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Variability in cacao accessions from the Brazilian, Ecuadorian, and Peruvian Amazons based on molecular markers

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ABSTRACT *- The objective of this work was to study the genetic variability of 19 clonal accessions of* Theobroma cacao *L. from the Brazilian, Ecuadorian, and Peruvian Amazons, based on RAPD and microsatellite markers. Six primers were used to obtain the RAPD markers and six pairs of primers for the microsatellite loci. The amplification products were submitted to electrophoresis on 1.2% and 3.0% standard agarose for RAPD and microsatellite markers, respectively. The generated molecular markers were analyzed separately, by transformation into numeric matrices, whereupon the genetic distances were calculated and the cocoa accessions grouped. The six primers used in each technique generated 56 RAPD and 45 microsatellite markers. In the cluster analyses, the groups involved accessions from different origins, with no clear evidence of regionalization of the genetic variability. The high genetic variability found in this work confirms the importance of the Amazon basin for germplasm collection.*

Key words: cocoa, genetic diversity, germplasm, RAPD and microsatellite markers.

INTRODUCTION

Natural genetic resources offer all requirements to improve many important and economic traits such as yield, adaptability, stability, resistance to plagues, and seed quality in varieties. The Amazon region holds an immense plant wealth, not duly explored yet in respect to its genetic resources. Exploratory collections in the Amazon that gathered natural genetic resources of cocoa (*Theobroma cacao* L.) have stimulated improvement programs of many research institutions. Pound (1938, 1943) found important wild cocoa collections along the Amazon River and some of its tributaries in Peru and Ecuador. The accessions of Pound's collection were taken to Trinidad and used in the local breeding program. As they presented a large genetic variability, several other collection expeditions have been realized since then in the Upper Amazon region (Soria 1970, Allen and Lass 1983, Ocampo 1984, Clement et al. 1988).

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Several cocoa collections also took place in the Brazilian Amazon. The most important of these collections since 1965 were the ones realized by CEPEC/CEPLAC (Vello and Medeiros 1965, Barriga et al. 1985, Almeida et al. 1987, Almeida et al. 1995).

Studies into the genetic diversity of cocoa accessions from natural as well as domesticated or genetically improved populations generate important and useful information for the maintenance and evaluation of germplasm collections, for the planning of future collections, and for the development of crossing plans in improvement programs and in the establishment of heterotic groups. Nowadays, different types of molecular markers have enabled such studies of genetic diversity in cocoa, allowing a rapid analysis of a limitless number of polymorphic markers at the DNA level, without any environmental influence (Laurent et al. 1993, 1994, Lerceteau et al. 1997a, N'Goran et al. 1994, Pires et al. 2000, Marita et al. 2001, Faleiro et al. 2001).

Some cocoa accessions from the Upper Amazon demonstrated high genetic diversity in studies realized with genetic markers based on isozymes, RAPD and RFLP markers (Laurent et al. 1993, 1994, N'Goran et al. 1994, Marita et al. 2001). In some cocoa accessions from Ecuador, Lerceteau et al. (1997b) verified a high allelic diversity by associating DNA and morphologic markers. Due to the large genetic variability found in the wild Ecuadorian cocoa populations, Cheesman (1944) described the forks of the rivers Napo, Caqueta, and Putumayo, tributaries of the Amazon River, as the most probable center of origin of cocoa species.

Objectives of the present work was the investigation of the genetic variability of cocoa accessions from the Brazilian, Ecuadorian, and Peruvian Amazons, based on RAPD and microsatellite markers and to establish conclusions on the information generated by each molecular marker type.

MATERIAL AND METHODS

A total of 18 *Theobroma cacao* L. accessions from the Brazilian, Ecuadorian, and Peruvian Amazons and a Trinitarian accession (ICS-1), were used in the present study (Table 2).

Leaf samples of each *T. cacao* L. accession were collected and stored at -80 °C. The genomic DNA was extracted from each sample individually by the CTAB method with some modifications (Faleiro et al. 2002a). After the extraction, the concentration of the DNA was determined by the spectrophotometer at 260 nm. The total genomic DNA was evaluated by electrophoresis in 0.8% agarose gel to test its integrity and purity. The DNA samples of good quality were diluted to a concentration of 10 ng mL^{-1}.

The amplification reactions for the RAPD and microsatellite markers were carried out according to the procedures reported by Faleiro et al. (2001). The sequences and other characteristics of RAPD and microsatellite primers used in this work are listed in Table 1. After the amplification, 3 uL of a mixture of bromophenol blue (0.25%) and glycerol (60%) was added to each sample. The amplified fragments were separeted in 1.2 and 3% agarose gels for RAPD and microsatellite markers, respectively. The electrophoresis was performed in TBE buffer (Tris-borate 90 mM, EDTA 1 mM) for approximately four hours, at 90 volts. After the electrophoresis, the gels were stained with ethidium bromide and photographed under ultraviolet light.

