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Chloroplast and mitochondrial DNA diversity in *Theobroma cacao*

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Abstract. The variability of cocoa (*Theobroma cacao*) cytoplasmic genomes has been investigated. A total of 177 cocoa clones was surveyed for restriction fragment length polymorphism (RFLP) in chloroplast DNA and in mitochondrial DNA using two restriction endonucleases and various heterologous cytoplasmic probes. A high level of polymorphism was found for the mitochondrial genome. This study points up a structuring of the species that fits with the distinction between the Criollo and Forastero populations. In contrast to all previous analyses, a higher level of polymorphism is found among the Criollo clones while the Forastero clones form quite a homogeneous group.

Key words: *Theobroma cacao* – RFLP – Mitochondrial genome – Chloroplast genome – Diversity study

Introduction

Cocoa trees, *Theobroma cacao*, are native to humid tropical regions of the American continent. They are classified into three different groups depending on geographic location and morphological characters (Cheesman 1944). The three morpho-geographical groups are designated Criollo, Forastero and Trinitario.

According to the geographical origin of the clones, the Forastero group is subdivided into Lower Amazon Forastero and Upper Amazon Forastero. The Lower Amazon Forastero trees were cultivated in the Amazon basin and were the first to be introduced to Africa. The Upper Amazon Forastero are highly diverse and are often used in breeding programs due to their vigor, precocity, and resistance to diseases.

The Criollo group is composed of trees with thick, white or rosy beans that yield the most flavoured and finest chocolate. They were the first cocoa trees to be domesticated. They have been cultivated in Central America for 2000 years, but are at present infrequently grown because of their weakness and their susceptibility to diseases and pests. All the Criollo clones that are currently known are either cultivated or sub-spontaneous forms, no truly wild-type has been identified up to now.

The high morphological variability found among the different populations in the Upper Amazon region has resulted in this region being considered as the center of origin of the species (Cheesman 1944). According to Cuatrecasas (1964), in early times, some trees of *Theobroma cacao* would have spread throughout the central part of the Amazon and northward to the South of Mexico developing in to the Criollo and Forastero populations separated by the Panama isthmus.

The Trinitario group is made up of hybrid forms of the first two groups. This population has apparently arisen spontaneously in Trinidad by hybridizations between the remaining Criollo of a plantation devastated by a natural disaster in 1727 and Forastero introduced in 1757 (Cheesman 1944).

Until now, all breeding strategies rely on crosses between clones of various groups to obtain fine-tasting chocolate from vigorous and productive trees. So there is a general need to improve our understanding of the relationships among cocoa populations. A previous enzymatic study has shown that the variability of the Upper Amazon Forastero embraces the global variability of the species, confirming that the Upper Amazon region could correspond to the first center of diversity (Lanaud 1987). The presence of rare alleles in individual populations

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Table 1. Constitution of the different chloroplastic and mitochondrial types

