1:1 were used to construct the linkage map. This segregation rate is expected for a first backcross population, since *C. arabica* is an amphidiploid species (Antony et al. 2001) with diploid behavior. Besides, the Híbrido de Timor used as parent is highly inbred as it had undergone at least four natural backcrosses with *C. arabica* (Pereira et al. 2002).

Linkage groups were identified by the two-point analysis, considering a maximum recombination value of 30% and a minimum LOD score of 3.0. Three-point and multipoint analyses were then used to find the most likely order of the markers within the linkage groups. Kosambi mapping function (Kosambi 1944) was used to convert the recombination rates into map distances (cM - centiMorgan).

The map markers data were also used to identify the BC_1 plants closest to the recurrent parent (Catuaí) in support of the selection of plants with rust resistance genes and a reduced proportion of the donor line (Híbrido de Timor). A matrix of genetic dissimilarity between cultivar Catuaí and the 59 plants of the BC_1 segregating population was created. The arithmetic complement for the Jaccard index was used to obtain this matrix. The grouping analysis was performed by the Single linkage method. The percentage of the recurrent parent in each plant was calculated based on the marker distribution of each parent.

RESULTS AND DISCUSSION

Polymorphism level and marker segregation

Of the1200 RAPD primers tested for polymorphic marker identification among the parents and F_1, 127 (10.6%) amplified consistent fragments in the Híbrido de Timor (non-recurrent parent) and F_1, which were absent in Catuaí Amarelo (recurrent parent). These RAPD fragments are of interest for mapping, once they distinguished the segregating plants in the backcross population. Considering the number of polymorphic fragments present in the recurrent parent (Catuaí) and in F_1, but absent in the donor parent (HT), the percentage of polymorphism was approximately 17.0%. Teixeira-

Cabral et al. (2004) found similar results with the parental Mundo Novo IAC 464-18 and Híbrido de Timor CIFC 2570, using 680 RAPD primers. In their study, the dominant allele (band) was present in Mundo Novo, while a recessive allele (band absence) characterized the Híbrido de Timor parent. Lashermes et al. (2001) studied two populations of 44 and 92 double-haploid *C. canephora* plants and found a polymorphism rate of approximately 12.0% between the RAPD primers.

In this study, the 127 informative primers originated 165 polymorphic RAPD markers (1.3 marker per primer), of which 124 (75.15%) segregated in the expected proportion of 1:1 and 41 (24.85%) diverged from it in the χ^2 test (P>0.05). This distortion rate was similar to the one found by Pearl et al. (2004) working with AFLP markers and a pseudo-F_2 mapping population from a Catimor x Mokka Hybrid cross. In *C. canephora*, Paillard et al. (1996) found a distortion rate of 20% for the RAPD marker and 12% for AFLP. Teixeira-Cabral et al. (2004) reported a distortion of 5.5% in a backcross population derived from the cross of Mundo Novo IAC 464-18 and the recurrent parent Híbrido de Timor CIFC 2570.

The polymorphism level revealed by the primer pair combination was below the level detected by individual primers. In 698 test combinations, 10 (1.43%) were polymorphic with consistent bands in the donor parent and in F_1 and no bands in the recurrent parent. A single polymorphic fragment was found in seven of these combinations and two in three combinations. Of the 13 polymorphic markers detected, 10 (76.9%) segregated 1:1 as expected and in three (23.1%) the distortion rate was similar to that found when using individual primers. The strategy of primer pair combinations was used to increase the number of RAPD polymorphic markers to be incorporated in the linkage map. However, this method was not successful in our study, contrasting with results of Corrêa et al. (1997). With RAPD primer pair combinations these authors obtained around 41% polymorphic bands between the parents used to construct an intra-specific map of soybean.

