Genetic variability in cultivated cacao populations in Bahia, Brazil, detected by isozymes and RAPD markers

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ABSTRACT

The objective of this work was to study genetic variability in accessions from the CEPLAC cacao germplasm collection, which constituted the parents of hybrids recommended by CEPLAC, to infer about the diversity of cacao plantations in Bahia. The 21 clones or accessions utilized were SIC, SIAL (local selections) groups and ICS, IMC, PA, SCA, TSA and UF groups (foreign selections). Molecular markers used were isozymes (MDH, IDH, and DIA enzyme systems) and RAPD (105 RAPD loci of 11 primers). Genetic similarities among accessions were determined by the NTSYS statistical package. A similarity matrix based on Dice´ s coefficient was calculated, and a dendrogram using unweighed pair-group method with arithmetic average (UPGMA) clustering was constructed. The accessions were classified into 7 groups. Four accessions (SIC 19, IMC 67, ICS 6 and ICS 8) did not cluster in any group. Other accessions formed small groups such as SCA 6, SCA 12, PA 30, PA 150 and ICS 1. The larger group was formed by 12 accessions, including SIC, SIAL, UF and TSA 644. Results indicate that the genetic base in Bahia plantations was quite narrow before using the recommended hybrids by CEPLAC. However, with the planting of hybrids, both the genetic variability and the chances of success from selection increased.

KEY WORDS: Breeding, biotechnology.

INTRODUCTION

Cacao (*Theobroma cacao* L.) is a major tropical crop, whose most valuable part is the bean (seed) used to produce chocolate. The cacao diversity center is the Orinoco and Amazon river basins (Cheesman, 1944).

In Bahia, cacao was introduced in 1746, as few seeds coming from the state of Pará (Vello et al., 1969). From these first introduction and, likely from other subsequent introductions from that region, several varieties were formed, which are usually referred to as "common" varieties. These common varieties were cultivated for centuries in Bahia and are still the prevalent varieties in plantations. Both farmers and Research Institutes have recently carried out mass selection in populations of common varieties which have become the main source of new plantings.

In 1950s, selections were done within the common varieties by the Experimental Station of Juçari, the Experimental Station of Uruçuca, in Bahia, and the Experimental Station of Goitacazes, in Espírito Santo which resulted in the well-known SIAL, SIC and EEG selections, respectively.

In 1960s, the recently created Cacao Research Center (CEPEC/CEPLAC), distributed great quantities of Catongo seeds (Toxopeus, 1987), a white seed mutant of the common variety. At the same time, CEPEC introduced clones from several other countries in order to produce interclonal hybrids (full-sib families) to be released to farmers. From 1960s to 1983 most of the plantings were made with a mixture of hybrids involving parents selected from common varieties (SIC, SIAL, and EEG clones) and parents introduced from Peru (IMC and SCA clone), Trinidad (ICS clones) and Costa Rica (UF clones) (Vello et al.,1969; 1972; Walter Santos Magalhães, personal communication).

The objective of recommending a mixture of hybrids was to increase the genetic diversity, to maintain stability in relation to disease resistance and to assure a good pollination, overcoming sexual incompatibility problems.

In 1986, based on more information on hybrid performances, the mixture of hybrids was restricted to crosses between common (SIAL and SIC) and ICS clones (Anonymous, 1989). Unfortunately, these hybrids were very susceptible

to a serious disease, "witches´ broom" caused by *Crinipellis perniciosa*, introduced in Bahia in 1989. In 1994, these varieties were substituted by a hybrid variety called Theobahia (SCA 6 x ICS 1), resistant to witches´ broom (Monteiro et al. 1995). Later, Theobahia I (ICS 6 x SCA 6) and Theobahia II (ICS 8 x SCA 6) were included in the recommendation. From 1995, CEPEC added to the recommendation clones derived from IMC 67, ICS 1 and SCA 6, coded as TSA, TSH, CEPEC and EET.

In 1993, both CEPEC scientists and farmers began to select resistant genotypes to witches´ broom in farms, mainly within hybrid populations, based on the assumption of high variability. Resistant and high yielding plants have been identified in these populations, in areas heavily infected, suggesting the existence of genetic diversity. In spite of the preliminary success in these selections, still remains the question of how much genetic variability exist in plantations.

