Genetic structure of Guianan wild cocoa (*Theobroma cacao* L.) described using isozyme electrophoresis

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Summary
Genetic structure of Guianan wild cocoa (*Theobroma cacao* L.) described using isozyme electrophoresis

Wild cocoa trees (*Theobroma cacao* L.) from southeast French Guiana, surveyed and collected between 1985 and 1995, represent an original group of germplasm that contributes significantly to the species' genetic structure. Nineteen populations (demes) from the areas around five rivers (Oyapok, Euleupousing, Yaloupi, Camopi and Tanpok) in the main two river basins of French Guiana are represented in collections maintained in French Guiana, in a number of cocoa producing countries and on some quarantine stations. This study describes the genetic structure of the Guianan group and the genetic parameters of the group as a whole (138 clones in total) and of the eight most abundantly represented populations. Isozyme electrophoresis based on six isozyme systems (nine loci) was used. The results revealed a high average number of alleles per locus (1.9), a high percentage of polymorphic loci (77.7) and low average heterozygosity (0.085). The various populations displayed highly variable allelic frequencies. Wright's fixation index, at around 20%, indicated substantial genetic differentiation among populations. The genetic structure of the group showed that populations from the Camopi river area fell into two sub-groups, confirming results obtained earlier with other descriptors, and that the populations from the other areas were significantly different, that of the Kérindioutou differing most from the others. The use of controls from other morpho-geographical groups (Upper and Lower Amazon Forastero, Trinitario) confirmed the uniqueness of the Guianan cocoa trees. The results of the investigations, including the low heterozygosity, suggest the potential for using clones derived from the Guianan genetic group for genetic improvement of the crop, and support arguments for continuing surveys throughout the Guianan shield.

Key words: French Guiana, isozyme electrophoresis, *Theobroma cacao*, wild cocoa trees

Résumé
Description de la structuration génétique des cacaoyers spontanés (*Theobroma cacao* L.) de Guyane Français étudiée par électrophorèse d'iso-enzymes

Les cacaoyers spontanés (*Theobroma cacao* L.) du sud-est de la Guyane Française, prospectés et collectés entre 1985 et 1995, constituent un groupe original et l'un des axes de la structuration génétique de l'espèce. Dix-neuf populations (demes) originaires de cinq rivières (Oyapok, Euleupousing, Yaloupi, Camopi et Tanpok) des deux principaux bassins fluviaux de la Guyane Française sont représentées en collections, en Guyane, dans de nombreux pays producteurs de cacao et en stations de quarantaine. L'étude présentée concerne la structuration génétique de ce groupe et les paramètres de génétique des populations, pour l'ensemble (soit 138 clones étudiés au total) et pour les huit populations les plus richement représentées. La méthode utilisée est l'électrophorèse d'iso-enzymes, à l'aide de six systèmes enzymatiques (neuf locus). Les résultats montrent un nombre moyen d'allèles par locus élevé (1,9), un pourcentage de locus polymorphes élevé (77,7) et une hétérozygotie observée moyenne faible (0,085). Les diverses populations présentent des fréquences alléliques très variables. L'indice de fixation de Wright, d'environ 20 %, indique une grande différentiation génétique entre populations. La structure génétique du groupe montre que les populations de la rivière Camopi se séparent en deux groupes, confirmant des résultats antérieurs obtenus par d'autres descripteurs, et que les populations des autres rivières sont différentes, celle de la Kérindioutou étant la plus éloignée des autres. L'utilisation de témoins appartenant à d'autres groupes morpho-géographiques (Forastero haut et bas Amazoniens, Trinitario) confirme l'originalité des cacaoyers guyanais. Ces résultats, y compris le caractère faiblement hétérozygote, montrent l'intérêt potentiel en amélioration génétique des clones issus de ce groupe et militent pour une poursuite des prospections dans l'ensemble du Plateau des Guyanes.