Cp Mt	Group	Origin	Clones	Cp Mt	Group	Origin	Clones
A 1	T	Ecuador	EQX94	A 16	L	Brazil	ERJOH(1, 2)
A 1	T	Colombia	SPEC54-2	A 18	C	Venezuela	Por(210, 211)
A 1	T	Indonesia	G23, WA40	A 19	L	Ivory Coast	IFC303
A 1	T	Costa Rica	CC39	A 19	U	Ivory Coast	UPA608
A 1	C	Venezuela	Porcelana3, POB	A 20	U	Peru	P32A
A 1	L	Venezuela	VEN(1, 4, 5, 11, 20, 31)	A 24	C	Nigeria	CD8/6
A 1	L	Ivory Coast	IFC(2, 5, 15)	A 30	U	Ecuador	LCT-EEN167
A 1	L	Guiana	GU144	A 31	U	Ecuador	LCT-EEN355
A 1	L	Brazil	ERJOH(1, 11, 15), SIAL(42, 70, 325)	A 32	C	Venezuela	Hernandez212
				A 34	T	Mexico	RIM8
A 1	U	Peru	P(1, 16), NA(32, 79), MO(9, 98), PA120, IMC(5, 78)	A 35	C	Venezuela	Procelana rojo, ZEA206
				A 36	C	Venezuela	PV6
A 1	U	Ecuador	LCT-EEN(37, 326)	A 37	U	Ecuador	LCT-EEN295
A 1	U	Colombia	EBC(5, 6, 10), SPA(5, 17)	A 38	T	Samoa	LAFI-7
A 1	U	Ivory Coast	UPA(413, 603)	A 39	C	Venezuela	POC
A 1	U	Ghana	T79/501	A 40	C	Venezuala	Providencia 201
A 2	T	Costa Rica	UF667	A 41	T	Ecuador	MOQ413
A 2	C	Venezuela	CATA211	A 42	T	Ivory Coast	IFC7
A 2	L	Brazil	ERJOH7	A 43	T	Grenada	GS29
A 3	T	Ghana	IFC304	A 44	C	Venezuela	IS201
A 3	L	Guiana	GU(154, 346, 349)	A 44	U	Peru	IMC31
A 3	L	Ivory Coast	IFC(307, 414)	B 1	T	Ecuador	EQX107
A 3	U	Ghana	T60/887	B 1	C	Costa Rica	LAF3
A 3	U	Peru	PA150	B 1	L	Brazil	ERJOH5
A 3	U	Ivory Coast	UPA(401, 402)	B 1	U	Ecuador	SCA6
A 4	T	Mexico	RIM(15, 19, 113)	B 1	U	Peru	PA13
A 4	T	Ivory Coast	IFC19	B 2	T	Trinidad	ACT2-11, ICS(6, 53, 75, 89, 98)
A 4	C	Costa Rica	LAF1	B 2	T	Costa Rica	UF10
A 4	C	Nicaragua	ICS48	B 2	T	Colombia	SC5, SPEC138-8
A 4	U	Ecuador	LCT-EEN127	B 2	T	Ivory Coast	IFC422
A 5	T	Ivory Coast	IFC(6, 420)	B 2	T	Nigeria	N38
A 5	T	Ecuador	MOQ647, EET59, EQX27	B 2	T	Panama	UF168
A 5	T	Venezuela	CHUAO24	B 2	T	Honduras	TJ1
A 5	T	Indonesia	DR1	B 2	T	Mexico	RIM76
A 5	C	Nicaragua	ICS40	B 2	C	Venezuela	PV2
A 6	T	Trinidad	ICS46	B 2	C	Indonesia	G8
A 6	T	Grenada	GS36	B 2	L	Costa Rica	Matina1-6
A 6	T	Ecuador	MOQ216	B 2	L	Brazil	SIC864, Para, Comun, IFC361
A 6	T	Cameroon	SNK12	B 2	L	Ivory Coast	SF23
A 6	C	Venezuela	OC61	B 3	U	Ecuador	SCA(9, 12)
A 6	C	Trinidad	ICS84	B 7	T	Honduras	MT1
A 6	C	Peru	PA35	B 8	T	Mexico	RIM105
A 6	L	Ivory Coast	IFC4	B 8	T	Venezuela	CNS22
A 7	T	Colombia	SC6	B 8	C	Venezuela	JS(202, 210), MTC201, CUM214
A 7	L	Brazil	ERJOH8				
A 7	U	Peru	P2	B 8	L	Costa Rica	Matina1-9
A 7	U	Ecuador	LCT-EEN325	B 15	C	Trinidad	ICS100
A 9	C	Venezuela	OC(63, 73), ZEA1	B 15	C	Mexico	La Esmida
A 9	C	Ghana	Q7	B 17	C	Venezuela	CUM209, OC77
A 9	U	Ecuador	Nacional	B 21	C	Venezuela	BO204
A 10	T	Ecuador	ECNR	B 22	T	Costa Rica	UF221
A 10	C	Venezuela	Por, CHUAO(49, 211)	B 23	C	Venezuela	JS206
A 11	T	Trinidad	ICS16	B 26	L	Brazil	ERJOH12
A 11	T	Cameroon	SNK109	B 27	C	Venezuela	CHO31
A 11	C	Colombia	SPEC185-4	B 29	T	Venezuela	CHUAO120
A 12	T	Ghana	K5	B 33	C	Costa Rica	LAF2
A 12	C	Nicaragua	ICS(39, 60)	? 4	L	Brazil	ERJOH14
A 13	T	Ivory Coast	IFC413	? 7	U	Ghana	T63/967
A 13	T	Colombia	SPEC160	? 7	U	Colombia	SPA11
A 14	T	Costa Rica	CC10	? 25	C	Venezuela	PV4
A 14	T	Ivory Coast	IFC11	? 29	T	Venezuela	CNS23

