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Microsatellite diversity and heterozygosity of parents of a cocoa breeding population

Milton Macoto Yamada[1*], Fábio Gelape Faleiro[2], Acassi Batista Flores[1], Uilson Vanderlei Lopes[1], José Luis Pires[1], Ronan Xavier Corrêa[3], and Reinaldo Figueiredo dos Santos[1]

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ABSTRACT – *The purpose of this study was to determine the genetic diversity and heterozygosity of 26 clones used as parents of 27 families. The populations are being evaluated by the Comissão Executiva do Plano da Lavoura Cacaueira (CEPLAC), in the state of BA. Nine of these clones are currently being recommended to farmers, while six others were used as control. The seven microsatellite generated 52 alleles with a mean of seven alleles/locus and genetic distance ranging from 0.17 to 0.90. This indicates a wide distribution of accessions and high variability. The heterozygosity ranged from 20% to 86%, and more than 50% of the loci were heterozygous in 79% of the clones. Although the selection of the parents for populations was not based on genetic distances, the high genetic diversity and heterozygosity of parents indicate highly segregating populations that make the selection of trees of interest possible, due to the variability.*

Key words: *Theobroma cacao*, molecular markers, breeding.

INTRODUCTION

After the confirmation of the occurrence of witches´ broom in Bahia, in 1989 (Pereira et al. 1989), the focus of the genetic breeding program of the Centro de Pesquisa do Cacau (CEPEC) has turned to the development of genotypes that are not only high-yielding, but also resistant to this disease caused by the fungus *Crinipellis perniciosa*. Although some selections are resistant to witches´ broom, the seeds are small to medium-sized and in other cases the infection level in fruits is high and will have to be improved. In spite of the resistance in fruits, some genotypes are susceptible to *Phytophthora* spp. which increases the costs by requiring fungicide application. With a view to join a great number of desirable agronomic traits in a single genotype, this experiment was conducted at the CEPLAC, with 27 interclonal families and four replications of 30 plants, totaling 120 progenies per family.

It is expected that genotypes with good agronomic traits can already be detected in the first generation (F_1). These will be tested at different locations for a subsequent recommendation to farmers. Others can be used in crosses to advance generations towards new breeding populations.

[1]Laboratório de Biotecnologia, CEPEC/CEPLAC, Km 22, Rodovia Ilhéus-Itabuna, C.P. 7, 45600-970, Itabuna, BA, Brazil. *E-mail: macoto@cepec.gov.br
[2]EMBRAPA Cerrados, BR 020, Km 18, CP 08223, Planaltina, DF, Brazil
[3]UESC, Rodovia Ilhéus-Itabuna, Km 16, 45650-000, Ilhéus, BA, Brazil

Diversity in cocoa has been characterized in germplasm accessions from many countries (Marita et al. 2001, Pires 2003), in specific populations like that in Criollos (Motamayor et al. 2002). In general, Forasteros from the Upper Amazon have the greatest genetic diversity (Pires 2003, Marita et al. 2001). The genetic base of the trees in Bahia cacao plantations was narrow until the CEPLAC began to recommend hybrids (Yamada et al. 2001), even at the beginning of the recommendation of clones Faleiro et al. 2001). Recently, Bertolde (2007) demonstrated the high genetic diversity in 38 recommended clones using microsatellite markers. In farm selections Faleiro et al. (2004) found that 50% of the clones had over 50% heterozygous loci. In the Parinari population, Yamada et al. (2003) found a heterozygosity of over 50% in about 40% of the accessions. The heterozygosity of 38 recommended genotypes ranged from 26 to 92% (Bertolde 2007).

We aimed to investigate the genetic diversity and heterozygosity of 26 clones used as parents that formed 27 F_1 families of experimental populations. Of these clones nine are already recommended to farmers while 6 are used as control in this experiment.