Resumen
Descripción de la estructura genética de los árboles silvestres de cacao en Guayana (*Theobroma cacao* L.) empleando electroforesis de isozimas

Los árboles silvestres de cacao (*Theobroma cacao* L.) de la zona sudoriental de la Guayana Francesa que fueron recogidos y examinados entre 1985 y 1995, forman un grupo original que contribuye de manera significativa a la estructura genética de la especie. Diecinueve poblaciones (demes) originarias de cinco ríos (Oyapok, Euleupousing, Yaloupi, Camopi y Tanpok) pertenecientes a las dos mayores cuencas fluviales de la Guayana Francesa están representadas en las colecciones mantenidas en dicho país, en varios países productores de cacao y en algunas estaciones de cuarentena. Este estudio describe la estructura genética del grupo guayanés y los parámetros genéticos del grupo entero (es decir 138 clones) y de las ocho poblaciones más abundantemente representadas. Para el análisis se empleó electroforesis de isozimas, con seis sistemas enzimàticos (nueve *loci*). Los resultados revelaron un elevado número promedio de aleles por *locus* (1,9), un alto porcentaje de *loci* polimórficos (77,7) y un bajo promedio de heterocigosidad observado (0,085). Las diversas poblaciones mostraron frecuencias alélicas altamente variables. El índice de fijación de Wright, de alrededor del 20%, indicó una diferenciación genética sustancial entre las poblaciones. La estructura genética del grupo mostró que las poblaciones del río Camopi se dividieron en dos subgrupos, confirmando los resultados obtenidos anteriormente con otros descriptores, y que las poblaciones de los otros ríos fueron significativamente diferentes, siendo la del río Kérindioutou la más diferente respecto de las otras. El empleo de testigos pertenecientes a otros grupos morfogeográficos (Alto y Bajo Amazonas Forastero, Trinitario) confirmaron la originalidad de los árboles de cacao guayaneses. Estos resultados, incluida la baja heterocigosidad, sugieren la posibilidad de usar clones derivados de este grupo genético con fines de mejoramiento genético, y demuestran la necesidad de continuar con los exámenes en todo el escudo guayanés.

Introduction

Cocoa (*Theobroma cacao* L.) is a preferentially allogamous Neotropical tree species of the Sterculiaceae (Cuatrecasas 1964). Cuatrecasas (1964) described two subspecies, *Theobroma cacao* subsp. *cacao* and *Theobroma cacao* subsp. *sphaerocarpum*, corresponding to the two original cultivated types, Criollo (mainly originating from Central America) and Forastero from South America. Within these two major types Cheesman (1944) and Cuatrecasas (1964) identified various sub-sets, dependent on their geographical origins, including, for example, Upper Amazon Forasteros and Lower Amazon Forasteros. A third type, a hybrid between Criollo and Forastero, resulting from human intervention, is known as Trinitario.

Knowledge of the genetic structure of the species has recently been further increased (Lanaud et al. 1999; Motamayor et al. 2002). The existence of two subspecies is challenged in favour of several morpho-geographical groups, though they have yet to be exhaustively specified.

The wild cocoa trees of southeast French Guiana, collected between 1985 and 1995 (Clément 1986; Lachenaud and Sallée 1993; Lachenaud et al. 1997) are considered to represent one of the four axes of the species' genetic structure (Lachenaud 1997; Lanaud et al. 1999). The mother-trees of the Guianan material that currently exist in several collections, or are maintained on a few quarantine stations (Ford et al. 2000), come from 19 populations (demes, according to Hartl and Clark 1997) originating from the areas around five rivers in the two main river basins of French Guiana (Figure 1): the Oyapok (its upper reaches are the Kérindioutou, along with its tributaries, the Euleupousing, Yaloupi and Camopi) and the Maroni (river Tanpok). The material, collected in its wild form as budwood or pods, was