For each clone, the cp type (A or B), mt type (1 to 44) classification (T = Trinitario, C = Criollo, L = Lower Amazon Forastero and U = Upper Amazon Forastero) and country of origin or selection are indicated

suggests that different modes of diversification might exist.

The usually uniparental mode of transmission of cytoplasmic genomes and their low rate of point mutations make the study of chloroplastic and mitochondrial genetic variability a powerful tool to investigate inter- and intra-specific relationships. The low rate of structural changes of the chloroplast (cp) genome is particularly suitable for inferring interspecific relationships (Palmer 1987) but intraspecific polymorphism has also been reported for various species such as barley (Holwerda et al. 1986) and *Eucalyptus* (Steane et al. 1991). Although the mitochondrial (mt) genome is subject to multimolecularity and recombinogeneity (Lejeune et al. 1988), the mtDNA shows a noteworthy stability that makes the mt genome useful to study relationships among and between species, such as sugarcane, that show few cp polymorphisms (D'Hont et al. 1993). In the present paper, mt and cp DNA analyses were used to assess cytoplasmic diversity within cocoa germplasm.

Materials and methods

Plant material

A sample of 177 cocoa clones from various areas belonging to the three different morpho-geographical groups (Table 1) was screened for chloroplastic and mitochondrial variability. Both cultivated and wild clones were represented. Dried leaves of the different clones were supplied by IDEFOR-DCC (Ivory Coast), CIRAD-CP (France), CRU (Trinidad), FONAIAP (Venezuela), CEPLAC (Brazil) and CATIE (Costa Rica).

DNA probes

DNA probes were hybridized to total DNA. Mitochondrial gene probes were pooled according to the function of the protein for which they code:
Mitochondrial mix 1: subunit α, subunit 6 and subunit 9 genes of sunflower ATP synthetase (*atp*) (Recipon 1989).
Mitochondrial mix 2: subunit I, subunit II and subunit III genes of wheat cytochrome oxidase (*cox*) (Lejeune, Laboratoire de Biologie Moléculaire Végétale, Paris XI, personal communication).
Mitochondrial mix 3: 26*s* gene (Falconet et al. 1985) and 18*s*+5*s* genes (Falconet et al. 1984) of wheat rRNA.

Chloroplastic probes:
The cytochrome f gene of pea (Willey et al. 1984). The Rubisco large subunit gene of spinach (Mache, Laboratoire de Biologie Moléculaire Végétale, Grenoble I, personal communication).

RFLP procedures

Approximately 0.5 g of dried leaves was ground and incubated for 1 h at 55 °C and then 10 min at 65 °C in 16 ml of extraction buffer (0.16 N sodium citrate, 62 m*M* NaEDTA, 1.2% SDS, 6.2 m*M* mercaptoethanol, 1.25 mg proteinase-K and 1 g PVP). The solution was then incubated at 0 °C for 10 min with 5 ml of 5 *M* potassium acetate. A volume of 9 ml of phenol:chloroform:isoamylalcohol (25:24:1) was added and the subsequent emulsion was centrifuged at 4200 *g* for 1 h. The aqueous phase was removed and mixed with 9 ml of isopropanol. The resulting DNA precipitate was immediately spooled out and resuspended in TE buffer (50 m*M* Tris-HCl pH 8, 10 m*M* NaEDTA). The solution was then incubated with 80 µg of RNAse T1 for 30 min at 37 °C. DNA was re-precipitated at 4 °C for 10 min with an 0.1 vol of 100 m*M* sodium acetate and 2.5 vol of ethanol. DNA was pelleted by centrifugation for 20 min in a microfuge. The DNA pellet was washed with 80% ethanol and resuspended in TE buffer. DNA was then purified by ultracentrifugation in a cesium chloride-ethidium bromide gradient.

