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Genetic diversity of cacao accessions selected for resistance to witches' broom based on RAPD markers

Fábio G. Faleiro[1*], Uilson V. Lopes[2], Milton M. Yamada[1], Gilson R.P. Melo[2], Wilson R. Monteiro[2], José L. Pires[2], José B. Rocha[2], Rita C.S. Bahia[1], Luana M.C. Gomes[1], Ioná S. Araújo[1] and Alessandra S.G. Faleiro[1]

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ABSTRACT - *To increase the genetic diversity sources of resistance to witches' broom disease, 59 cacao trees of commercial plantations of the State of Bahia, Brazil, were selected and cloned. The diversity of these clones was evaluated with RAPD markers. Their pedigree was investigated by comparison to traditional resistance sources used as parents for most hybrid mixtures cultivated in the State. One hundred and six RAPD bands were obtained, 85.8% of which were polymorphic. Genetic distances among the clones varied from 0.04 to 0.41. The MDS plot showed large genetic variability among clones. Analyses based on rare markers inherent to Scavina-6 and Scavina-12 clones, which represent important resistance sources, showed a close relationship of these sources to most accessions. However, some accessions did not present any of the rare Scavina markers, suggesting potentially different alleles, which could contribute to broaden the gene pool of breeding programs for resistance to witches' broom.*

Key words: *Theobroma cacao* L., disease resistance, *Crinipellis perniciosa*, cacao germplasm, RAPD markers.

INTRODUCTION

Witches' broom, a cacao disease caused by *Crinipellis perniciosa* (Stahel) Singer, has caused serious economic, social, and ecological losses in Bahia, Brazil, since its arrival in 1989 (Pereira et al. 1989). Yield losses have reached 100% on some farms.

An efficient control of witches' broom involves integrated pest management with special emphasis on the use of resistant varieties, due to their low price, efficiency, and accessibility to cacao producers. The identification of genotypes with high yield and resistance to witches' broom is the first step in the breeding program. Stell (1933) and Pound (1938, 1943) were the first to search for resistance of cacao to witches' broom. After years of evaluation, two sibling plants, called Scavina-6 and Scavina-12 (Baker and Holliday 1957) were originally selected as immune to witches' broom. These sources of resistance, together with others like IMC, Cruzeiro do Sul, and RB have been widely used in several breeding programs including the one carried out by the CEPEC - Cacao Research Center. Consequently, many cacao trees used on farms come from crosses involving these sources, recommended for planting since the 1960s.

In 1993, the CEPEC set a large-scale program in motion to select high yield and resistant cacao genotypes on commercial plantations heavily infected with witches' broom (Pinto and Pires 1998) and aiming at a high diversity, in order to increase the durability of the resistance. By 2001, about one thousand trees had been selected, cloned, and coded as VB selections. They were established in areas with high incidence/severity of the disease for further evaluation. Although the resistance to witches' broom in many of these VB selections has been confirmed, it is important to assess the genetic diversity among them, in order to increase the

[1] Laboratório de Biotecnologia.
[2] Seção de Genética, Centro de Pesquisas do Cacau, C.P. 07, 45.600-000, Itabuna, BA, Brasil. *E-mail: ffaleiro@cpac.embrapa.br, Present address: Embrapa Cerrados, C.P. 08223, 73.301-970, Planaltina, DF, Brasil

chances of combining different genes for resistance and other traits. Only a few of these clones were subjected to preliminary studies. Yamada and Lopes (1999), using isozymes, verified that the most likely parents of 39 VB selections were the clones Sca-6, Sca-12, IMC-67, and Pa-150, particularly, the first two.

Yamada et al. (2001), using isozymes and RAPD markers, detected a high diversity among parents of the hybrid mixtures (full-sib families) recommended for farmers by the CEPEC, suggesting the existence of a high diversity in commercial plantations. Yamada et al. (2001), Pires et al. (2000), and Cascardo et al. (1993) compared some of these parents with clones of the local variety 'Comum' and verified that the diversity among the former is much higher than that among the latter. These results, associated with the detection of trees with low witches' broom severity have stimulated both breeders and farmers to search for resistant types in plantations of the State of Bahia.

This study aims at an evaluation of the genetic diversity among VB selections by RAPD molecular markers and of the origin of VB selections based on their relationship with traditional resistance sources. Another objective was to assess how much the selection process reduced the diversity compared to that of the original population represented here by the parents of all full-sib families planted in Bahia.