This research was aimed to quantify the genetic variability, and to access the genetic similarity among the genotypes used as parents of hybrids released by CEPLAC to farmers, by using isozymes and RAPD markers. Genetic diversity in commercial plantations is inferred from these results.

MATERIAL AND METHODS

Genotypes

For the studies of genetic diversity, 21 clones were used, namely: ICS 1, ICS 6, ICS 8, IMC 67, PA 30, PA 150, SCA 6, SCA 12, SIAL 70, SIAL 169, SIAL 325, SIAL 505, SIC 17, SIC 19, SIC 328, SIC 329, SIC 813, TSA 644, UF 168, UF 613 and UF 667. These clones were used as parents of hybrids recommended by the Cacao Research Center (CEPEC) to farmers, from 1960´s to 2001. All these clones belong to the CEPEC´s germplasm collection, in Ilhéus, Bahia, Brazil.

The selections ICS (Imperial College Selections) were made by the Imperial College of Tropical Agriculture, in Trinidad and Tobago. The IMC (Iquitos Mixed Calabacillo), PA (Parinari) and SCA (Scavina) selections were made in the Peruvian Amazon, and the TSA (Trinidad Selection Amazon) in Trinidad, by the Ministry of Agriculture, Trinidad. The SIC ("Seleção do Instituto de Cacau") and SIAL ("Seleção do Instituto Agronômico") selections carried out in populations of common varieties, in Bahia, by the "Instituto de Cacau da Bahia" and by the "Instituto Agronômico do Leste". The UF selections (United Fruit) were made in Costa Rica by the United Fruit Company.

The SIC and SIAL selections were included in this study not only because they participated in the formation of hybrids but also because they represent common varieties cultivated in Bahia for centuries.

Isozymes

Young leaves from each of the 21 accessions were collected and enzymes extracted as described by Yamada and Guries (1994). The MDH, IDH and DIA systems with the buffer designated AC (Yamada and Guries, 1989) was used. In the eletrophoresis, the gel run was about 4 hours, using 50 mA and 150 V as upper limits. The methodology is described in details in Yamada and Guries (1994). The presence/absence of bands were scored for statistical analyses. Some heterozygous genotypes produced 2 or 3 bands, depending on the quaternary structure of the enzyme considered.

RAPD

Leaves from each genotype were collected and stored at –80 ºC for DNA extraction. The genomic DNA of each material was extracted using the CTAB method (Doyle and Doyle, 1990) with some modifications (Araújo et al., 2000). After extraction, the concentration of DNA was estimated by spectophotometer at 260 nm (Sambrook et al., 1989). Bands of total genomic DNA separated by electrophoresis in 0.8% agarose gel were used to check the integrity and purity of the extracted DNA. After quantification, the DNA samples were diluted to 10 ng / µL.

DNA samples of each genetic material were amplified by RAPD (Random Amplified Polymorphic DNA) technique. Amplification reactions were made in a volume of 25 ml, adding Tris-HCl 10 mM (pH 8.3), KCl 50 mM, $MgCl_2$

2mM, 100µM of each one of the desoxinucleotides (dATP, dTTP, dGTP and dCTP), 0.4 mM of a primer (Operon Technologies Inc., Boulevard, CA, USA), one unit of the enzyme Taq DNA polymerase and approximately 30 ng of DNA. Eleven primers were used to obtain RAPD markers (Table 1). The amplification was made in a termocycler for 40 cycles (94 ºC for 15 sec, 35 ºC for 30 sec and 72 ºC for 90 sec) followed by a stage of final extension of 72 ºC for 7 minutes. After that, the temperature was lowered to 4 ºC. After the amplification, 3 µl of a mixture of bromophenol blue (0.25%), glycerol (60%) and water (39.25%) was added to each sample. Those samples were applied in agarose gel (1.2 %), submerged in TBE (Tris-borate 90 mM, EDTA 1 mM). The electrophoresis was run for approximately 4 hours at 90 volts. At the run completion, the gel was stained with ethidium bromide and photographed under ultraviolet light.