Five micrograms of total DNA were digested overnight by 3 UE/µg of the restriction endonucleases *Dra*I and *Eco*RI. Restriction fragments were separated by electrophoresis in an 0.7% agarose gel in TAE buffer (Sambrook et al. 1989) for 16 h at 1.04 V/cm. Fragments were fixed on nylon Hybond N+ membranes in 0.4 N NaOH by Southern blotting. Probes were labeled with ^{32}P by random priming. Prehybridizations and hybridizations were performed at 68 °C overnight in 6 × SSC, 5 × Denhart, 0.5% SDS, 25 µg/ml herring sperm DNA. Blots were washed twice at 68 °C for 30 min in 2 × SSC followed by 30 min in 2 × SSC, 0.1% SDS and finally 30 min in 0.1 × SSC, 0.1% SDS. Autoradiographs were exposed over-night at −80 °C.

Data and phylogenetic analysis

Each polymorphic band was scored for presence and absence. A factorial analysis of correspondences (Benzecri 1973) was performed with these variables.

Results

Hybridization with the cytochrome f chloroplast probe did not reveal any polymorphism among the 177 clones digested by *Dra*I and *Eco*RI in contrast to hybridization with the Rubisco LS chloroplast probe which produced two polymorphic bands on *Dra*I digests (Fig. 1).

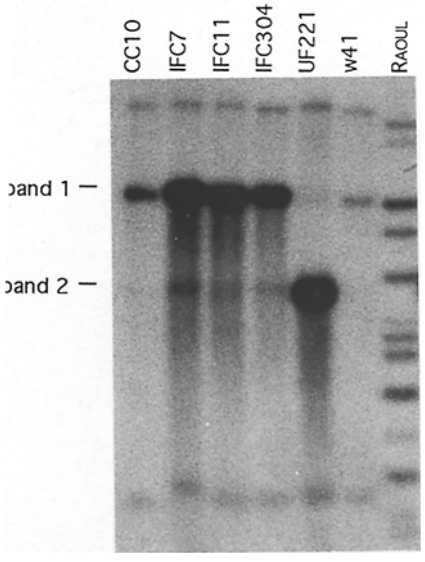

Fig. 1. Example of restriction patterns obtained with the chloroplast Rubisco LS probe for *Dra*I digest of several Trinitario clones. Polymorphic bands are indicated on the left. Each lane contains internal weight markers (i.e., 1.5 kb and 24.8 kb) in order to vizualise migration distorsion. Raoul sizes are 18.5, 14.9, 9, 7.4, 5.6, 4.4, 3.9, 3.5, 2.9, 2.3, 1.8, 1.4, and 1.2 kb