planted (as clones or open pollinated progenies) in the reference collection at the CIRAD (Centre de Coopération Internationale en Recherche Agronomique pour le Développement, Montpellier, France) Paracou-Combi station, at Sinnamary (French Guiana), where it was studied and characterized. This stage is a prerequisite for its use in genetic improvement programmes. It is important to determine the diversity of this material and, to this end, the diversity of certain populations has already been described using biochemical, morphological and agronomic descriptors (Lanaud 1987, 1999, 2001). The study described here covered 17 of the 19 known populations using the established and reliable method of isozyme electrophoresis (Sounigo et al. 1999). It also aimed to determine conventional population genetics parameters for the Guianan material as a whole and for the adequately represented populations, and to evaluate genetic differentiation among populations.

Material and methods
Planting material

The planting material comprised 138 clones of Guianan cocoa trees and four control clones belonging to other main morpho-geographical groups.

The Guianan clones belonged to the Oyapok (Borne 7, Ker, Pina, Oya), Camopi (Cam 0, 1, 3, 7, 8, 9, 10, 11, 12, 13), Euleupousing (Elp), Yaloupi (Yal) and Tanpok (Tan) populations. Despite the geographical scatter of the trees on the Oyapok banks downstream of Pina, we considered them as belonging to a single population, termed 'Oya' in the present paper (Figure 1). The numbers of individuals studied per population are given in Table 1. The control clones were
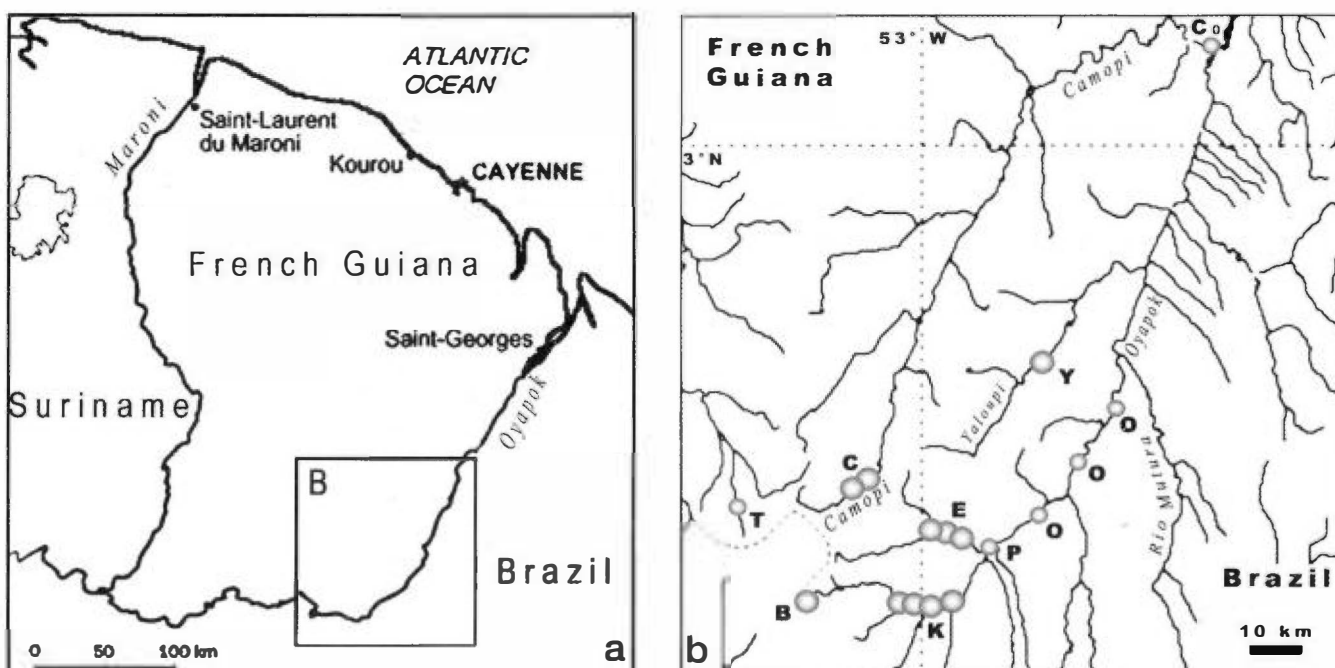