Statistical Analyses

The RAPD and isozyme markers generated were transformed into binary data, with 0 representing the absence and 1 the presence of bands. The NTSYS statistical analysis package was used (Rolf, 1993) to determine genetic similarities among accessions. A genetic similarity matrix based on Dice´s coefficient was calculated by:

$$GS(i,j) = 2a/(2a + b + c),$$

where GS(i,j) is the genetic similarity between the i^{th} and j^{th} accessions, *a* is the number of bands present in both accessions, *b* is the number of bands present only in the i^{th} accession and *c* is the number of bands present only in the j^{th} accession. The genetic similarity coefficients obtained were used to construct a phenogram by the unweighed pair-group method, with the arithmetic averages option (UPGMA).

Table 1 - Primers used in the studies of genetic diversity in cacao, their respective sequences of bases and number of polymorphic and monomorphic bands.

		Nº of bands	
Primer	Sequence 5´→3´	Polymorphics	Monomorphics
OPA03	AGTCAGCCAC	8	6
OPA04	AATCGGGCTG	5	3
OPA07	GAAACGGGTG	7	3
OPA13	CAGCACCCAC	3	5
OPC05	GATGACCGCC	9	3
OPC13	AAGCCTCGTC	7	4
OPD08	GTGTGCCCCA	2	4
OPD16	AGGGCGTAAG	3	2
OPE05	TCAGGGAGGT	10	0
OPE14	TGCGGCTGAC	10	1
OPE15	ACGCACAACC	6	4
Total of bands		70	35

RESULTS AND DISCUSSION

The three isozyme systems used resulted in 10 bands. MDH, which is a dimer with 3 alleles, resulted in 5 bands; IDH, a dimer with 2 alleles, 3 bands; and DIA, a monomer with 2 alleles, 2 bands. The 11 RAPD primers resulted in 105 bands, 35 monomorphic and 70 polymorphic (Table 1). Therefore, considering isozymes and RAPD, 80 bands were generated for analysis.

Clone Grouping

At 86% of similarity, 21 cacao accessions were classified into seven groups (Figure 1). The first group includes the two scavina clones (SCA 6 and SCA 12), with 93% of similarity. This grouping was already expected, considering that the two clones are siblings originated from seeds of the same fruit, collected in Peru. Similar results were reported in other studies using molecular markers (Marita, 1998).

A second group was formed by SIC, SIAL and UF clones. Most of the clones included in this group belong to the common variety (SIC and SIAL clones) from Bahia, except the SIC 19, (Figure 1). However, UF clones selected in Costa Rica were also clustered in this group. Mariano and Bartley (1979), studying several progenies,