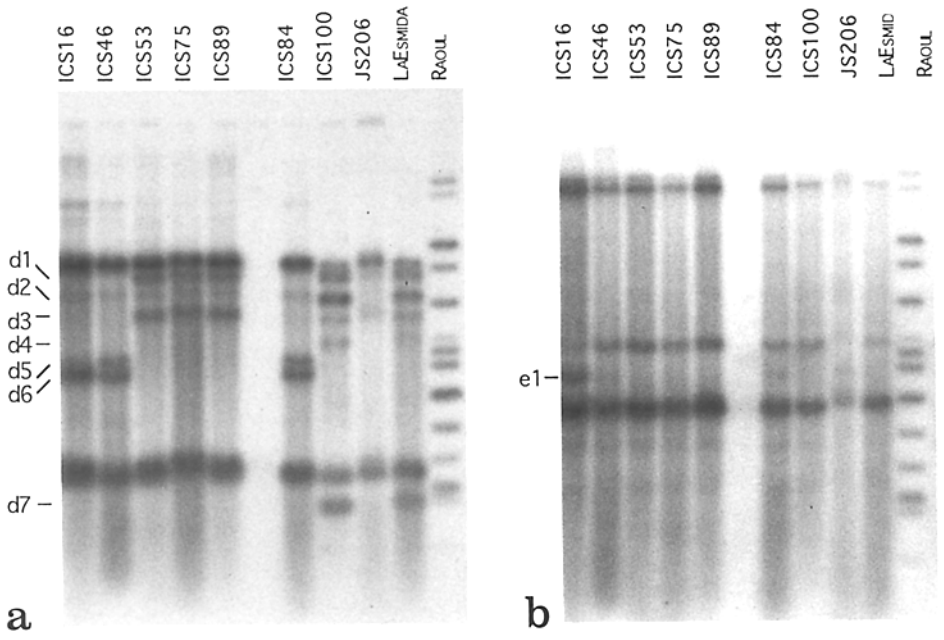

Fig. 2a, b. Example of restriction patterns obtained with the mitochondrial *atp* probes for *Dra*I (**a**) and *Eco*RI (**b**) digests of several Trinitario (*ICS16–ICS89*) and Criollo (*ICS84* to *La Esmida*) clones. Polymorphic bands are indicated on the left. Raoul sizes are 18.5, 14.9, 9, 7.4, 5.6, 4.4, 3.9, 3.5, 2.9, 2.3, 1.8, 1.4, and 1.2 kb

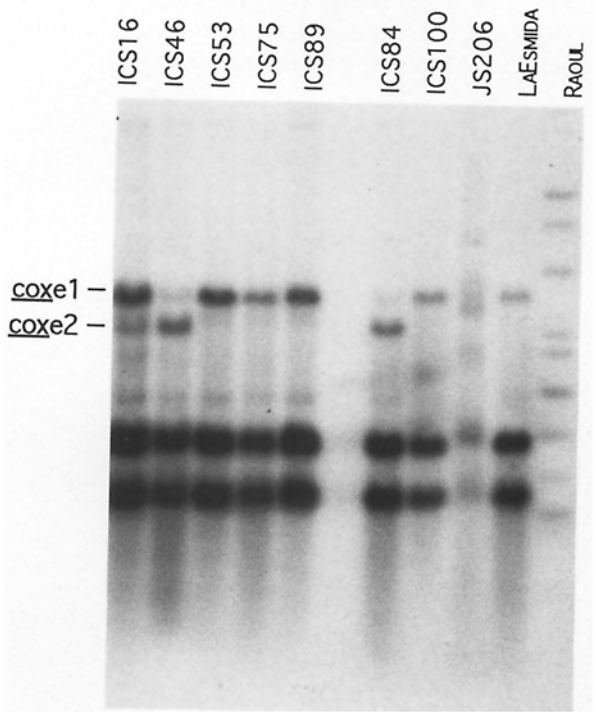

Fig. 3. Example of restriction patterns obtained with the mitochondrial *cox* probes for *Eco*RI digest of several Trinitario (*ICS16–ICS89*) and Criollo (*ICS84* to *La Esmida*) clones. Polymorphic bands are indicated on the left. Raoul sizes are 18.5, 14.9, 9, 7.4, 5.6, 4.4, 3.9, 3.5, 2.9, 2.3, 1.8, 1.4, and 1.2 kb

These two bands did not appear simultaneously for an individual. After a longer time exposure of the blots (Fig. 1) faint bands could be revealed in some individuals. Such faint bands are suspected to reflect an occasional heteroplasmy of some individuals. Due to the faintness of these bands, compared to the main signal, they were not taken into account in this analyis. Seventy per cent of the clones studied are characterized by the presence of band 1 (type A). This type is composed of clones belonging to the three morpho-geographical groups. The remaining clones all exhibit band 2 (type B). They are mainly Trinitario and Criollo clones. A few related Lower Amazon Forastero clones (Comun, Para, Catongo, Matina) and Upper Amazon clones (SCA) are also characterized by type B (Table 1).

Mitochondrial *cox* and *atp* probes revealed some polymorphism in contrast to the mitochondrial rRNA genes which did not reveal any polymorphism among the clones studied.