Genetic linkage map

The 134 polymorphic RAPD markers from DNA amplifications with individual primers and primer pair combinations that segregated 1:1 were used for the construction of the partial linkage map. Seventeen of these markers (12.7%) were not linked to any linkage group, while the other 117 markers were assigned to 11 linkage groups, comprising a total of 803.2 cM (Figure 1). To construct other partial maps for *C. arabica*, Teixeira-Cabral et al. (2004) evaluated 82 RAPD markers and Pearl et al. (2004) used 368 AFLP markers and found 6.1and 51.9% non-linked markers, respectively. These results show that many genome regions of *C. arabica* are yet unexplored by mapping studies and need more in-depth evaluations.

The maximum distance between adjacent markers was 26.9 cM in group 11, and only seven intervals exceeded 20 cM (Figure 1). The length of linkage groups ranged from 11.85 to 172.11 cM. The linkage group size was highly correlated with marker number per group (r=0.954), indicating random distribution of markers within groups. A correlation coefficient of the same magnitude (0.959) was found when developing the *C. canephora* map (Paillard et al. 1996). Teixeira-Cabral et al. (2004) also observed high values for this correlation (r=0.887) in *C. arabica*. The mean distance between two markers, considering all linkage groups, was 7.5 cM - very close to the 7.3 cM observed by Teixeira-Cabral et al. (2004). In each linkage group the mean distance between two markers ranged from 4.94 cM (group 1) to 26.93 cM (group 11).

The linkage map obtained here, in spite of gaps found in some linkage groups, is useful for QTL mapping, since most intervals between markers (93.4%) were less than 20 cM. This is considered the threshold value by most of the statistical mapping models of QTL. Three linkage groups (GL8, GL10 and GL11) consisted of only two markers that could be part of other linkage groups. The gaps in the map can be filled by increasing the number of plants in the mapping population and by adding other markers. These strategies could increase the number of markers in the small linkage groups or even identify them as part of other known groups. Non-linked markers can be integrated in the linkage groups as well. In *Trifolium repens* L., Jones et al. (2003) joined two linkage groups by the addition of AFLP markers to SSR data.

Any mapping study of *C. arabica* (2n=44) should address the identification of 22 linkage groups, corresponding to the haploid number of chromosomes of the species. It is important to emphasize that there is no ideal population to generate a highly informative