Figure 1. (a) Location of French Guiana and (b) location of the cocoa populations studied (for more precise localization see Lachenaud and Sallée 1993 and Lachenaud et al. 1997), where: B=Borne 7, Co=Cam 0, C=Cam 1 to 13, E=Elp, K=Ker, P=Pina, O=Oya, T=Tan and Y=Yal. The river Oyapok represents the border between French Guiana and Brazil; elsewhere the border is indicated by _ _ _ _

Upper Amazon Forasteros Pa 120 and Pa 121, an Amelonado type Lower Amazon Forastero IFC 1 and the Trinitario ICS 1 (Enriquez and Soria 1967; Ford et al. 2000).

Methods

Isozyme electrophoresis

The method of analysis was isozyme electrophoresis on starch gel (Lanaud 1986a,b). Six isozyme systems were used in the study: glucose phosphate mutase (GPM), glucose-phosphate isomerase (GPI), malate dehydrogenase (MDH), acid phosphatase (ACP), alcohol dehydrogenase (ADH) and isocitrate dehydrogenase (IDH). Some of these systems involved several genes: two for GPM (GPMA and GPMB) and three for MDH (MDHA, MDHB and MDHC). A total of nine loci were therefore considered.

The technique used (grinding, development, buffer solutions) was described by Lanaud (1986a). However, as a portable laboratory was used (Lebrun and Chevellier 1990), a few adaptations were made: the run time was fixed at 8 instead of 18 h and the migration tanks of the portable laboratory were smaller than those used by Lanaud (1986a).

Statistical methods and genetic parameters

Cluster analysis, genetic parameters and distance calculations were performed using Popgene 32 software (Yeh et al. 1999). Nei distances (D_{AB}, Nei 1972) between populations A and B were calculated as follows.

First, at the level of each locus, the Nei similarity index between populations A and B (I_{AB}) was calculated, according to the formula:

$$I_{AB} = \frac{\Sigma_k (p_{kA} \, p_{kB})}{\Sigma_k [(p_{kA}^2 \cdot p_{kB}^2)]^{1/2}}$$

where p_k is the frequency of allele k in the population. Then the Nei distance between populations A and B was calculated as:

$$D_{AB} = - \ln \overline{I}_{AB}$$

where $\overline{I}_{AB}$ is the arithmetic mean of the I_{AB} values calculated for each locus.

The genetic parameters calculated were:

1. Shannon's diversity index (Shannon and Weaver 1949): for each locus, this value (H) was calculated as:

$$H_l = -\Sigma_{kl} \ln p(kl)$$

where $p(kl)$ = frequency of allele k of locus l in the population. The mean value of these indices was then calculated for all the loci: $H = \overline{H}_l$

Table 1. Distribution per population of Guianan cocoa clones included in this study

Basin	Sub-basin	Population	Number of analysed clones
Oyapok	Oyapok	Borne 7	6
		Pina	1
		Oya	2
		Ker	8
	Euleupousing	Elp	11
	Yaloupi	Yal	3
	Camopi	0	1
		1	19
		3	15
		7	12
		8	2
		9	35
		10	1
		11	1
		12	5
		13	13
Maroni	Tanpok	Tan	3
Total			138

2. Wright's fixation index (F) is "the reduction in heterozygosity expected with random mating at any one level of a population hierarchy relative to another, more inclusive level of the hierarchy " (Hartl and Clark 1997), i.e. a higher level. In our study, we defined F_{ST} as the fixation index of the populations (demes) relative to the total combined population:

$$F_{ST} = (H_T - H_S) / H_T$$

where H_T is the total (expected) heterozygosity and H_S is the average (expected) heterozygosity among populations. These two H values may be replaced by Shannon's indices if random mating is not verified.