A total of 11 polymorphic bands was obtained with the mitochondiral probes. *Cox* probes revealed three variable bands on an *Eco*RI digest (Fig. 2A); *atp* probes produced seven variable bands on a *Dra*I digest (Fig. 2B) and one on an *Eco*RI digest (Fig. 3). The combination of the different restriction patterns obtained for the various mitochondrial probes defined 44 different mt types (see Table 1). Thirty-five of them are composed of less than five clones each. These minor types include both Criollo and Trinitario clones. Among the remaining nine mt types, type 1 contains a quarter of the clones which are

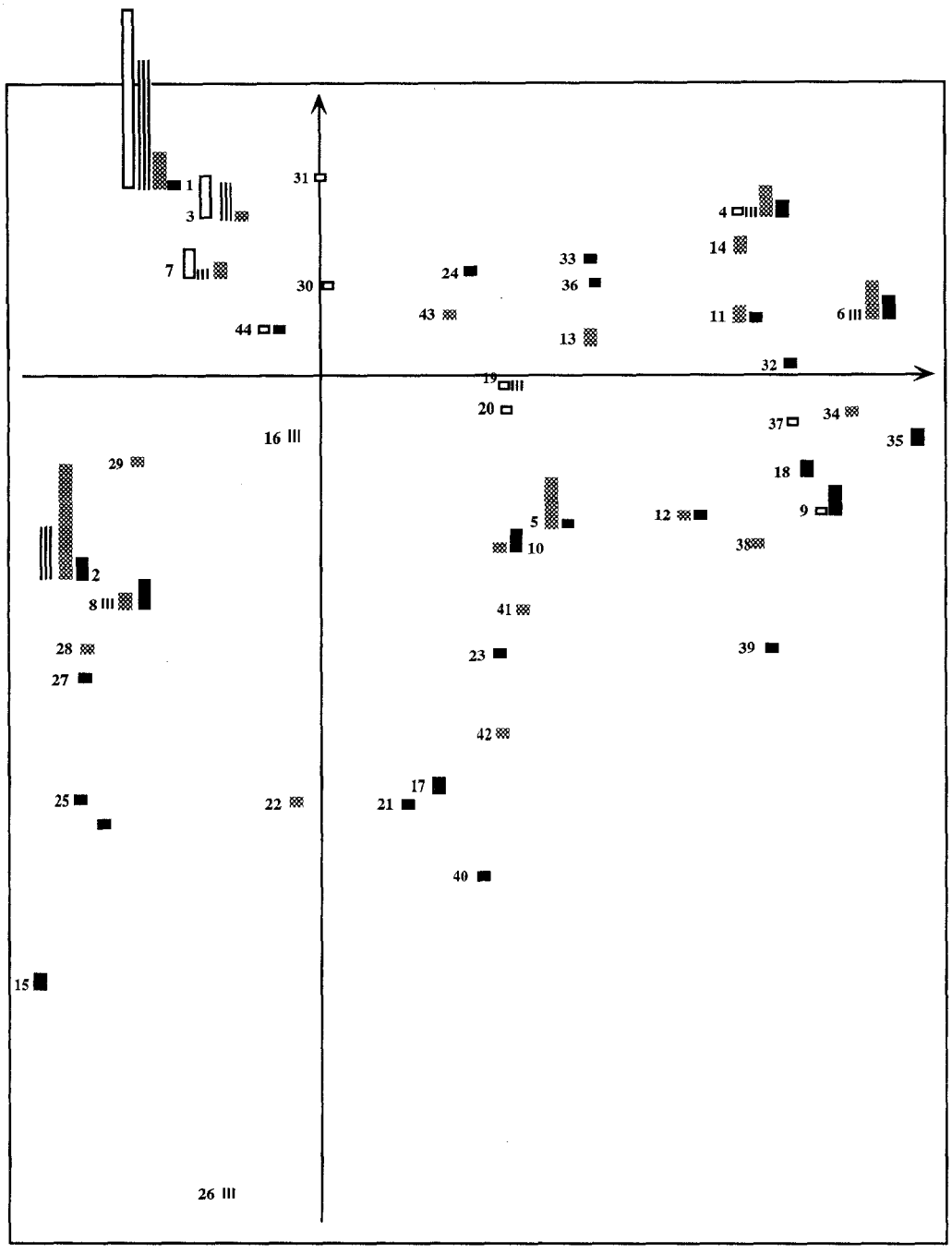

Fig. 4. Distribution of the various mt types on the first FAC plane. The relative distribution of clones of the various morpho-geographical groups (▨ = Trinitario, ■ = Criollo, ▥ = Lower Amazon Forastero and □ = Upper Amazon Forastero) is indicated for each mt type

mainly Forastero and type 2 is composed of 26 clones, mainly Trinitario along with a few Lower Amazon Forastero related to the Para variety.