The RAPD and microsatellite markers generated were analyzed independently, by transforming them into coded numeric matrices, from which genetic distances were calculated between the accessions according to the procedures reported by Faleiro et al. (2001).

Genetic distances obtained from RAPD and microsatellite markers were displayed, independently, in a biplot based on the multidimensional scaling, using the principal coordinates analysis discussed by Dias (1998). Similarity groups of accessions obtained by each marker type were established based on cluster analysis using the UPGMA method as grouping criterion. The 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.

To compare the results obtained with the two types of molecular markers, the polymorphic information content (PIC) (Smith et al. 1997) was calculated by the following expression:

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

p_i and p_j are the frequencies of i^{th} and j^{th} alleles at a locus and n is the allele number.

The Pearson correlation coefficient among the genetic distances and the coincidence of the cocoa accessions in the groups obtained by each marker type were calculated.

RESULTS AND DISCUSSION

The six primers used in each technique generated 56 RAPD loci (45 polymorphic) and 45 microsatellite alleles (45 polymorphic). The DNA amplification products for the 19 accessions obtained by the techniques used in this study are shown in Figure 1. The number of polymorphic loci and polymorphic alleles per locus depend on the genetic variability of the analyzed accessions. The average 7.5 loci

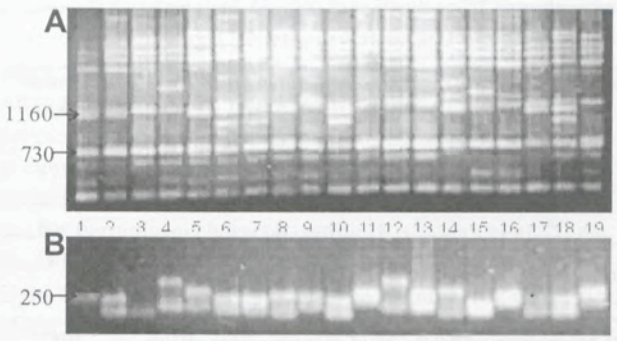

Figure 1. Amplification products of genomic DNA from 19 cocoa accessions, by using the RAPD primer OPH8 (A) and the microsatellite mTcCIR3 (B). The arrows indicate markers with 1160, 730, and 250 pb. Accessions coded as in Table 2.

per primer for RAPD and the average 7.5 alleles per locus for microsatellite in this study are above average compared to other studies (Risterucci et al. 2000a, Marita et al. 2001). The average alleles per locus for microsatellite could be lager if high resolution gels were used for the electrophoresis.

The genetic distances among the different accessions based on RAPD markers ranged from 0.10 to 0.40, while the ones based on microsatellite markers varied from 0.25 to 1.00. Although the two techniques brought forth the same number of polymorphic bands for the studied accessions, microsatellite markers were more efficient at differentiating the cocoa accessions (Figure 2). The number of loci analysed by RAPD was higher than by microsatellite markers (Table 1). On the other hand, the polymorphic information content (PIC) is higher with microsatellite than RAPD markers (Table 1). According to Risterucci et al. (2000a), the efficiency of genotype identification with different kinds of molecular markers depends on the number of alleles that can be identified at a single locus. The larger efficiency of the microsatellite is explained once it is a codominant and multi-allelic marker, providing more genetic information per locus, when compared to RAPD (Litt and Luty 1989, Russell et al. 1997).

Microsatellites have been isolated and characterized in cocoa (Lanaud et al. 1999) and applied in genetic studies (Risterucci et al. 2000a, Risterucci et al. 2000b). Twenty microsatellite loci were utilized to construct the genetic map