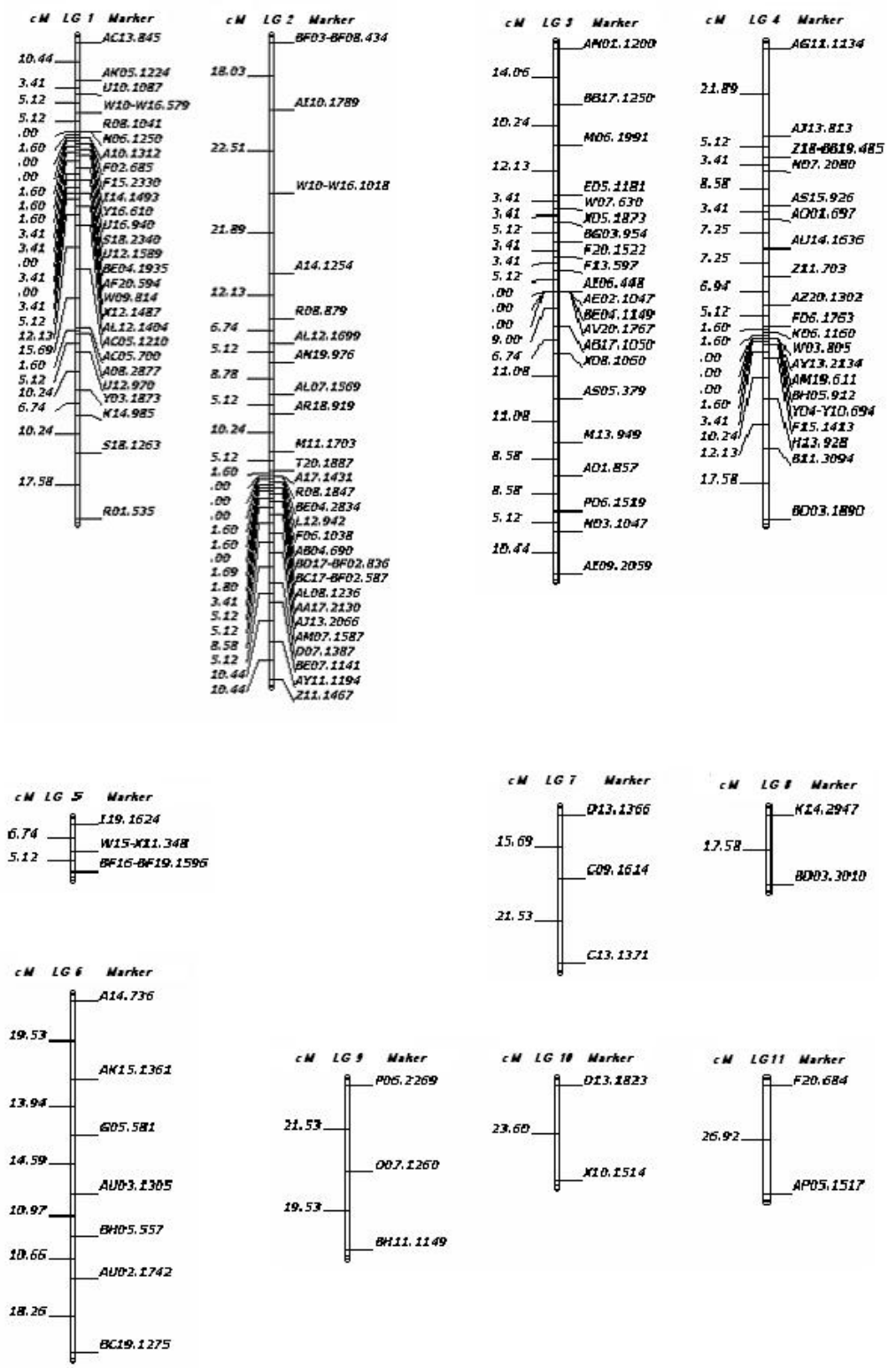

Figure 1. Partial linkage map of *Coffea arabica* L. based on RAPD markers, constructed with a minimum LOD score of 3.0 and maximum recombination frequency of 30%

linkage map of *C. arabica*. The genetic diversity of this species is low, which has been attributed to the small number of ancestor plants that originated it as well as the reproductive biology (Antony et al. 2002, Steiger et al. 2002).

The Híbrido de Timor UFV 445-46, descendant of a natural hybrid between *C. arabica* and *C. canephora*, was used as one of the parents of the mapping population to increase the number of polymorphic markers in the map. Besides, this genotype has different rust resistance genes which can be further identified and localized in the linkage map, aiming at marker-assisted selection.

Recovery of the recurrent parent in the segregating population

The markers detected in the linkage map were used to assist coffee tree selection in the segregating population. The selected plants are to be used in the next backcross generation, for rust resistance gene introgression in coffee breeding program. For this purpose, BC_1 plants were inoculated with *Hemileia vastatrix* race II and genotyped with molecular markers to identify the resistant plants genetically closest to the recurrent genome.

The inoculated plants within the progeny presented variable resistance/susceptibility degrees. Of these plants, 47 were fully resistant trees with no disease symptoms, three were highly infested plants with intense sporulation, similar to the susceptible control (Catuaí Amarelo - IAC 30), and nine plants presented an intermediate response to the disease. According to the adopted scale, seven of these plants were classified as resistant, and two susceptible.

The molecular analyses showed that one of the 50 BC_1 rust-resistant plants recovered only 55% of the recurrent parent genome (Catuaí), 17 (34%) recovered between 70 and 80%, 22 (44%) between 62 and 69%, and seven (14%) between 81 and 85%. In the plant group closest to Catuaí there was one plant with 88% and two with 92% of the genome of this parent (Figure 2). Faleiro et al. (2004) used RAPD markers to accelerate the introgression of anthracnose and rust resistance genes into common bean and identified plants with a higher percentage of the recurrent parent recovery than the average BC1 population in a BC_1 progeny.