MATERIAL AND METHODS

Three groups of accessions of the 26 parents and controls used for the experiment (Table 1) were analyzed at the experimental station Joaquim Bahiana of the Comissão Executiva do Plano da Lavoura Cacaueira (CEPLAC). The control accessions (ICS 95, ICS 1, SCA6, SCA 12 and IMC 67, RIO ENG); recommended accessions (TSH 565, EET 392, CEPEC42, CCN 10, CCN 51, TSH 1188, VB 514, VB 1151, SJ 02) and others (RB 39, SIC 19, SGU 54, CC10, MAC 1, CEPEC 523, PA 169, EET 45, EET 62, CEPEC 94, PA 285, CEPEC 515, SGU 26 , VB 184, VB 1139, CASA, and CSG 70). Aside from ICS 95 and RIO ENG, the controls are clones that were largely used for hybrid production in the past. The recommended genotype are normally the ones with resistance to witches´ broom and good yield, and some also have good seed size, such as CCN51 and CCN10. The others, including other germplasm, as of Forastero and farm selections, are genotypes with resistance to witches´ broom or *Phytophthora spp.*

DNA extraction

Leaves from each accession were collected for genomic DNA extractions by the CTAB method (Doyle and Doyle 1990) with some modifications (Araújo et al. 2000). After the extractions the DNA concentration was estimated by a sphectophotometer at 260 nm (Sambrook et al. 1989). Bands of total genomic DNA were separated by electrophorese in 0.8% agarose gel to evaluate the integrity and purity of the extracted DNA. After the quantification, the DNA samples were diluted to a final concentration of 10ng μL^{-1}.

Microsatellite markers

The amplifications reactions were prepared in a total volume of 15 μL, including Tris-HCl 10 nM (pH 8.3), KCl 50mM, MgCl2 2.4mM, 150mM of each of the desoxynucleotides (dATP, dTTP, dGTP and dCTP), 1.5 pM primers labeled F and 1.5 primers labeled R, one unit of enzyme Taq polymerase and 30 ng DNA. The amplifications were performed in a thermocycler as follows: 4 minutes at 94 °C + 10 cycles (30 sec at 94 °C + 60 sec at 60 °C – 1 °C for each cycle + 90 sec at 72 °C) + 30 cycles (30sec at 94 °C + 60 sec at 48 °C+ 90 sec at 72 °C) + 6 minutes at 72 °C. Then, the samples were cooled down to 4 °C.

For the loading buffer on the polyacrylamide gel, 250 μL of formamide were used, 50 μL size standard (GeneScan ROX-500). and 25 μL loading buffer for 100 samples. The multiplex system and details of methodology are described by Yamada et al. (2007).

Statistical analyses

The microsatellite markers were converted into a numeric matrix, where: 0 for allele absence, 1 for the presence of one allele copy and 2 for the presence of two allele copies (Faleiro et al. 2001). Based on the numeric matrix, the heterozygosis levels and genetic distances were calculated and clustering analyses performed.

The genetic distances were calculated by the following expression

$GD_{ij} = 1 - (NCL/TNL)$,

Where, $G D_{ij}$ = genetic distance between the varieties i and j; NCL = number of coincident loci; TNL = total number of loci.

NCL is the sum of allelic coincidences of each analyzed locus, where each coincidence is assigned

Table 1. Accessions and estimated heterozygosity

No.	Accessions	Estimated heterozygosity (%)	Accession group
1	ICS 95	20	Control
2	ICS 1	67	Control
3	RB 39	67	Other parents
4	SIC 1'9	43	Other parents
5	SGU 54	57	Other parents
6	CC 10	57	Other parents
7	MAC 1	83	Other parents
8	CEPEC 523	71	Other parents
9	PA 169	71	Other parents
10	EET 45	71	Other parents
11	TSH 565	43	Other parents
12	EET 62	86	Other parents
13	EET 392	71	Recommended
14	SCA 6	43	Control
15	CEPEC 94	33	Other parents
16	SCA 12	71	Control
17	IMC 67	50	Control
18	PA 285	71	Other parents
19	RIO ENG	71	Control
20	CEPEC 42	57	Recommended
21	CCN 10	43	Recommended
22	CEPEC 515	71	Other parents
23	TSH 1188	86	Recommended
24	CCN 51	57	Recommended
25	SGU 26	57	Other parents
26	VB 184	71	Other parents
27	VB 1139	60	Other parents
28	VB 514	57	Recommended
29	VB 1151	57	Recommended
30	SJ 02	71	Recommended
31	CASA	83	Other parents
32	CSG 70	57	Other parents

value 1 for the matches (2 2); 0.5 for the matches (2 1), (1 2) and (1 1) and 0 for the matches (0 1), (1 0) and (2 0) Faleiro et al. (2001)