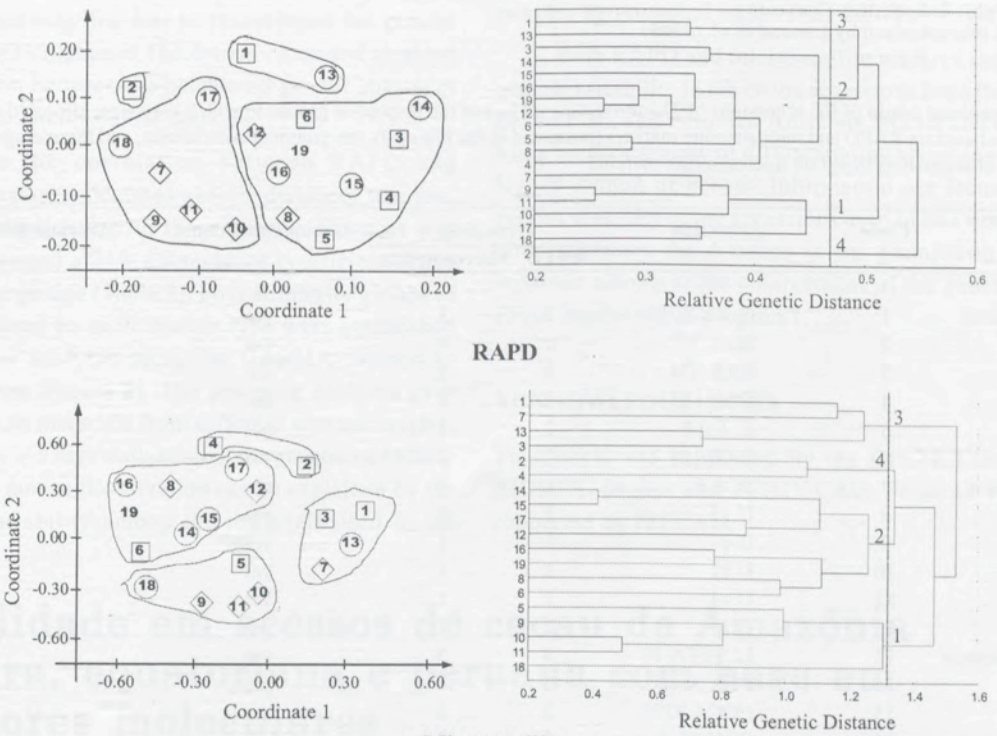

Figure 2. Dispersion analysis of 18 cocoa accessions coming from the Brazilian (1-6□), Peruvian (7-12◇) and Ecuadorian (13-18 ○) Amazons plus a Trinitario (19). The similarity groups of accessions were established based on cluster analysis using the UPGMA method as clustering criterion. Accessions coded as in the Table 2

Table 1. Primers utilized for RAPD and microsatellite markers and the number of respective polymorphic and monomorphic bands (loci and alleles, respectively)

RAPD Primer	Sequence (5'→3')	Nº of polymorphic loci	Nº of monomorphic loci	PIC mean
OPF1	ACGGATCCTG	6	2	0.220
OPF2	GAGGATCCCT	0	0	-
OPF3	CCTGATCACC	6	4	0.178
OPG6	GTGCCTAACC	6	0	0.151
OPH8	GAAACACCCC	17	4	0.204
OPH12	ACGCGCATGT	10	1	0.187
Total		45	11	-
Microsatellite Primer*	**Sequence (5'→3') F and R**	**Nº of polymorphic alleles**	**Nº of monomorphic alleles**	**PIC**
mTcCIR3	CATCCCAGTATCTCATCCATTCAGT CTGCTCATTTCTTTCATATCA	9	0	0.834
mTcCIR4	CGACTAAAACCCAAACCATCAA AATTATTAGGCAACCCGAACTT	6	0	0.658
mTcCIR6	TTCCCTCTAAACTACCCTAAAT TAAAGCAAAGCAATCTAACATA	8	0	0.836
mTcCIR9	ACCATGCTTCCTCCTTAC ACATTTATACCCCAACCA	8	0	0.714
mTcCIR11	TTTGGTGATTATTAGCAG GATTCGATTTGATGTGAG	6	0	0.752
mTcCIR12	TCTGACCCCAAACCTGTA ATTCCAGTTAAAGACCAT	8	0	0.773
Total		45	0	-

PIC (Polymorphic Information Content)
*Isolated and characterized by Lanaud et al. (1999)

Table 2. Geographical origin of the accessions of *Theobroma cacao* L. and the respective groups to which they were allocated according to the cluster analysis based on RAPD and microsatellite markers (presented in the Figure 2), the grouping coincidence, and the average heterozygosity of each group of accessions based on microsatellite markers

Origin	Code	Accession	Grouping		Group coincidence	Average heterozygosity
			RAPD	Microsatellite		
					— % —	— % —
Brazilian Amazon	1	C. Sul 3	3	3	yes	64
	2	SIAL 70	4	4	yes	
	3	CAB 324	2	3	no	
	4	CAB 191	2	4	no	
	5	C. Sul 8	2	1	no	
	6	CAB 148	2	2	yes	
PeruvianAmazon	7	SCA 6	1	3	no	61
	8	U 32	2	2	yes	
	9	U 6	1	1	yes	
	10	U 11	1	1	yes	
	11	U 14	1	1	yes	
	12	U 10	2	2	yes	
Ecuadorian Amazon	13	LCTEEN 37	3	3	yes	61
	14	EQX 3360	2	2	yes	
	15	COCA 3370	2	2	yes	
	16	EQX 3348	2	2	yes	
	17	EQX 107	2	2	yes	
	18	EQX 3161	2	1	no	
Trinidad	19	ICS 1	2	2	yes	66
Total					74	62