Due to the high phenotypic similarity between BC_1 plants evaluated here, the identification of coffee trees with a high proportion of the genome background of the commercial cultivar might be difficult without DNA marker support. It is expected that BC_1 plants will contain on average 75% of the recurrent parent's genome. We found resistant plants with 88 and 92%, which demonstrates the high usefulness of the marker to speed up the introgression of desirable genes, such as the ones associated with coffee leaf-rust resistance.

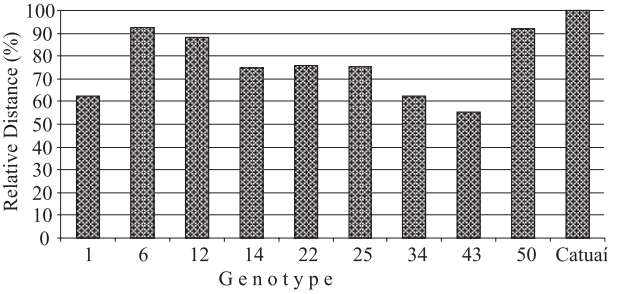

Figure 2. Genetic distance (%) of BC_1 progeny genotypes resistant to *Hemileia vastatrix* race II, compared to the recurrent parent Catuaí Amarelo IAC 30. Genotypes 6, 12 and 50 were selected to integrate the following backcross cycle

The multivariate data analysis separated the individuals genetically closest to each other and in relation to the recurrent parent. The Single Linkage method indicated the plants of the segregating population genetically closest to the recurrent parent (Figure 3). Coffee tree number 33, previously included in the group nearest Catuaí, was not used in the fo llowing backcross cycles due to the susceptibility to *H. vastatrix* race II.

In conclusion, a reasonable number of polymorphic and consistent RAPD markers were revealed in the study population, allowing the construction of a partial linkage map of *C. arabica*. The integration of this partial map with others will make it more informative and useful in genetic analyses of agronomic traits of *Coffea*, especially those of more complex inheritance. Furthermore, RAPD markers were helpful to identify plants with the highest genome proportions of the recurrent parent in the backcross population.