The matrix of genetic distances was used for cluster analyses via dendrogram, by the UPGMA method (Unweighted Pair Group Method with Arithmetic Mean) as grouping criterion. The genetic distance matrix was shown in a scatter plot based on multidimensional scaling, using the principal coordinate analysis method, as discussed by Dias (1998). The analysis and plot construction were performed using Software SAS (SAS Institute Inc. 1989) and Statistica (Statsoft Inc. 1999). The heterozygosity levels were calculated based on the relation between the number of loci in heterozygosis and the total number of analyzed loci.

RESULTS AND DISCUSSION

The seven microsatellite loci generated 52 alleles with a mean of 7 alleles/locus (Table 2). This number is higher than the 5.6 alleles/locus found by Risterucci et al. (2000) or 3.7 alleles/locus found by Yamada et al. (2003) using agarose gel, indicating the high variability in the parent accessions used here (Figure1) The genetic distance ranged from 0, 17 to 0.90 (data not shown) and shows great dispersion of accessions (Figure 2), also indicating high variability.

The accessions TSH 565, EET 392, CEPEC 42, CCN 10, TSH 1188, CCN 51, VB 514, VB 1151 and SJ02 are clones that are already recommended to farmers, and were used to form this population of 27 families. The distribution of these clones is broad (Figure 2), in agreement with Bertolde (2007). The purpose of the inclusion of a recommended genotype in a breeding program is to improve only few traits because this genotype already contains many good traits.

The variability in the other clones or selections was high, confirming the choice of the parents to form the population (Figure 2).

The variability in the six clones used as control was low (Figure 2), which can be explained by the narrow genetic base when the clones were first recommended (Faleiro et al. 2001) which were derived from the ancestors ICS 1, Sca 6, Sca 12 and IMC 67. The heterozygosity ranged from 20% to 86% (Table1), and in 79% more than 50% loci were heterozygous. This value is high, compared with other reports (Faleiro et al. 2004, Yamada et al. 2003, Bertolde 2007). Clone ICS 95 was one of the least, and EET 62 and TSH 1188 were the most heterozygous. Similarly, Bertolde (2007) reported that the heterozygosity of clone TSH 1188 was one of the highest. The selections MAC 1 and CASA were collected on farms. The clones CASA and EET 62 were used in this study due to their high yield, probably explained by heterosis, being very heterozygous.

Although the selection of the parents for populations was not based on genetic distances, the high genetic diversity and heterozygosity of the parents indicate highly segregating populations so the high variability makes the selection of trees of interest possible. This population is interesting with a view to individual selection for clones, rather than for the selection of families for hybrid production, due to the heterozygosity of the parents.

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Table 2. Microsatellite primers, their sequence and respective number of polymorphic alleles

Loci	Sequence of primers F and R (5' and 3')	Number of polymorphic alleles
CIRAD 35	TTTCCTTGTATTGACCTA ATATAAACACACTTCAGAGAT	6
CIRAD 10	ACAGATGGCCTACACACT CAAGCAAGCCTCATACTC	7
CIRAD 15	CAGCCGCCTCTTGTTAG TATTTGGGATTCTTGATG	10
CIRAD 7	ATGCGAATGACAACT GGT GCTTTCAGTCCTTTGCTT	9
CIRAD 2	CAGGGAGCTGTGTTATTGGTCA AGTTATTGTCGGCAAGGAGGAT	4
CIRAD 17	AAGGATGAAGGATGTAAGAGAG CCCATACGAGCTGTGAGT	6
CIRAD 13	CAGTCTAACAAAGGTGAG TGCCCCACTTGACAACTA	10
TOTAL		52

* Isolated and characterized by Lanaud et al. (1999)

