CBAB
CROP BREEDING AND
APPLIED BIOTECHNOLOGY

Variability in cacao selected by producers for resistance to witches´ broom based on microsatellite markers

Alessandra S G Faleiro[1], Fábio G Faleiro[2*], Uilson V Lopes[1], Gilson R P Melo[1], Wilson Reis Monteiro[1], Milton Macoto Yamada[1], Rita C S Bahia[1], and Ronan X Corrêa[3]

ABSTRACT - *This study aimed at a microsatellite marker-based assessment of the genetic variability of 30 accessions of* Theobroma cacao *selected for resistance to witches´ broom on commercial plantations in the cocoa region of Bahia State and to compare them with traditional sources of resistance to witches´ broom (SCA-6 and IMC-67) and other parents (ICS-1 and SIC-19) used in the breeding program of the Cocoa Research Center (CEPEC). Sixteen pairs of specific primers for microsatellite loci were used, generating 64 alleles. The genetic distance between the analyzed accessions ranged from 0.13 to 0.71 and the heterozygosis levels from 23 to 76%. The cluster analyses grouped 18 accessions (60%) together with SCA-6. Accessions selected on farms for their high levels of resistance to witches´ broom, good agronomic traits, which are genetically distant from SCA-6, have a high potential for the breeding program or even for direct recommendation to farmers.*

Key words: *Theobroma cacao*, molecular markers, *Crinipellis perniciosa*, resistance genes, participatory breeding.

INTRODUCTION

The cacao, *Theobroma cacao* L., is an allogamous and perennial species of great social and economic-ecological importance in the producing regions of Brazil, where close to 3 million people depend directly or indirectly on this crop. Cacao is an important source of receipts, income and employment, moving, in beans and derivatives, nearly 1.5 billion dollars an year. The South of Bahia is the main cacao producing region of Brazil, where practically 100 counties are economically cacao based. The cacao is cultivated on some 29 thousand farms, covering an area of over 700 thousand hectares (Souza and Dias 2001).

Witches' broom disease, caused by the fungus *Crinipellis perniciosa* (Stahel) Singer, has engendered a dramatic economical, ecological and social impact on the cacao region of Bahia and throughout Brazil in the last years. In the Bahia cacao region, the disease was introduced in 1989 (Pereira et al. 1989) causing yield losses of up to 100% on some farms.

There are possibilities to control the pathogen efficiently with integrated methods focusing on resistance, which is a cheap, efficient, and accessible method for producers. The identification of different genotypes with adequate resistance to witches' broom is a basic step in improvement programs aiming at resistance. Pound (1938,

1943) realized the first studies in search of resistance sources to the fungus *C. perniciosa*. The studies to identify resistance sources were intensified in the Bahia cacao region from the moment of the introduction of the disease (Pires et al. 2000a, Pires et al. 2000b). Results of these studies allowed an identification of different resistance sources and also supported the recommendation of clones derived from the accessions SCA-6, IMC-67, and ICS-1, designated TSA, TSH, CEPEC, and EET, which allow the recovery of the Bahia cacaoculture.

From 1960 on, the CEPEC/CEPLAC commenced a program to obtain seeds of genetically improved hybrid varieties, obtained by gene combinations between accessions from different geographic origins, including sources of resistance to witches' broom such as SCA-6, SCA-12, IMC-67, and other plant materials from the Upper Amazon. The use of these seeds of hybrid varieties by the farmers on their plantations, or the collection of materials for planting from neighboring farms led to a mixture of genotypes with great variability for different characteristics, including resistance to witches' broom in the Bahia cacao region. Based on this potential of genetic variability, the CEPEC initiated in 1993 a project to identify witches' broom resistant trees with good yield characteristics on commercial plantations of the Bahia cacao region. Currently, there are over 1000 trees selected by researchers and producers, which have been cloned and multiplied for evaluations in experimental designs.