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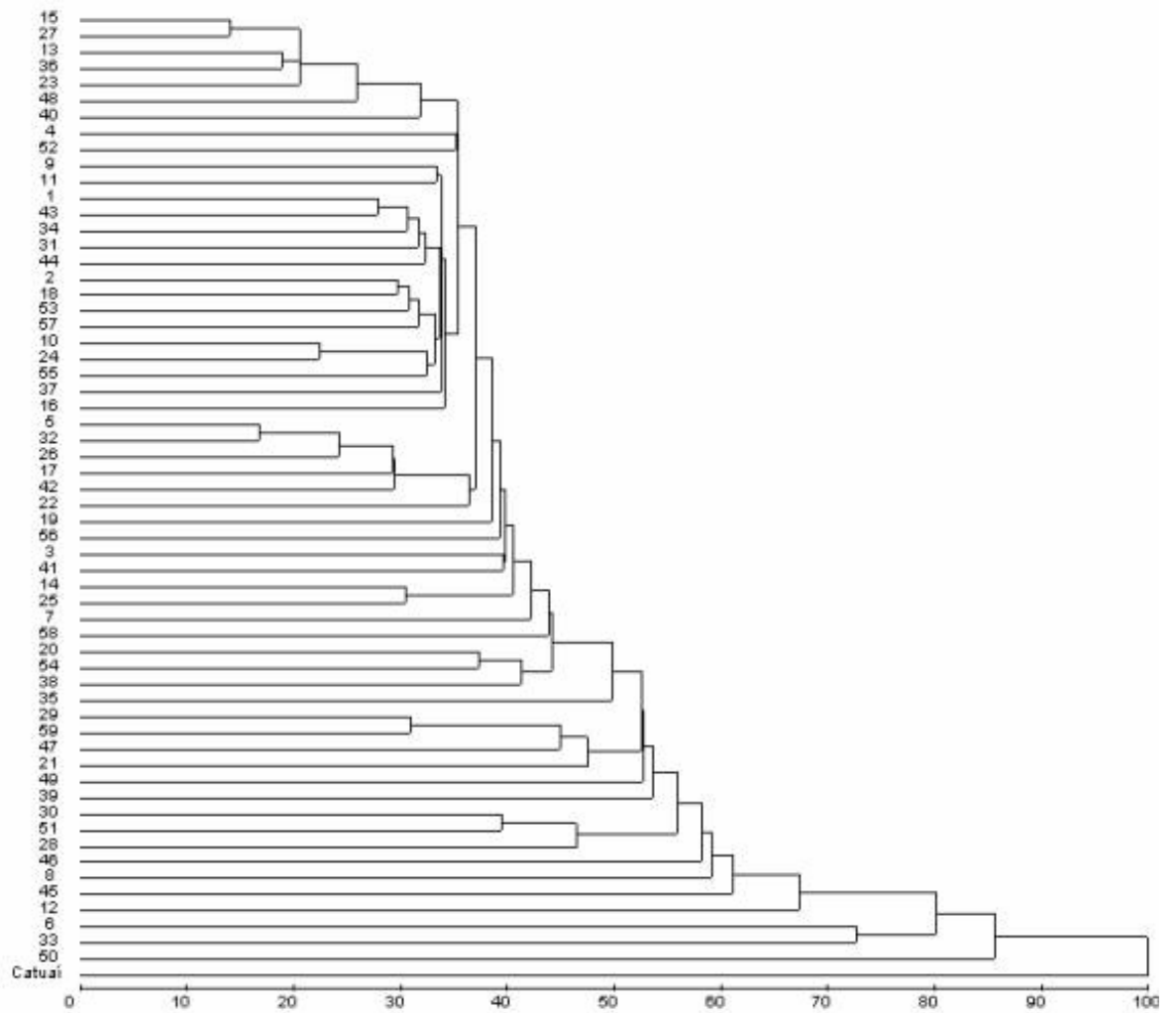

Figure 3. Dendrogram constructed from RAPD data for the recurrent parent (Catuaí Amarelo - IAC 30) and 59 plants of the BC_1 segregating population. The data were analyzed by the Single Linkage Method using Jaccard Index

Mapa parcial de *Coffea arabica* L. e recuperação do genitor recorrente em progênies de retrocruzamentos

RESUMO - *Um mapa parcial para* Coffea arabica *L. foi elaborado utilizando uma população de retrocruzamentos e marcadores RAPD. Foram avaliados 178 marcadores, dos quais 134 apresentaram segregação 1:1 (P>0,05) e 44 desviaram dessa proporção, não sendo aproveitados no mapa. Dezessete marcadores não estavam ligados, enquanto 117 formaram 11 grupos de ligação, cobrindo uma distância de 803,2 cM do genoma. Os grupos de ligação apresentaram boa densidade de marcadores, com intervalo máximo de 26,9 cM entre duas marcas adjacentes, sendo que apenas sete intervalos excederam 20 cM. Os marcadores foram utilizados também para assistirem a seleção de indivíduos mais próximos do genitor recorrente, visando acelerar a introgressão de genes de resistência à ferrugem em programa de melhoramento genético. Três plantas RC_1 resistentes à raça II de* H. vastatrix *e com similaridade genética ao 'Catuaí' muito acima da média esperada, para o primeiro retrocruzamento, foram selecionadas para integrar os próximos ciclos de retrocruzamentos.*

Palavras-chave: Café, marcadores moleculares, RAPD, seleção assistida, ferrugem.

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