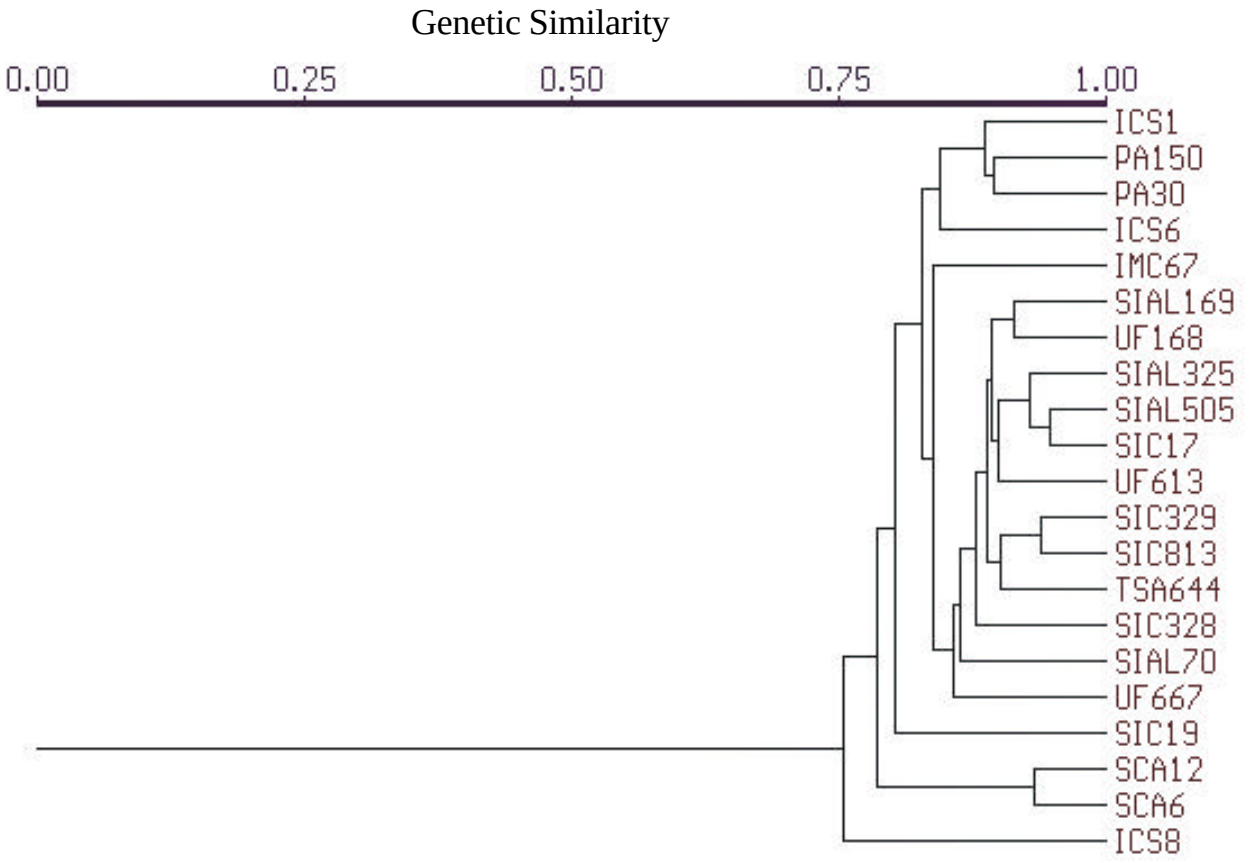

Figure 1 - Dendrogram for 21 accessions of *Theobroma cacao* L. based on Dice´s coefficient of similarity, using UPGMA method.

did not find differences among SIC and SIAL clones as parents of hybrids, probably due the high similarity of those clones as shown in this study. In addition, Bartley et al. (1982), did not observe heterosis when crossing SIC and SIAL clones with UF clones, specially the UF 613. One of the reasons for that is the high genetic similarity of SIC and SIAL with UF clones.

A third group was formed by Parinari clones (PA 30 and PA 150), selected in the Peruvian Amazon (Figure 1). The ICS 1 clone , however, selected in Trinidad was also included in this group. This high genetic similarity between the PA 30 and the PA 150 (89%, not shown) confirms the lower genetic diversity within Parinari clones using isozymes (Yamada and Guries, 1994) and incompatibility tests (Yamada et al. 1996). In such tests, only three phenotypic incompatibility groups have been observed.

The other four "groups" were formed by only one clone each (SIC 19, IMC 67, ICS 6 and ICS 8). Great diversity was observed within ICS selections, in special the ICS 8, which was very different from all other materials studied (Figure 1) and presented the lowest genetic similarities (0.66 to 0.83, not shown) with other clones. In general, ICS 8 produces high yield hybrids, having a good general combining ability (Monteiro et al. 1988). The CEPEC´s decision to recommend the ICS 6 and the ICS 8, besides ICS 1, in crosses with SIC and SIAL clones and later with Scavina, was correct to maintain diversity, considering that the three ICS clones are genetically distinct, mainly ICS 8 (Figure 1).

Clones selected in Peru (PA 30, PA 150, IMC 67, SCA 6, SCA 12) were put in three different groups (Figure 1). Marita (1998) observed a high genetic diversity among Upper Amazon clones using molecular markers. Clones selected in Trinidad (ICS clones) also showed the same trend of high diversity observed among Peruvian clones.