Different criteria are being applied for the selection of cacao accessions on commercial plantations. These criteria have two basic aims: yield and witches' broom resistance (Pinto and Pires 1998). Besides, this selection process tries to identify cacao accessions genetically different from each other and from traditional witches' broom resistance sources. In this sense, the study of the genetic diversity, as well as the inference on the ascendance of such selected accessions is extremely important when they are to be used in improvement programs or to be multiplied for the distribution to producers. For example, Yamada and Lopes (1999), in a preliminary study based on isoenzymatic markers, showed that selected accessions on commercial plantations in Bahia come from different sources, including SCA-6, SCA-12, IMC-67, and Pa-150.

Currently, different molecular marker types of DNA facilitate studies into genetic diversity and pedigree (N'Goran et al. 1994, Lerceteau et al. 1997, Pires et al. 2000a, Risterucci et al. 2000, Faleiro et al. 2001, Marita et al. 2001, Faleiro et al. 2004a, Faleiro et al. 2004b), since they allow analyses of practically unlimited numbers of polymorphic markers without environmental influence in a short time. Among the different molecular marker types, the microsatellites, codominant and multiallelic, present optimum abilities of

differentiation of the cacao accessions (Risterucci et al. 2000, Faleiro et al. 2001, Faleiro et al. 2004b) and, since they are reproducible, facilitate the exchange of information among different research institutions.

This study aimed at: i) an evaluation of the genetic diversity in 30 accessions of *T. cacao*, selected for resistance to witches' broom on commercial plantations of the Bahia cacao region, based on microsatellite markers, ii) an analysis of the genetic inter-relationship of the selected accessions with the traditional witches' broom resistance sources, SCA-6 and IMC-67 (Upper Amazon type) besides the ICS-1 (Trinitarian type) and SIC-19 (Common Bahia type).

MATERIAL AND METHODS

Plant material

In the present study, 30 accessions of *T. cacao* selected by producers for their resistance to witches' broom on commercial plantations in the Bahia cacao region were evaluated (Table 1). Four accessions from different geographic origins were also included in the analyses – two from the Upper Amazon (SCA-6 and IMC-67), one Trinitarian (ICS-1) and one Lower Amazon type (SIC-19). The responsible producers for the selections have efficiently contributed to this study for the recovery of cacaoculture, taking part in the measures of the participatory breeding, as recommended by Dias and Resende (2001). Besides the selection of accessions, these farmers carry out installations, the conduction, and evaluations of the experiments on their own farms.

DNA extraction

Leaves of each *T. cacao* accession were collected and stored at –80 °C. The genomic DNA of each accession was extracted by the CTAB method (Doyle and Doyle 1990) with some modifications cited by Faleiro et al. 2002. After the extraction, the concentration of the DNA was estimated by spectrophotometry at 260 nm (Sambrook et al. 1989). Total genomic DNA bands, separated by electrophoresis in 0.8% agarose gel were used as indicators of the integrity and purity of the extracted DNA. After the quantification, the DNA samples were diluted to a concentration of 10ng mL^{-1}.

Production of the microsatellite markers

The amplification reactions for microsatellites were carried out in a total volume of 15 mL, containing Tris-HCl 10 mM (pH 8.3), KCl 50 mM, MgCl$_2$ 2.4 mM, 150 mM of each one of the deoxyribonucleotide (dATP, dTTP, dGTP, and dCTP),

Table 1. List of the 34 cacao (*Theobroma cacao* L.) accessions used in the present study and the respective origins