3. Proportion of observed heterozygosity: the proportion of heterozygous genotypes was calculated for each locus and the mean value was calculated for all loci.

4. Mean number of alleles per locus: included all the alleles, irrespective of their frequencies.

5. Number of polymorphic loci included all the loci with more than one allele, irrespective of the frequencies of the alleles.

Factorial Analyses of Correspondences (FAC) were performed using WINSTAT software developed at CIRAD. Cluster analyses were performed using the UPGMA procedure (Unweighted Pair Group procedure with Arithmetic Means, Sneath and Sokal 1973).

Results

Genetic parameters

For the set of 138 clones studied and the eight populations represented by a minimum of six clones in this study, Tables 2 and 3 indicate the numbers of polymorphic loci, the alleles present and their frequencies, the average number of alleles observed per locus, the observed and calculated heterozygosities (assuming panmixia) and Shannon's diversity index values.

Two loci were monomorphic (MDH B and MDH C) and the entire set of material studied displayed allelic frequencies that did not conform to Hardy–Weinberg equilibrium. However, two populations, Cam 1 and Cam 7, appeared to display panmixia.

The average number of alleles per locus ranged between 1.22 for populations Cam 1 and Cam 7 to 1.89 overall. Allelic frequencies differed substantially among populations with, for example, that of allele 3 of the ACP locus ranging from 0.06 in the Ker population to 0.62 in Cam 7, and that of allele 1 of IDH from 0.19 in Ker to 0.88 in Cam 13.

The recorded overall heterozygosity (Nei index) was 8.4% on average, with extremes ranging from 3.7% (Borne 7) to 11.1% (Cam 1); Table 3 indicates an overall expected heterozygosity of 15.2% assuming panmixia. Shannon's diversity index was 0.25 overall, ranging from 0.14 (Cam 1 and Cam 7) to 0.21 in the Elp population.

When Wright's fixation index was calculated from the expected heterozygosities for the 8 main populations and the set they constituted (119 clones), a value of $F_{ST}=0.197$ was obtained. Using Shannon's indices, the value was 0.227. It can therefore be deduced that around 20% of diversity is among the populations and 80% within.

Tables 2 and 3 also show that the contribution of the populations represented by five or fewer individuals (Tan, Cam 0, Cam 8, Cam 10, Cam 11, Yal, Oya, Pina) was considerable: two additional alleles (involving a further two polymorphic loci, GPMA and GPI), increased the average number of alleles per locus from 1.67 to 1.89 and Shannon's index from 0.22 to 0.25.

Figure 2 is a dendrogram (based on Nei's genetic distances) of the eight most abundantly represented populations. The Camopi populations were grouped into two sub-sets: Cam 1-7 and Cam 3-9-13. The Ker population differed most from the others.

Figure 3 represents the plane defined by the first two axes of the FAC using data for all clones and controls. The first axis accounted for 33% of total diversity, the second for 20%. The graph shows strong grouping of the Cam populations (particularly along the first axis) and the uniqueness of certain representatives of the Ker, Yal, Oya and other populations represented by fewer than five clones (termed 'others' in the legend), such as Cam 0. The Amelonado control (IFC 1) was very different from virtually all the wild Guianan material.

Discussion

When comparing our results with those of Lanaud (1987) on the same loci and on 332 clones representing the different

Table 2. Alleles present and allelic frequencies for the eight loci studied (see text), for the set of Guianan cocoa materials studied (138 clones) and the main eight populations included in this study