Genetic Diversity

Before the introduction of clones from other countries in Bahia, the diversity in plantations was low, as shown by the high similarity (mean= 0.87, range= 076-0.95) among representatives of the

population of the common variety (SIC and SIAL) (Figure 2a). If the clone SIC 19, one of the most genetically distant of the other common clones, is dropped of the analysis, the average similarity increases to 0.89 (not shown). Similar results were found by Cascardo et al. (1993) when studying the genetic diversity of 78 selections of the common population, using 14 RAPD primers (108 polymorphic bands). Those authors observed an average similarity of 0.93 (range 0.86 to 0.99).

From 1960 on, the Cacao Research Center (CEPEC) introduced many clones from several countries. Many of these clones were included in the CEPEC's breeding program, and some of them (ICS 1, ICS 6, ICS 8, IMC 67, PA 30, PA 150, SCA 6, SCA 12, TSA 644, UF 168, UF 613 and UF 667) were released to farmers as crosses with clones of the common variety (SIAL 70, SIAL 169, SIAL 325, SIAL 505, SIC 17, SIC 19, SIC 328, SIC 329, SIC 813). The introduced clones presented higher diversity (mean similarity = 0.81, range=0.66-0.93) than the clones of the local (common) variety (Figure 2c). They also presented low genetic similarity with clones of the local variety (mean=0.83, range=0.72-0.93) (Figure 2b). With the introduction of the new clones, the overall similarity reduced from 0.87, in the common variety, to 0.83 (Figure 2d).

Besides increasing the overall diversity, with the introduction of new clones, specific genes of interest for breeding were also introduced. For example, most clones of the common variety are highly susceptible to a serious disease of cacao - the witches' broom disease (*Crinipellis perniciosa*). On the other hand, the scavinas selected in Peru, are highly resistant to that disease (Toxopeus, 1987). The ICS clones, introduced from Trinidad, are highly productive and have a high general combining ability (Monteiro et al, 1988). This high diversity in cacao plantations in Bahia, associated to the quality of the materials introduced, are probably the reasons for the success of selection in plantations by the Cacao Research Center and farmers. This diversity is also very important in reducing the effects of the witches' broom disease in the region.

ACKNOWLEDGEMENTS

This work was partly supported by the Common Fund for Commodities, supervised by the International Cocoa Organization and executed by the Government of Brazil (CEPLAC). IBECAU also contributed for employing Fábio G. Faleiro.

RESUMO

Variabilidade genética em populações de cacaueiros cultivados na Bahia, Brasil, detectada por isoenzimas e marcadores RAPD

O objetivo do presente trabalho foi estudar a variabilidade genética dos acessos da coleção do germoplasma que constituiram progenitores de híbridos recomendados pela CEPLAC, para inferir sobre a diversidade dos cacaueiros da Bahia. Os 21 acessos ou clones utilizados foram das séries SIC, SIAL (variedades locais), ICS, IMC, PA, SCA, TSA e UF (introduzidos). Os marcadores moleculares foram isoenzimas (sistemas de enzimas, MDH, IDH e DIA) e RAPD (105 locos RAPD de 11 primers). O pacote estatístico NTSYS foi usado para determinar a similaridade genética entre os acessos. A matriz de similaridade baseada no coeficiente de Dice foi calculada e o dendrograma construido usando o método de UPGMA. Os acessos foram classificados em 7 grupos: 4 acessos (SIC 19, IMC 67, ICS 6 e ICS 8) não se agruparam. Outros formaram pequenos grupos como SCA 6 e SCA 12 e PA 30, PA 150 e ICS 1. Um grupo maior foi formado com 12 acessos das séries SIC, SIAL, UF e o TSA 644. Os resultados deste trabalho indicam que a variabilidade genética nas populações de *T. cacao* plantados na Bahia era muito estreita, contudo com a recomendação dos híbridos pela CEPLAC aumentou consideravelmente. Aumentando, portanto, as chances de sucesso com a seleção nestas plantações.