MATERIAL AND METHODS

Plant material

Fifty-nine VB selections and 21 parents of the hybrids (full-sib families) recommended for planting by the CEPEC (Table 1) were used in this study, making up a total of 80 cacao accessions. Of the 59 VB selections, 49 had been selected by the CEPEC and 10 by local farmers.

DNA Extraction

Leaves of each one of the 80 accessions were collected and stored at -80 °C until the DNA extraction. The genomic DNA of each accession was extracted by the CTAB method (Doyle and Doyle 1990) with some modifications (Faleiro et al. 2002). After the extraction, the DNA concentration was assessed by spectrophotometry at 260 nm (Sambrook et al. 1989). Bands of the total genomic DNA separated by agarose gel eletrophoresis at 0.8% were used as indicator of the integrity and purity of the extracted DNA. After the quantification, samples of DNA were diluted to 10 ng μL^{-1}.

RAPD Markers

DNA samples of each clone were amplified by the RAPD technique (Random Amplified Polymorphic DNA) (Williams et al. 1990). The amplification reactions were realized in a total volume of 25 µL, which contained 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 2 mM of $MgCl_2$, 100 µM of each deoxyribonucleotide (dATP, dTTP, dGTP, and dCTP), 0.4 mM of a primer (Operon Technologies), one unit of the Taq polymerase enzyme, and, approximately, 30 ng of DNA. Eleven decamer primers were used for the PCR amplification (Table 2). The amplifications were carried out in a thermocycler programmed for 40 cycles, with the following sequence each: 15 seconds at

Table 1. Cacao accessions

Nº [1]	Accession	Nº	Accession	Nº	Accession	Nº	Accession
1	ICS-1	21	UF-667	41	VB-0515	61	VB-1146
2	ICS-6	22	VB-0002	42	VB-0533	62	VB-1149
3	ICS-8	23	VB-0003	43	VB-0566	63	VB-1150
4	IMC-67	24	VB-0024	44	VB-0663	64	VB-1151
5	Pa-150	25	VB-0090	45	VB-0679	65	VB-1152
6	Pa-30	26	VB-0113	46	VB-0681	66	VB-1153
7*	Sca-12	27	VB-0114	47	VB-0892	67	VB-1154
8*	Sca-6	28	VB-0117	48	VB-0900	68	VB-1155
9	SIAL-169	29	VB-0118	49	VB-0902	69	VB-1159
10	SIAL-325	30	VB-0184	50	VB-0903	70	VB-1160
11	SIAL-505	31	VB-0186	51	VB-0906	71	SJ-02
12	SIAL-70	32	VB-0189	52	VB-0918	72	FSU-18
13	SIC-17	33	VB-0195	53	VB-0920	73	FSU-77
14	SIC-19	34	VB-0201	54	VB-0930	74	América-1
15	SIC-328	35	VB-0276	55	VB-0931	75	América-2
16	SIC-329	36	VB-0309	56	VB-1139	76	Camacã-1
17	SIC-813	37	VB-0316	57	VB-1142	77	SM-06
18	TSA-644	38	VB-0343	58	VB-1143	78	SF-423
19	UF-168	39	VB-0430	59	VB-1144	79	RT-09
20	UF-613	40	VB-0514	60	VB-1145	80	BB-1.33

[1] 1 to 21 – Parents of hybrid mixtures recommended for planting by the Cacao Research Center in Bahia, Brazil (*traditional sources of resistance to witches' broom); 22 to 70 – VB selections made by Cacao Research Center; 71 to 80 – VB selections made by the farmers.

Table 2. RAPD primers, their sequences of bases, and number of polymorphic and monomorphic bands associated to them

Primer	Sequence 5´→3´	Number of bands	
		Polymorphic	Monomorphic
OPA03	AGTCAGCCAC	13	2
OPA04	AATCGGGCTG	5	3
OPA07	GAAACGGGTG	9	1
OPA13	CAGCACCCAC	5	3
OPC05	GATGACCGCC	10	2
OPC13	AAGCCTCGTC	10	1
OPD08	GTGTGCCCCA	6	0
OPD16	AGGGCGTAAG	4	1
OPE05	TCAGGGAGGT	10	0
OPE14	TGCGGCTGAC	11	0
OPE15	ACGCACAACC	8	2
Total of bands		91	15

94 ºC, 30 seconds at 35 ºC and 90 seconds at 72 ºC. After the 40 cycles, a final extension stage of 7 minutes at 72 ºC was realized and finally, the temperature was reduced to 4 ºC. After the amplification, 3 ml of a mixture of bromophenol blue (0.25%), glycerol (60%), and water (39.75%) was added to each sample. These samples were applied on agarose gel (1.2%) and submerged in lid TBE (Tris-borate 90 mM, EDTA 1 mM). The electrophoretic separation lasted approximately four hours, at 90 volts. At the end of the process, the gels were stained with ethidium bromide and photographed under ultraviolet light.