The first plane of the factorial analysis of correspondences (Fig. 4) is essentially defined by *atp* (e1, d1 and d6) and *cox* (e1 and e2) bands for the first axis and by *atp*d3 and *atp*d5 for the second one. This combination of variables covers 49% of the total variability. The remaining bands contribute to the third and forth axes, they did not radically change the relative distributions of the individuals on corresponding planes 1–3 and 1–4 (data not shown).

On the first plane, most of the Forastero clones are localized in a reduced part of the plane (top left) which is defined by the combined absence of *atp* (e1, d3, d6) and *cox*e2 bands and presence of *atp* (d1, d5) and *cox*e1

bands. Criollo, Trinitario and few Forastero clones are scattered in the rest of the plane.

Discussion

Nuclear, chloroplastic and mitochondrial genomes are known to exchange sequences (Palmer, 1987) and hybridization of cytoplasmic probes on total genomic DNA can induce some cross hybridization between the different genomes.

This study of the mitochondrial and chloroplast cocoa genomes was performed on total DNA to simplify the DNA extraction procedure. Such a procedure is commonly applied and has been proven to give specific hybridization with cytoplasmic genomes in a number of studies (Breiman 1987; Polans et al. 1990). In our analysis, the particular structuring of species obtained with the mitochondrial probes suggests that mt probes have hybridized to the mitochondrial genome rather than to the nuclear one; however, cross hybridizations between organellar genomes cannot be ruled out.

The two chloroplast genes, cytochrome f and Rubisco LS, correspond to a very reduced part of most of the angiosperm cp genome (Palmer 1987) and thus could not account for the general organization of the cp molecule. The corresponding region of the cp genome (i.e., large single copy) has, however, been reported to be highly variable in *Nicotiana* (Kung et al. 1982) and *Oryza* species (Kanno and Hiraï 1992).

Two types, a major one and a minor one, can be detected by the Rubisco probe. The major type (A) includes clones of the three different morpho-geographic groups of very different origin whereas the minor type (B) includes a majority of Trinitario and Criollo clones and only a few Forastero. The presence of a preponderant cp type has already been reported for various species such as *Lupinus texensis* (Banks and Birky 1985) and pea (Teeri et al. 1985). This major type is considered to reflect an ancestral cp DNA molecule from which all the others have been derived by mutations and/or rearrangements. For the cocoa cp genome, the representation of all the morpho-geographical groups in type B and the presence of related clones in both A and B types suggest that similar changes may have occurred independently in Criollo and in Forastero. This hypothesis leads to the assumption that the changes involved in the formation of type B are more likely to have been rearrangements than point mutations. Indeed the rate of parallel mutations at a specific site of the chloroplast genome has been shown to be low in comparison with structural variations (Palmer et al. 1985). Moreover, several identical rearrangements have been reported to occur at specific sites of the chloroplast genome for species such as pea (Teeri et al. 1985) and wheat (Ogihara et al. 1988).

The mitochondrial probes used are interspersed in the mitochondrial genome of *Brassica campestris* (Makaroff and Palmer 1987) although several of them code for similar functions and can be expected to be distributed on a large part of the cocoa mt genome.

The mitochondrial genome of cocoa shows high variability with 44 distinct mt types identified. The structuring of this variability is in general accordance with the distinction between the two morpho-geographical types Criollo and Forastero. Indeed the various mt types identified are nearly specific for either the Criollo or Forastero group. Moreover, this study points up a relative high level of polymorphism of Criollo. Forastero clones are mainly distributed in three closely related major types (1, 3 and 7) with very few other clones. In contrast, Trinitario and Criollo clones are represented by seven major types (2, 4, 5, 6, 8, 9 and 10) and include several minor ones as well.