Code	Accession	Origin	Code	Accession	Origin
1	SCA-6	Farm Sabina, Equador	18	NV-21	Farm Nova Vida, Itabuna
2	ICS-1	River Estate, Trinidad	19	NV-22	Farm Nova Vida, Itabuna
3	SIC-19	Baixo Amazonas	20	PB-617	Farm Pedra Branca, Gandú
4	IMC-67	Pará and Iquitos, Peru	21	PH-109	Farm Porto Híbrido, SJV
5	AM-01	Farm América, Uruçuca	22	PH-16	Farm Porto Híbrido, SJV
6	AM-02	Farm América, Uruçuca	23	PH-92	Farm Porto Híbrido, SJV
7	TR-35	Farm Tororó, Buerarema	24	PS-1319	Farm Porto Seguro, Uruçuca
8	FSU-117	Farm Santa Úrsula, Camacã	25	PS-57111	Farm Porto Seguro, Uruçuca
9	FSU-127	Farm Santa Úrsula, Camacã	26	RT-06	Farm Reunidas Thiara, Ibirataia
10	FSU-151	Farm Santa Úrsula, Camacã	27	RT-106	Farm Reunidas Thiara, Ibirataia
11	FSU-23	Farm Santa Úrsula, Camacã	28	RT-15	Farm Reunidas Thiara, Ibirataia
12	FSU-7	Farm Santa Úrsula, Camacã	29	SJ-02	Farm São José, Itajuípe
13	FSU-77	Farm Santa Úrsula, Camacã	30	SM-02	Farm Santa Mônica, Camacã
14	LP-24	Farm Lagoa Pequena, Uruçuca	31	SM-06	Farm Santa Mônica, Camacã
15	M-05	Farm Massaranduba, Itajuípe	32	TR-012	Farm Tororó, Buerarema
16	NV-00	Farm Nova Vida, Itabuna	33	TR-36	Farm Tororó, Buerarema
17	NV-02	Farm Nova Vida, Itabuna	34	TR-15	Farm Tororó, Buerarema

Accessions 1-4: controls; Accessions 5-34: plants selected by producers for their resistance to witches' broom on commercial cacao plantations in the Bahia cacao region; SJV – São José da Vitória

3 pM of each one of the two primers (F and R), one unit of Taq polymerase enzyme, and 30 ng of DNA. The amplifications were carried out in a thermocycler, in the sequence: 4 minutes at 94 ℃; 10 cycles of 30 seconds at 94 ℃; 60 seconds at 60 ℃ with a reduction of 1 ℃ at each cycle and 90 seconds at 72 ℃; followed by 30 cycles of 30 seconds at 94 ℃; 60 seconds at 48 ℃ and 90 seconds at 72 ℃; with a final step of 6 minutes at 72 ℃. After the amplification, the temperature of the samples was reduced to 4 ℃. Sixteen *primer* pairs identified by the characteristics and sequences of Lanaud et al. (1999) were used (Table 2).

After the amplification, 3 mL of a mixture of bromophenol blue (0.25%), glycerol (60%), and water (39.75%) was added to each sample. Theses samples were applied on 3% agarose gel for the separation of the microsatellite fragments. Electrophoresis was realized in a TBE buffer (Tris-Borate 90 mM, EDTA 1 mM), for approximately four hours at 100 volts. Thereafter, the gels were stained with ethidium bromide and photographed under ultraviolet light.

Statistical analyses

The microsatellite markers were converted into a numeric matrix, using the following code/codification: 0 for allele absence, 1 for the presence of one copy of the allele and 2 for the presence of two copies of the allele (Faleiro et al. 2001).

From the numeric codified matrix, the polymorphic information content per locus, heterozygosis levels, and genetic distances were calculated and clustering analyses realized.

The genetic information content per locus (Smith et al. 1997) was calculated according to the following expression:

$$PIC = 1 - \left(\sum_{i=1}^{n} p_i^2 \right) - \left(\sum_{i=1}^{n-1} \sum_{j=l+1}^{n} 2p_i^2 p_j^2 \right)$$

where:

p_i and p_j are the frequencies of the ith and j^{th} allele in the locus.

The genetic distances, calculated based on the microsatellite markers, were based on the following expression used by Faleiro et al. (2001):

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

GD_{ij} = genetic distance between the varieties i and j;

NCL = number of coincident loci;

TNL = total number of loci.

NCL is the sum of the allelic coincidences of each analyzed locus, where each coincidence assumes 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).