Statistical Analyses

The obtained RAPD was transformed into a matrix of binary data, from which genetic distances based on the complement of the similarity coefficient of Nei and Li (1979) were calculated with the software Genes (Cruz 1997). The matrix of genetic distances was displayed in a biplot based on the multidimensional scaling (MDS), using the principal coordinates analysis method (Balasch et al. 1984). The advantages of this method were discussed by Dias (1998). The softwares SAS (SAS Institute Inc. 1989) and Statistica (StatSoft Inc. 1999) were used for the analysis and plot construction.

RESULTS AND DISCUSSION

Totally 106 RAPD bands were generated with the 11 decamer primers (a mean of 9.6 bands per primer). Among the 106 bands, 91 (85.8%) were polymorphic and 15 (14.2%) monomorphic (Table 2).

The genetic distances among the VB selections ranged from 0.04 to 0.41 (Table 3). The resistance and genetic diversity among these selected clones can be explained by the history of the breeding program carried out at the CEPEC (Vello et al. 1967). During this program, created in 1960, several clones, including different sources of resistance to witches' broom, were used for the generation of full-sib families (hybrid mixture) to be distributed to farmers.

The graphic analysis, based on the genetic distances, shows that the VB selections are very dispersed in the biplot (Figure 1), evidencing a high genetic variability among these selections. A similar dispersion pattern was observed for the parents of the hybrid mixtures recommended by the CEPEC breeding program.

The range of genetic distances among VB selections was wider than that observed among the hybrid parents (Table 3). Assuming that the genetic diversity in plantations is similar to that among the hybrid parents used in the formation of these plantations, as studied by Yamada et al. (2001), the selection

Table 3. Average (Ave), minimum (Min) and maximum (Max) genetic distances within and between parents of hybrid mixtures (PHM), VB selections by the CEPEC (VBC) and by farmers (VBF)

		PHM	VBC	VBF
PHM	Ave	0.141		
	Min	0.020		
	Max	0.280		
VBC	Ave	0.160	0.162	
	Min	0.040	0.040	
	Max	0.390	0.390	
VBF	Ave	0.190	0.190	0.210
	Min	0.080	0.060	0.100
	Max	0.400	0.400	0.410

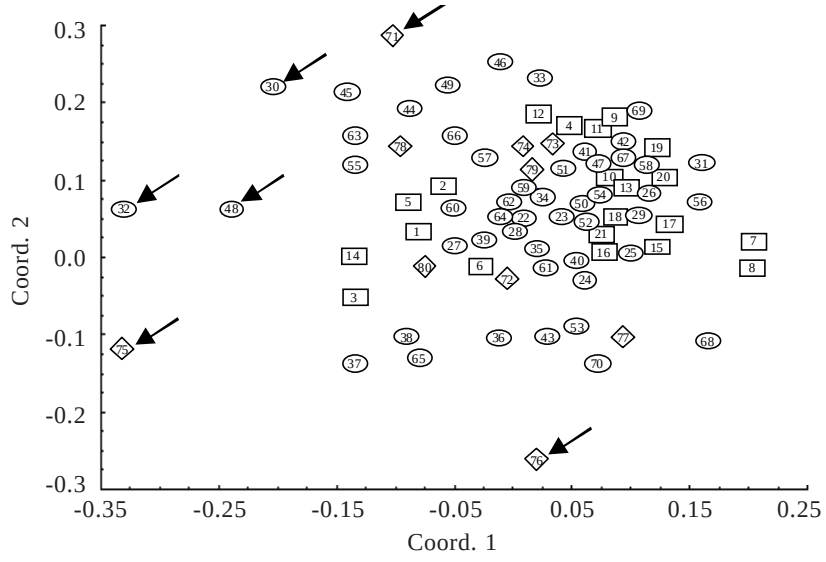

Figure 1. MDS plot of 80 cacao clones constituted by 59 VB selections made on plantations by the Cacao Research Center (⬭) and farmers (◊), and 21 parents of the hybrid mixtures (□), based on the matrix of genetic distances computed from RAPD markers. The arrows point selections that presented a high genetic distance from Scavina-6 (7) and Scavina-12 (8) and presented few rare Scavina markers. Accessions coded as presented in the Table 1.

for resistance did not reduce the variability. It should also be pointed out that the clones used as hybrid parents represent a significant part of the variability existing in *Theobroma cacao* (Marita 1998, Pires et al. 2000). Therefore, the diversity among the most resistant clones selected on farm plantations is as high as the diversity within the species. Considering that Scavina-6 and Scavina-12 came from seeds of the same pod (Baker and Holliday 1957), the genetic distance between these two clones was, as expected, very low (0.05).