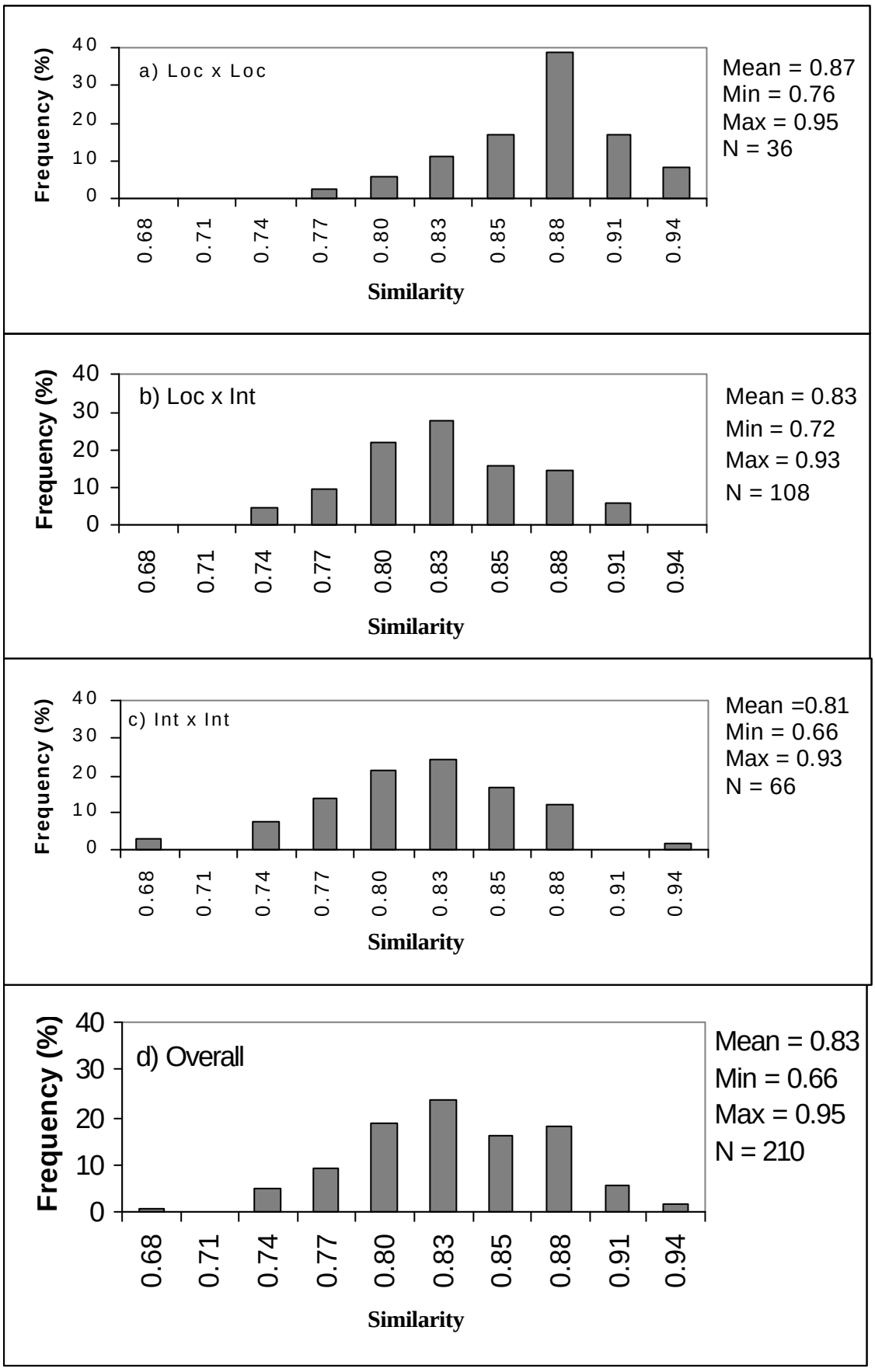

Figure 2 – Frequency distribution of similarity coefficients between cacao clones: a) local clones, b) local and introduced clones, c) introduced clones, and d) local and introduced clones. Also presented the mean, minimum, and maximum similarity coefficients, considering all N pairs of clones in a group.

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Received: March 21, 2001;
Accepted: September 03, 2001.