The matrix of genetic distances was used to realize clustering analyses via dendrogram, using the UPGMA method

2.   ,   ,   ( )

*	(5 → 3)		
6	( )$_7$( )$_{13}$	6	0.727
8	( )$_5$ ( )$_{17}$ ( )$_4$	4	0.455
9	( )$_{8\ 15}$( )$_{5\ 9}$( )$_{10}$	5	0.705
11	( )$_{13}$	4	0.658
12	( )$_{4\ 18}$( )$_6$	4	0.523
13	( )$_{13}$	2	0.360
15	( )$_{19}$	6	0.751
18	( )12	4	0.496
19	( )$_{28}$	3	0.112
26	( )$_9$ ( )$_4$ ( )$_{11}$	4	0.508
29	( )$_{10}$	4	0.594
32	( )$_2$ ( )$_6$	3	0.454
43	( )$_{14}$	3	0.421
44	( )$_{10}$	2	0.212
46	( )$_{15}$( )$_{12}$	6	0.664
48	( )$_9$	4	0.539
		64	

*   . (1999)

(Unweighted Pair Group Method with Arithmetic Mean) as grouping criterion. The matrix of genetic distances was displayed in a scatter plot based on the multidimensional scaling, using the principal coordinates analysis method discussed by Dias (1998). Softwares SAS (SAS Institute Inc. 1989) and Statistica (Statsoft Inc. 1999) were used for the analysis and plot construction. The heterozygosis level was also calculated based on the relation between the number of loci in heterozygosis and the total number of analyzed loci.

RESULTS AND DISCUSSION

The sixteen specific *primer pairs* for microsatellite loci used in this study brought forth a total of 64 alleles. The number of alleles per locus varied from 2 to 6, with a mean of 4 alleles per locus (Table 2). The content of polymorphic information per locus (PIC) varied from 0.112 to 0.751 (Table 2). The PIC value showed that the loci mTcCIR6, mTcCIR9, and mTcCIR15 were the most informative. Highest means of
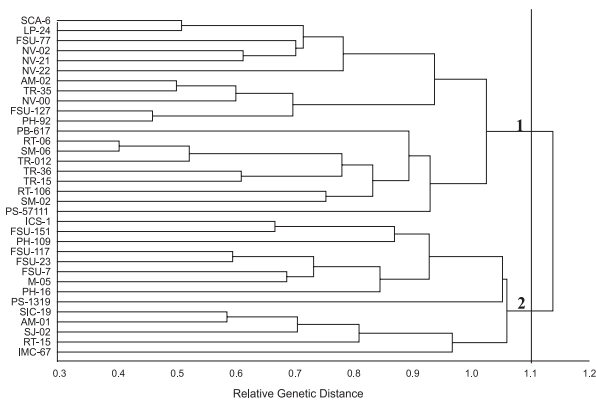
Figure 1. Clustering analyses of 30 cacao trees selected for their resistance to witches' broom and four controls, based on the matrix of genetic distances calculated based on microsatellite markers. The UPGMA method was applied as grouping criterion
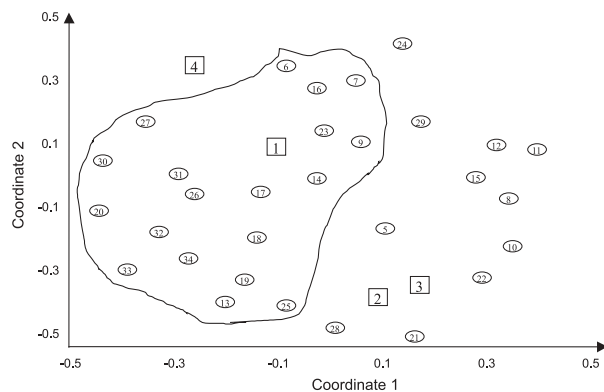

Figure 2. Dispersion analysis of 30 cacao trees selected for their resistance to witches' broom (O) and four controls ( ), based on the matrix of genetic distances calculated based on microsatellite markers. The numbers refer to the codes listed in Table 1. The grouping apparent in this figure refers to similarity group 1 of Figure 1

alleles per locus and PIC have been obtained with electrophoresis in polyacrylamide gels. For instance, Risterucci et al. (2000) used 20 microsatellite loci to characterize 28 cacao accessions and obtained a mean of 5.6 alleles per locus. According to these authors, the use of 8 microsatellite loci would be sufficient for an initial characterization of the cacao accessions, however the use of 15 loci would be necessary for a differentiation of genetically strongly related accessions.