Populations	GPMA	GPMB	GPI	MDHA	MDHB	MDHC	ACP	ADH	IDH
Overall	1 (0.02) 2 (0.98)	1 (0.75) 2 (0.25)	1 (0.99) 2 (0.01)	2 (0.98) 3 (0.02)	1	1	2 (0.64) 3 (0.36)	1 (0.91) 2 (0.09)	1 (0.85) 2 (0.14) 4 (0.01)
Borne 7	2	1 (0.50) 2 (0.50)	1	2	1	1	2	1 (0.83) 2 (0.17)	1 (0.83) 2 (0.17)
Cam 1	2	1 (0.71) 2 (0.29)	1	2	1	1	2 (0.42) 3 (0.58)	1	1
Cam 13	2	1 (0.85) 2 (0.15)	1	2	1	1	2 (0.54) 3 (0.46)	1	1 (0.88) 2 (0.12)
Cam 3	2	1 (0.90) 2 (0.10)	1	2	1	1	2 (0.50) 3 (0.50)	1	1 (0.77) 2 (0.23)
Cam 7	2	1 (0.67) 2 (0.33)	1	2	1	1	2 (0.38) 3 (0.62)	1	1
Cam 9	2	1 (0.83) 2 (0.17)	1	2	1	1	2 (0.74) 3 (0.26)	1	1 (0.86) 2 (0.14)
Elp	2	1 (0.45) 2 (0.55)	1	2 (0.91) 3 (0.09)	1	1	2 (0.77) 3 (0.23)	1 (0.86) 2 (0.14)	1
Ker	2	1 (0.94) 2 (0.06)	1	2	1	1	2 (0.94) 3 (0.06)	1 (0.12) 2 (0.88)	1 (0.19) 2 (0.62) 4 (0.19)

Values in bold indicate cases where Hardy–Weinberg equilibrium was verified.

Table 3. Diversity parameters for the main eight cocoa populations and for the whole set of Guianan cocoa material included in this study