Such a structuring of the species into groups corresponding to the morpho-geographical Criollo and Forastero types has also been found for nuclear rDNA probes (Laurent et al. 1993). In contrast, isozyme analysis of the species showed that the variability of the Upper Amazon Forastero clones embraces the global variability of the species (Lanaud 1987) and fits with the fact that the Upper Amazon region is considered as the center of origin of the species. However, the lack of distinction of Criollo from Forastero could be the result of the small number of Criollo clones screened. According to the Cheesman hypothesis (1944), the origin of Criollo would have relied on the human selection of some genotypes with white beans in the Upper Amazon region. After man-aided crossing of the Andean Cordillera, the species would have spread again naturally until it reached Central America where it was domesticated by the Mayas and the Aztecs. Both mtDNA and rDNA results (Laurent et al. 1993) show some discrepancy with this hypothesis. The prominent discrimination of the Criollo types those of Forastero at both nuclear (unpublished results) and mitochondrial levels suggests that Criollo and Forastero have diverged independantly on both sides of the Andean barrier rather than the Criollo populations having originated from some Upper Amazon Forastero clones.

The discrepancy between the level of polymorphism among Criollo and Forastero would thus be the result of different selection pressures in the two regions.

Contrary to the nuclear rDNA study, pointing to a hybrid origin of the Trinitario clones (Laurent et al. 1993), the present study of the mt genome groups Trinitario clones together with those of Criollo. This could be a reflection of the maternal mode of inheritance of the mitochondrial cocoa genome (unpublished results). This hypothesis involves the assumption that Criollo clones were used, at least in Trinidad, as the female parent of the

Trinitario clones and that, at present, most of new Trinitario clones are obtained from crosses involving Trinitario as the female parent. Thus the original Criollo mt genomes could have been conserved.

The hypothesis of an independent differentiation of the Criollo and Forastero populations is in general accordance with the structuring of variability of the cytoplasmic genomes. However, at the individual level, some discrepancies appear between the morpho-geographical classification of some clones and their cytoplasmic types. Such a discrepancy is illustrated by the clustering of some Lower Amazon Forastero clones related to the Para variety, Comun, Para, Catongo [IFC361] and Matina (Soria 1970), with the Criollo clones (cp type B, mt type 2). This result is quite surprising considering the pooling of these clones into the Forastero group for the nuclear rRNA genes (Laurent et al. 1993). Moreover, other clones of the Comun variety (SIAL) are, in contrast, clustered with the Forastero type (cp type A, mt type 1). A better knowledge of the origin of these clones would help us to understand what this phenomenon reflects.

In other cases, cytoplasmic types grouped together clones with the same morphological characteristics or rDNA types. Indeed, the clustering of the Ecuadorian Nacional clone, which bears large plumped beans and comes from the western slopes of the Andes, with the Criollo (mt type 9) supports the assumption that the Nacional variety is a Criollo rather than a Forastero type (Enriquez, 1992). It also supports our hypothesis of two distinct populations separated by the Andean barrier. However, many uncertainties remain about the classification of these clones. Indeed, the Nacional clone is characterized by very special nuclear rRNA genes which show no similarity to the Criollo type (Laurent et al. 1993). Another example is found in the clustering of three Criollo clones, LAF3, POB and IS201, with the Forastero clones for both rDNA and mtDNA types (mt type 1 and 44). This could indicate that these clones could be Trinitario rather than Criollo.

Both chloroplast and mitochondrial results suggest that Criollo and Forastero may have diversified independently during evolution of the species. The common ancestral form could be an unknown cocoa type or a related species of *Theobroma cacao*. This hypothesis should be verified by further studies of inter- and intra-specific variability. The determination of the mode of inheritance of the cocoa chloroplast genome, the checking of the specificity of molecular hybridization, and the use of a larger number of probes to study the variability of the chloroplast genome will give a more accurate description of that genome. The presence of an ancestral cp molecule and the corresponding hypothesis of a common ancestry of the two groups, Criollo and Forastero, could then be checked.

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