The genetic distances between the evaluated cacao accessions in this study varied from 0.13 and 0.71, indicating a high genetic variability. Faleiro et al. (2004a) used RAPD markers to study the genetic diversity of 59 accessions selected on cacao plantations of the South of Bahia, where he also found a high genetic variability. According to these authors, the characteristics of resistance and genetic variability of the selected accessions on the farms are explained by the history of the cacao improvement program developed by the CEPEC (Vello et al. 1967), which used cacao accessions from different sources of resistance to witches' broom to produce interclonal hybrids, from 1960 on, i.e., before the arrival of witches' broom in Bahia in 1989 (Pereira et al. 1989).

The cluster analysis based on genetic distances showed the existence of genetic variability among the 30 selected accessions by producers and the formation of two groups of similarity (Figure 1). Based on the cluster analysis by the UPGMA method, 18 accessions (60%) would enter in the same group as the SCA-6 (main source of resistance to witches' broom) (Figure 1 and 2). Among the four controls of the study, accession SCA-6 presented the smallest mean distance in relation to the selected accessions on the farms (Figure 3), corroborating other studies. For example, analyses
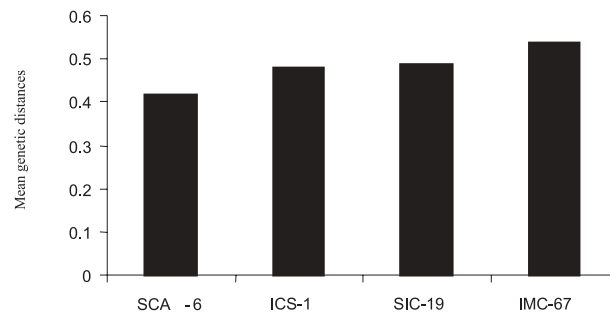

Figure 3. Mean genetic distances of the control accessions in relation to the accessions selected by producers for their resistance to witches' broom on commercial plantations of the Bahia cacao region

based on isoenzymatic and RAPD markers demonstrated the genetic inter-relationship of the selections realized on cacao farms in the South of Bahia for resistance to witches' broom with accession SCA-6 (Yamada and Lopes 1999, Araújo et al. 2003, Faleiro et al. 2004a), which is the genetic resistance base of the presently recommended varieties by the CEPEC (Faleiro et al. 2001). Inheritance studies show that the accession SCA-6 has a major "gene" that controls the resistance to witches' broom (Queiroz 2003).