Populations	No. polymporphic loci (%)	Average no.alleles observed per locus	Observed heterozygosity	Expected heterozygosity (Nei)	Shannon index
Overall	7 (77.7)	1.89	0.0845	0.1520	0.2508
Borne 7	3 (33.3)	1.33	0.0370	0.1173	0.1771
Cam 1	2 (22.2)	1.22	0.1111	0.0999	0.1425
Cam 13	3 (33.3)	1.33	0.0769	0.1068	0.1641
Cam 3	3 (33.3)	1.33	0.0963	0.1153	0.1735
Cam 7	2 (22.2)	1.22	0.0648	0.1015	0.1442
Cam 9	3 (33.3)	1.33	0.0762	0.1012	0.1598
Elp	4 (44.4)	1.44	0.0808	0.1387	0.2142
Ker	4 (44.4)	1.56	0.0833	0.1102	0.1962
Total 8 pop.	5 (55.5)	1.67	0.0822	0.1387	0.2219
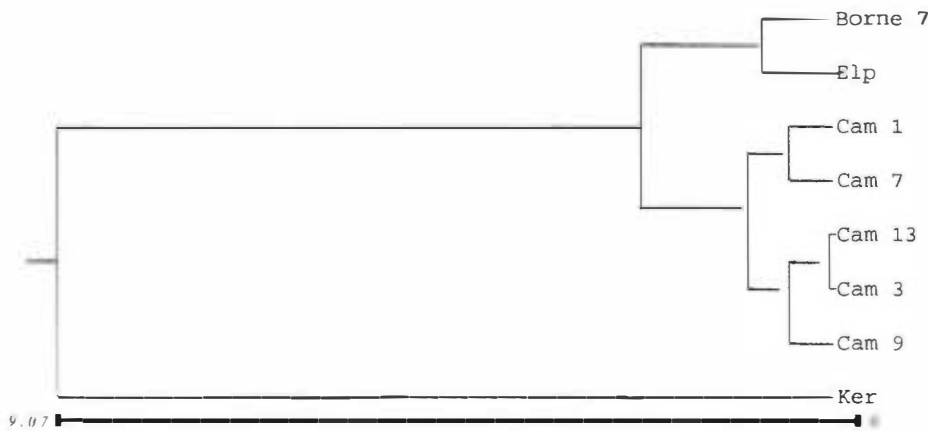
Figure 2. Dendrogram based on Nei's genetic distances (UPGMA).
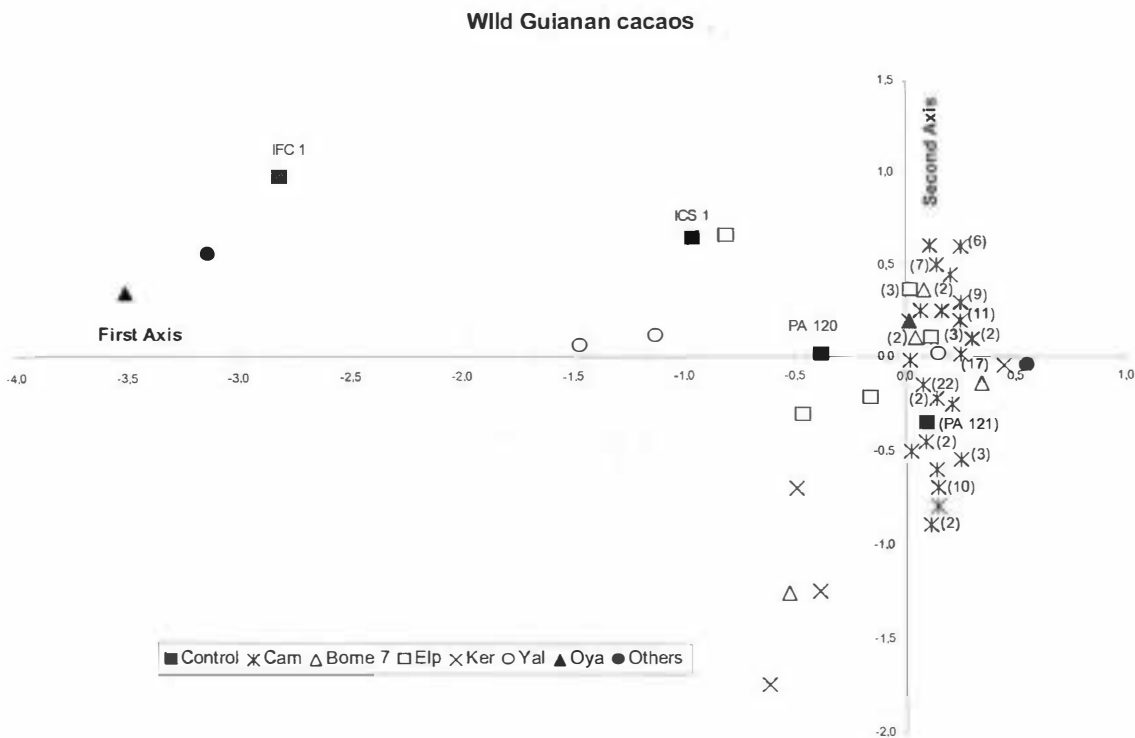
Wild Guianan cacaos

Figure 3. Plane of the first two FAC axes for all the cocoa clones (Guianan and controls). The numbers of clones from the same population sharing the same coordinates on the plane are included in parentheses.

morpho-geographical groups in the species, the average number of alleles per locus for the set of wild Guianan clones (1.9) was high. It was higher for example than that of the hybrid group of Trinitarios cultivated in America (American Trinitarios, 1.7), about the same as that for Trinitarios cultivated in Africa (African Trinitarios, 2.0), but lower than that for Upper Amazon Forasteros (between 2.0 and 2.6). The number of polymorphic loci was high, and the percentage (77.7) equal to or greater than that for all the groups studied by Lanaud (1987), except for certain Upper Amazon Forasteros (EBC and G0). However, the average observed heterozygosity (0.0845) was low, indicating substantial fixation of certain alleles. Wright's fixation index (F_{ST}), at around 20%, indicated considerable genetic differentiation (Hartl and Clark 1997) through a highly substantial reduction in heterozygosity and is in line with the values reported by Ronning and Schnell (1994) and Sounigo et al. (1996). Shannon's genetic diversity index for the whole set (0.25) was typical of allogamous perennial plants (Hamrick et al. 1992). However, a study of individuals grown from seed and maintained in a collection under suitable growing conditions tends to increase the survival of homozygotes and could therefore bias the observed heterozygosity rate (Hamrick et al. 1993).