Another important analysis is the comparison of VB selections with the Scavina-6 and Scavina-12 clones, the traditional sources of resistance. At this stage, three RAPD markers found in Scavina-6 and Scavina-12, but with low frequency in other parents (less than 32%), were used to assess the relationship of these resistance sources with the VB selections (Table 4), as suggested by Pires et al. (2000). Table 4 shows that such markers occur in at least 54% of the selections, evidencing the relationship of these VB selections with Scavina clones. Similar results were found by Yamada and Lopes (1999), when analyzing the paternity of 39 VB selections with isozymes. They verified that Scavina-6 and Scavina-12 were the most likely parents of 18 of these selections. When studying the origin of five VB selections, Araújo et al. (2000) also verified that four of them showed some genetic relationship with Scavina-6.

In many other studies, Scavina clones have presented a high level of resistance. Moreover, recently a major QTL for resistance to witches´ broom was found in Scavina-6 (Queiroz et al. 2003). Therefore, considering that many selections descend from Scavina clones, as supported by the high frequency of "specific" markers of Scavina on these selections, and considering the high diversity among these selections and between them and the parents, the Scavina resistance is stable across different genetic backgrounds. Also, taking into account that the selections were realized on plantations scattered over the cocoa region with its diverse fungal population (Anderbrhan et al. 1999), this resistance appears to be stable across different populations or pathotypes of the fungus.

Some selections, however, presented a high genetic distance from Scavina-6 and Scavina-12 and presented few rare Scavina markers. Among these selections are América-2 (75), VB-189 (32), VB-184 (30), VB-900 (48), Camacã-1 (76), and SJ-02 (71). This fact does not guarantee the presence of non-Scavina alleles in these selections, but suggests it strongly. This assumption is reinforced by the fact that other sources of resistance were used as parents of hybrid varieties from which the selections were derived.

Table 4. Scavina-6 and Scavina-12 specific RAPD markers and frequency among parents of hybrid mixtures (PHM) and among VB selections of the CEPEC (VBC) and of farmers (VBF)

RAPD Markers[1]	Frequency (%)	
	PHM	VBC+VBF
$OPA13_{1230}$	15.79	54.24
$OPA13_{580}$	31.59	54.90
$OPE05_{660}$	31.59	67.35

[1] The size of the RAPD fragment is indicated with the reference of RAPD primer.

Molecular marker studies reveal that the clonal varieties currently recommended by the CEPEC are genetically interrelated (Faleiro et al. 2001). The inclusion of the best VB clones in the programs of breeding and direct recommendation to farmers could contribute to broaden the restricted genetic basis of the clonal varieties currently recommended to farmers by the CEPEC in Bahia. The high genetic variability found among the VB selections stimulates working on a selection program for high yield and resistant cacao genotypes in commercial plantations.

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Diversidade genética por marcadores RAPD de acessos de cacaueiros selecionados para resistência à vassoura-de-bruxa

RESUMO - *Visando ampliar a base genética da resistência à vassoura-de-bruxa, 59 cacaueiros de plantações comerciais da Bahia foram selecionados e clonados. Avaliou-se a diversidade genética desses clones por marcadores RAPD. Inferiu-se sobre o pedigree desses acessos por comparação com as fontes tradicionais de resistência usadas como genitores das misturas híbridas cultivadas no estado. Foram analisadas 106 bandas RAPD, 85,8% das quais foram polimórficas. As distâncias genéticas entre acessos variaram de 0,04 a 0,41. O biplot de escala multidimensional evidenciou expressiva variabilidade genética. Análises de marcas raras dos clones Scavina-6 e Scavina-12, tradicionais fontes de resistência, mostraram o estreito relacionamento deles com grande parte dos clones selecionados. Parte dos acessos selecionados, entretanto, não apresenta as marcas raras, sugerindo alelos de resistência potencialmente diferentes, o que poderá contribuir para ampliar a base genética do programa de melhoramento para resistência à vassoura-de-bruxa.*

Palavras-chave: *Theobroma cacao* L., resistência a doença, *Crinipellis perniciosa*, germoplasma de cacau, marcadores RAPD.

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