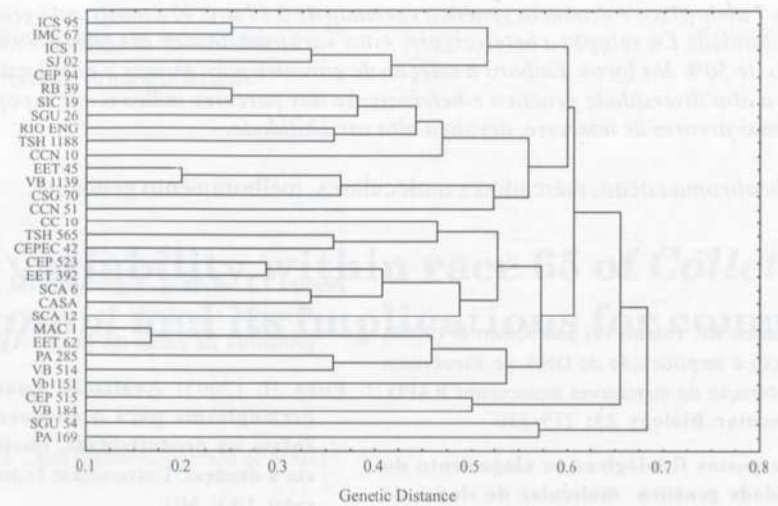

Figure 1. Cluster analysis of 32 cacao accessions based on a genetic distance matrix calculated using 52 microsatellite alleles. The UPGMA method was used as clustering criterion

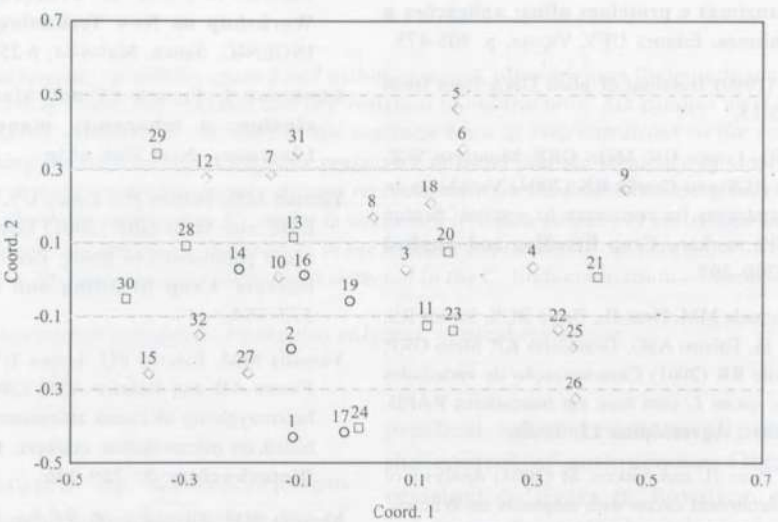

Figure 2. Dispersion analysis of 32 cocoa accessions based on genetic distance matrix calculated using 52 microsatellite alleles. The numbers correspond to accessions listed in Table 1, where control accessions (○); recommended accessions (□) and other parents (◇)

Diversidade genética e heterozigose de parentes de população de melhoramento para resistência a doenças em cacau usando marcadores microssatélites

RESUMO - *O objetivo deste trabalho foi avaliar a diversidade genética e heterozigose de 26 clones usados como parentes de 27 famílias que estão sendo avaliadas na Estação Experimental "Joaquim Bahiana" da CEPLAC, sendo 9 desses clones já é recomendado como clones para os produtores com 6 usado como controle. Os sete locos microssatélites geraram 52*

alelos com a média de 7 alelos/loco e distância genética variando de 0.17 a 0.90 e mostrando grande dispersão dos acessos e indicando alta variabilidade.Em relação a heterozigose, estas variaram de 20% até 86%,e 79% dos clones apresentaram heterozigotos em mais de 50% dos locos. Embora a seleção de parentes para formar a população não ter sido baseada em distancias genéticas, a alta diversidade genética e heterozigose dos parentes indicam que a população será segregatinge possibilitando selecionar árvores de interesse, devido a alta variabilidade.

Palavras-chave: *Theobroma cacao*, marcadores moleculares, melhoramento genético.

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