On the other hand, results of this study show that the selections AM-01, FSU-117, FSU-151, FSU-23, FSU-7, M-05, PH-109, PH-16, PS-1319, SJ-02, PS-57111, and RT-15, which made up 40% of the studied selections, were not part of the group of SCA-6 showing the possibility of the existence of potentially different witches' broom resistance genes, according to conclusions of Faleiro et al. (2004a).
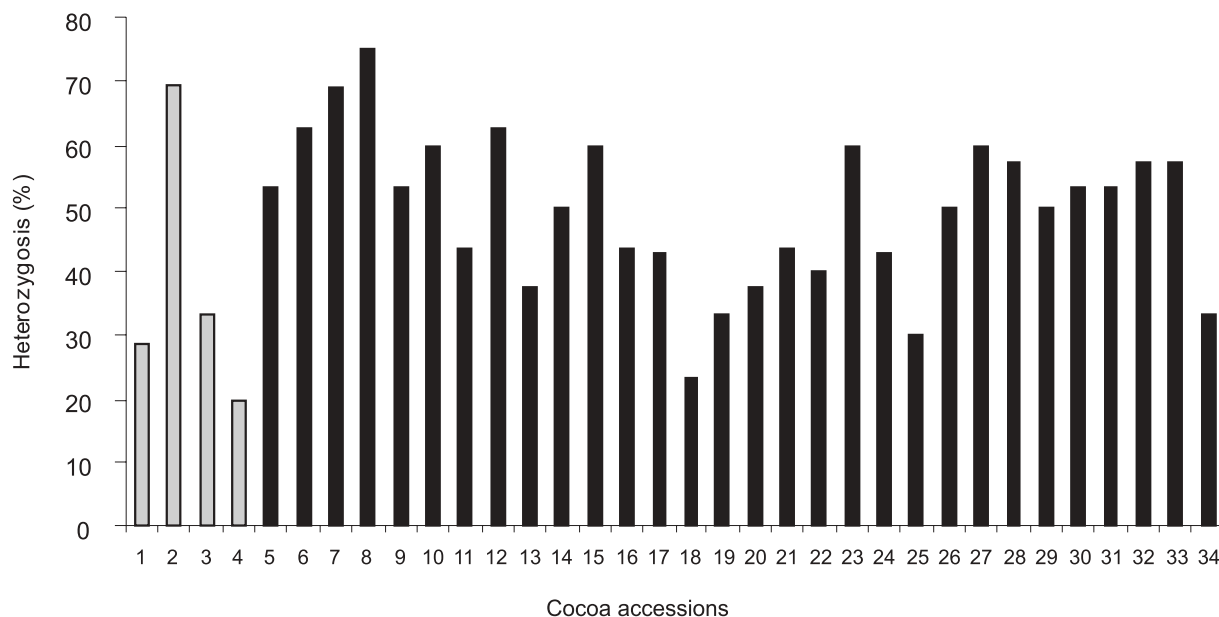
Figure 4. Heterozygosis of 30 cacao trees selected for their resistance to witches' broom (5-34) and of four controls (1- SCA-6; 2- ICS-1; 3- SIC-19; 4- IMC-67) calculated based on 16 microsatellite loci. The numbers on the cocoa accessions refer to the codes listed in Table 1

The accessions used as controls (SCA-6, IMC-67, ICS-1, and SIC-19), which are from different origins, represented a considerable part of the genetic variability in cacao (Pires et al. 2000, Marita 2001) and appeared dispersed along the scatter plot (Figure 2). The use of these and other accessions introduced from other countries in crossings with accessions of the Common Bahia variety in the hybrid variety production program developed by the CEPEC contributed significantly to the amplification of the genetic base of the cacao improvement program in Brazil (Yamada et al. 2002). Today, this program of hybrid variety production is of great importance since it is possibly the main source of the selections realized on cacao farms in the South of Bahia for resistance to witches' broom. High heterozygosis levels found in some selections (Figure 4) underpin this hypothesis. Fifty percent of the analyzed selections have over 50% of the loci in heterozygosis. The selections AM-02, TR-35, FSU-117, and FSU-7 with over 60% loci in heterozygosis are the most outstanding. These results show another desirable characteristic of the selections carried out by the farmers: the positive effect of heterosis in resistance and yield traits of cacao (Atanda 1973).

Finally, the selections on farms for high resistance to witches' broom, good agronomic traits, and high genetic diversity in relation to SCA-6 have an excellent potential for the improvement program and even for a direct recommendation to producers, thus contributing to an amplification of the genetic base of the clonal varieties presently recommended for planting in the South of Bahia. An amplification of the genetic resistance base is urgent, since the present-day resistant clonal varieties, recommended by the CEPEC, are genetically interrelated (Faleiro et al. 2001).

ACKNOWLEDGEMENTS

Variabilidade em cacaueiros selecionados por produtores para resistência à vassoura-de-bruxa com base em marcadores microssatélites

RESUMO - *Avaliou-se a variabilidade genética de 30 acessos de* Theobroma cacao *selecionados para resistência à vassoura-de-bruxa em plantações comerciais da região cacaueira baiana, com base em marcadores microssatélites, para compará-la com as fontes tradicionais de resistência à doença (SCA-6 e IMC-67) e importantes genitores (ICS-1 e SIC-19) usados no programa de melhoramento do CEPEC. Foram utilizados 16 pares de* primers *específicos para locos de microssatélites, os quais geraram 64 alelos. A distância genética entre acessos variou de 0,13 a 0,71 e o nível de heterozigosidade entre 23 e 76%. Pela análise de agrupamento, 18 acessos (60%) agruparam-se com o SCA-6. Seleções realizadas em fazendas para alta resistência à vassoura-de-bruxa, boas características agronômicas e alta diversidade genética em relação ao SCA-6 têm potencial para integrar o programa de melhoramento e até mesmo para serem recomendadas diretamente aos produtores.*

Palavras-chave: *Theobroma cacao*, marcadores moleculares, *Crinipellis perniciosa*, genes de resistência, melhoramento participativo.