Among the alleles revealed in our study, the presence of an allele different from the two major alleles IDH 1 and IDH 2 is worth noting. We named this allele IDH 4, but have been unable to verify whether it is original, or is identical to allele IDH 3 described by Lanaud (1987) in certain LCT-EEN clones of Upper Amazon Forasteros from Ecuador (Allen 1983; Allen 1987). This allele IDH 4 was found in the Ker population at a frequency of 0.19, whereas it was rare or absent in the set of populations studied by Lanaud (1987).

Lanaud (1987) used the same method to study cocoa populations from the Camopi (Cam 1, 3, 7, 9, 12, 13) and a comparison with her results shows that the surveys of 1990 and 1995 in three other valleys (Lachenaud and Sallée 1993; Lachenaud et al. 1997) led to considerable enrichment of wild Guianan cocoa tree representation. The average number of alleles per locus increased from 1.3 to 1.9, the percentage of polymorphic loci increased from 25.0 to 77.7 and average heterozygosity from 0.058 to 0.085. This enrichment can clearly be seen in the plane defined by the first two axes of the FAC, where the Oyapok populations (especially Oya and Ker) and the Yaloupi population (Yal) appear very distinct from the Camopi populations (Cam).

Grouping the various Cam populations indicated by the dendrogram in Figure 2 tallies with the results obtained using floral (Lachenaud et al. 1999) and agronomic descriptors (Lachenaud et al. 2001). Populations Cam 1 and 7 are clearly distinct from group 3-9-13, in which Cam 3 and 13 are very close. Likewise, the Ker population appears to be different from population Borne 7, which is located further up the same river. Charters and Wilkinson (2000) published a comparable dendrogram, isolating Ker clones from Cam and Borne 7 clones.

The plane defined by the first two axes of the FAC shows that most of the Guianan clones are very distinct from the Trinitario (ICS 1) and Amelonado (IFC 1) controls. On the other hand, one of the two Upper Amazon controls (PA 121) is observed close to several CAM clones.

Conclusion

This study of clones representing virtually all the cocoa populations surveyed in southeast French Guiana between 1985 and 1995 shows their overall richness (average number of alleles per locus, % of polymorphic loci, rare alleles), diversity (highly variable allelic frequencies) and the fixed nature of most of the alleles (low average heterozygosity, high F_{ST}). The results obtained therefore make it possible to moderate the conclusions obtained earlier on the Cam populations alone (Lanaud 1987; Sounigo et al. 1996) as to the diversity encountered in wild Guianan cocoa trees. The inclusion of six additional populations (Elp, Borne 7, Ker, Pina, Oya, Yal) shows that genetic diversity is substantial, at least in the area considered (around 7000 km^2), when compared with that for Upper Amazon G0 or LCT-EEN (Pound 1938, 1943; Allen 1983; 1987) collected from much larger areas. This observation suggests continuing surveys, both in French Guiana, where there are other zones with wild cocoa tree stands (Lachenaud et al. 1997), and also throughout the Guianan shield (Cuatrecasas 1964).

There is substantial among-population diversity (around 20%), which is typical of allogamous perennial plants that have been subjected to substantial genetic mixing, and particularly for cocoa, which occurs in its wild state in demes, where considerable fixation occurs. The wild cocoa trees of French Guiana display a low overall rate of recorded heterozygosity (0.085), which could be a considerable advantage for their use as parents in genetic improvement programmes, to obtain more uniform progenies and to maximize potential heterosis effects.

Acknowledgements

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