of cocoa (Risterucci et al. 2000b). The use of this marker type to analyze cocoa genetic diversity and the germplasm characterization have been discussed (Risterucci et al. 2000a). Advantages of the microsatellite markers are the high polymorphism levels per locus, random distribution in the genome, and different alellic frequencies – aspects which make this marker an appropriate choice for diversity analyses and for germplasm characterization (Risterucci et al. 2000a, Yamada et al. 2003). According to Risterucci et al. (2000a), eight microsatellite loci would be enough for an initial characterization of cacao accessions, but 15 loci would be necessary for the differentiation of genetically strongly related accessions. However, the of RAPD marker technique will continue to be used for different genetic studies owing to its feasibility, speed, and relatively lower cost than other techniques (Figueira and Cascardo 2001, Yamada et al. 2001, Faleiro et al. 2002b). Actually, the availability of laboratory infrastructure and the study target define whether the use of RAPD or microsatellite is preferable.

In this work, the correlation between RAPD and microsatellite marker-calculated genetic distances was low ($r = 0.27$, $P < 0.01$). This low correlation could be explained by the differences in the analyzed loci number (45 RAPD x 6 microsatellites). Faleiro et al. (2001) used 117 polymorphic RAPD and 9 microsatellite loci to characterize the genetic diversity of the 10 varieties of *Theobroma cacao* and obtained a 0.69 correlation between the calculated genetic distances using RAPD and microsatellite markers.

Although the correlation between RAPD and microsatellite marker-calculated genetic distances was low, the grouping established with these genetic distances was similar and presented a 74% coincidence coefficient of the accessions in the groups (Table 3). Four similarity groups of accessions obtained by each marker type were established based on cluster analysis using the UPGMA method as grouping criterion (Figure 2). The grouping analyses gave rise to groups with materials from different amazon origins, with no tendency to a regionalization of the genetic variability (Figure 2). This non-regionalization can be explained by the high genetic variability among the materials used in the present study. A high genetic variability has been also verified among other Amazon accessions (Pires et al. 2000, Marita et al. 2001). Other observations that also corroborate the high genetic variability of the analyzed materials are the high means of the observed heterozygosity, i.e., 64, 61, and 61% of the genetic materials from the Brazilian, Ecuadorian, and Peruvian Amazon, respectively (Table 2). This heterozygosity could be larger if high resolution gels were used for the electrophoresis. The high genetic diversity among plants from the Upper Amazon, evidenced by molecular, agronomic, and morphologic markers, has given support to the hypothesis that the Upper Amazon is the most probable center of diversity for the *Theobroma cacao* L species (Cheesman 1944, Figueira et al. 1994).

Smallest genetic distances were verified among the accessions of the Ucayali series from the Peruvian Amazon, for both molecular markers. This fact can be verified by the proximity of the accessions of this series in the scattered diagram (Figure 2). This genetic similarity within the Ucayali series can be explained by the fact that these accessions had been collected in a restricted area belonging to the lower part of the Ucayali River basin. This type of regionalization of the genetic diversity associated to a river has also been detected with RAPD markers (Figueira et al. 1994) and morpho-agronomical traits (Dias 2001).

Both RAPD and microsatellite markers showed broad genetic variability in the cocoa accessions from the Brazilian, Ecuadorian, and Peruvian Amazons. Microsatellite markers had a higher capacity of differentiating accessions due to the higher content of genetic information per locus. The high genetic diversity of the accessions used in this work confirms how important the Amazon is for germplasm collection missions aiming at the enlargement of the genetic base for cocoa improvement programs.

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Variabilidade em acessos de cacau da Amazônia brasileira, equatoriana e peruana com base em marcadores moleculares

RESUMO - *O objetivo desse trabalho foi estudar a variabilidade genética de 19 acessos clonais de* Theobroma cacao *L. oriundos das amazônias brasileira, equatoriana e peruana com base em marcadores RAPD e microssatélites. Foram utilizados*

6 primers para obtenção de marcadores RAPD e 6 pares de primers específicos para locos microssatélites. Os produtos da amplificação foram submetidos a eletroforese em gel de agarose 1,2% e 3,0% para marcadores RAPD e microssatélites, respectivamente. Os marcadores moleculares gerados foram analisados separadamente e convertidos em matrizes numéricas, a partir das quais foram calculadas distâncias genéticas e realizadas análises de agrupamento dos acessos de cacaueiro. Os 6 primers utilizados em cada técnica geraram 56 marcas RAPD 45 microssatélites. As análises de agrupamento mostram a formação de grupos contendo materiais provenientes das diferentes origens, não mostrando uma regionalização clara da variabilidade genética. A alta variabilidade genética encontrada neste trabalho, confirma a importância da amazônia para a coleta de recursos genéticos.

Palavras-chave: cacau, diversidade genética, germoplasma, marcadores RAPD e microssatélites.

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