REFERENCES

Araújo IS, Bahia RCS, Faleiro FG, Gomes LMC, Melo GRP, Ahnert D, Lopes UV, Yamada MM and Valle RR (2003) Caracterização da diversidade genética e ascendência de fontes de resistência de *Theobroma cacao* à vassoura-de-bruxa com base em marcadores moleculares RAPD. In: **Proceedings of the 13th International Cocoa Research Conference**. Cocoa Producers' Alliance, Kota Kinabalu, p. 613-618.

Atanda OA (1973) Heterosis in crosses of *Theobroma cacao*. **Experimental Agriculture 9**: 23-29.

Dias LAS (1998) Análises multidimensionais. In: Alfenas AC (ed.) **Eletroforese de isoenzimas e proteínas afins**. Editora UFV, Viçosa, p. 405-475.

Dias LAS and Resende MDV (2001) Estratégias e métodos de seleção. In: Dias LAS (ed.) **Melhoramento genético do cacaueiro**. FUNAPE, Viçosa, p. 217-287.

Doyle JJ and Doyle JL (1990) Isolation of plant DNA from fresh tissue. **Focus 12**: 13-15.

Faleiro FG, Lopes UV, Yamada MM, Pires JL, Bahia RCS, Santos RS, Gomes LMC, Araújo IS, Faleiro ASG, Gramacho KP, Melo GRP, Monteiro WR and Valle RR (2001) Caracterização de variedades clonais de *Theobroma cacao* L. com base em marcadores RAPD, AFLP e microssatélites. **Agrotrópica 13**: 79-86.

Faleiro FG, Santos IS, Bahia RCS, Santos RF, Yamada MM and Anhert D (2002) Otimização da extração e amplificação de DNA de *Theobroma cacao* L. visando a obtenção de marcadores RAPD. **Agrotrópica 14**: 31-34.

Faleiro FG, Lopes UV, Yamada MM, Melo GRP, Monteiro WR, Pires JL, Rocha JB, Bahia RCS, Gomes LMC, Araújo IS and Faleiro ASG (2004a) Genetic diversity of cacao accessions selected for resistance to witches' broom disease based on RAPD Markers. **Crop Breeding and Applied Biotechnology 4**: 12-17.

Faleiro FG, Pires JL, Monteiro WR, Lopes UV, Yamada MM, Piedra AG, Moura AD, Arévalo-Gardini E., Marques JRB, Gramacho KP, Faleiro ASG and Santos MCM (2004b) Variability in cocoa accessions from the Brazilian, Ecuadorian, and Peruvian Amazons based on molecular markers. **Crop Breeding and Applied Biotechnology 4**: 227-233.

Lanaud C, Risterucci AM, Pieretti I, Falque M, Bouet A and Lagoda PJL (1999) Isolation and characterization of microsatellites in *Theobroma cacao* L. **Molecular Ecology 8**: 2141-2143.

Lerceteau E, Robert T, Pétiard V and Crouzillat D (1997) Evaluation of the extent of genetic variability among *Theobroma cacao* accessions using RAPD and RFLP markers. **Theoretical and Applied Genetics 95**: 10-19.

Marita JM, Nienhuis J, Pires JL and Aitken WM (2001) Analysis of genetic diversity in *Theobroma cacao* with emphasis on witches' broom disease resistance. **Crop Science 41**: 1305-1316.

N'goran JAK, Laurent V, Risterucci AM and Lanaud C (1994) Comparative genetic diversity studies of *Theobroma cacao* L. using RFLP and RAPD markers. **Heredity 73**: 589-597.

Pereira SL, Ram A, Figueiredo IM and Almeida LCC (1989) Primeira ocorrência da Vassoura de Bruxa na principal região produtora de cacau do Brasil. **Agrotrópica 1**: 79-81.

Pinto LRME and Pires JL (1998) **Seleção de plantas de cacau resistentes à vassoura-de-bruxa.** CEPLAC/CEPEC, Ilhéus, 34p. (Boletim Técnico 181).

Pires JL, Marita JM, Lopes UV, Yamada MM, Aitken WM, Melo GRP, Monteiro WR and Ahnert D (2000a) Diversity for phenotypic traits and molecular markers in CEPEC's germplasm collection in Bahia, Brazil. In: **Proceedings of the International Workshop on New Technologies and Cocoa Breeding**. Ingenic, Kota Kinabalu, p. 72-88

Pires JL, Monteiro WR, Luz EDMN, Silva SDVM, Pinto LRM, Figueira A, Gramacho KD, Lopes UV, Albuquerque PSD, Yamada MM, Anhert D and Brugenerotto MI (2000b). Cocoa breeding for witches's broom resistance at CEPEC, Bahia, Brasil. In: **Proceedings of the 12th International Cocoa Research Conference**. Cocoa Producers' Alliance, Salvador, p. 24-25.

Pound FJ (1938) **Cacao and witches' broom disease (*Marasmius perniciosa*) of South America, witch notes on other species of *Theobroma*. Report on a visit to Ecuador, the Amazon Valley and Colombia, April 1937 – April 1938**. Yulle's Printerie, Port of Spain, 58p.

Pound FJ (1943) **Cacao and witches' broom disease (*Marasmius perniciosa*). Report on a recent visit to the Amazon territory of Peru, September, 1942 – February, 1943**. Yulle's Printerie, Port of Spain, 14p.

Queiroz VT, Guimarães CT, Ahnert D, Schuster T, Daher RT, Pereira MG, Miranda VRM, Loguercio LL, Barros EG and Moreira MA (2003) Identification of a major QTL in cocoa (*Theobroma cacao* L.) associated with resistance to witches´ broom disease. **Plant Breeding 122**: 268-272.

Risterucci AM, Eskes B, Fargeas D, Motamayor JC, Lanaud C (2000) Use of microsatellite markers for germplasm identity analysis in cocoa. In: **Proceedings of the 16th-17th International Workshop on New Technologies and Cocoa Breeding**. Ingenic, Kota Kinabalu, p. 25-33.

Sambrook J, Fritsch EF and Maniats T (1989) **Molecular cloning: a laboratory manual.** 2nd ed., Cold Spring Harbor Laboratory, New York, 653p.

Smith JSC, Chin ECL, Shu H, Smith OS, Wall SJ, Senior ML, Mitchell SE, Kresovich S, and Ziegele J (1997) An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): comparisons with data from RFLPs and pedigrees. **Theoretical and Applied Genetics 95**: 163-173.

Souza CAS and Dias LAS (2001) Melhoramento ambiental and sócio-economia. In: Dias LAS (ed.) **Melhoramento genético do cacaueiro**. FUNAPE, Viçosa, p. 1-47.

SAS Institute Inc. (1989) **SAS/STAT user´s guide**. Version 6, 4th ed., vol. 2, SAS Institute, Cary, 846p.

StatSoft Inc. (1999) **Statistica 6 for Window**. http://www.statsoft.com.

Vello F, Mariano AH, Garcia JR, Nascimento TF and Magalhães WS (1967) O programa de melhoramento genético do cacau na Bahia. In: **II Conferência Internacional de Pesquisas em Cacau**. Cocoa Producers' Alliance, Lagos, p. 43-55.

Yamada MM and Lopes UV (1999) Paternity analysis of cacao trees selected for resistance to witches' broom disease in plantations of Bahia, Brazil **Agrotrópica 11**: 83-88.

Yamada MM, Faleiro FG, Lopes UV, Pires JL, Bahia RCS, Gomes LMC and Melo GRP (2002) Genetic variability in cultivated cacao populations in Bahia, Brazil, using isozymes and RAPD markers. **Crop Breeding and Applied Biotechnology 1**: 377-384.