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Variability for nuclear ribosomal genes within *Theobroma cacao*

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Restriction fragment length of the rRNA genes was studied in *Theobroma cacao* using heterologous rDNA probes. One hundred and ninety-two individuals including both cultivated and wild clones were analysed. *Dra*I and *Eco*RI restriction sites were mapped. Both length heterogeneity and restriction site polymorphism have been found. Fifteen different types of rDNA units have been characterized. As opposed to previous enzymatic studies, the rRNA gene analysis indicates a clear distinction of the three genetic groups Criollo, Forastero and Trinitario within cocoa and points out the hybrid origin of Trinitario.

Keywords: genetic diversity, RFLP, ribosomal RNA genes, *Theobroma cacao.*

Introduction

Cocoa trees, *Theobroma cacao*, which are native to humid tropical regions of the American continent, are classified into three different groups depending on geographic location and morphological characteristics (Cheesman, 1944). The three groups are designated Criollo, Forastero and Trinitario. The Criollo group is composed of trees with thick, white or rosy beans which yield the most flavoured and finest chocolate. They were the first cocoa trees to be domesticated. Criollo have been cultivated in Central America for 2000 years but they are, at present, grown infrequently because of their weakness and their susceptibility to disease. The Forastero group represent more than 80 per cent of the world's production. The Forastero are split into Lower Amazon Forastero and Upper Amazon Forastero but very different populations remain pooled in these two subgroups. The Lower Amazon Forastero were cultivated in the Amazon basin and were the first cocoa trees to be introduced into Africa. The Upper Amazon Forastero, which were collected more recently, are highly diversified and are often used in breeding programmes due to their strength, precocity and resistance to disease. The third group, Trinitario, is made up of hybrid forms of the first two groups. However, the morphological distinction between Criollo and Trinitario is not obvious and many uncertainties exist about the classification of individuals in Criollo or Trinitario groups.

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The high morphological variability found among the different populations in the Upper Amazon has resulted in this region being considered to be the centre of origin of the species (Cheesman, 1944). Similarly, isozyme analyses designated this area as the primary centre of diversity (Lanaud, 1987).

Until now, most crop improvement programmes were based on crosses between Trinitario and Upper Amazon Forastero or between Lower Amazon Forastero and Upper Amazon Forastero to obtain fine tasting chocolate from vigorous and productive trees. Since all breeding strategies are based on crosses between clones of various groups, it is of special importance to understand the structure of the genetic variability of the species for refining the current classification. The first genetic markers used for studying a large number of cocoa genotypes were isozymes (Lanaud, 1987). Molecular markers and particularly RAPD have been recently applied on a smaller number of cocoa genotypes (Wilde *et al.*, 1992; Figueira *et al.*, 1992).

In this paper, we analyse the polymorphism of the ribosomal RNA genes (rDNA) within 192 clones of *T. cacao*. In plants, nuclear genes encoding ribosomal RNA are present in high copy number and are arranged in arrays of tandem repeats located at a few loci in the genome usually associated with the nucleolar organizers. Each unit is composed of an intergenic spacer (IGS) and a region coding for the 18S, 5.8S and 25S rRNAs (Rogers & Bendich, 1987). Coding regions are highly conserved between different species and genera (Appels & Dvorak, 1982) while the IGS shows intra and interspecific variation.

Materials and methods

Plant material

A sample of 192 cocoa clones from various areas belonging to the three different groups (Table 1) was surveyed. Both cultivated and wild clones were represented. Dried leaves of the different clones were obtained from IDEFOR-DCC (Ivory Coast), IRCC (France), CRU (Trinidad), CENIAP (Venezuela), CEPLAC (Brazil) and CATIE (Costa Rica).

The classification proposed in Table 1 takes into account that some clones originated from open pollination and, therefore, that some discrepancy might have occurred between the parental type and the progeny. All hybrid forms involving Forastero and Criollo are considered as Trinitario whatever the number of generations since the initial cross.

DNA probes

Several rDNA probes were used for hybridization. The pTA71 probe corresponds to the entire rRNA gene of wheat (Gerlach & Bedbrook, 1979). Two probes derived from pTA71 were also used. They correspond to a 3.7 kb *Bam*HI fragment located within the coding sequence (pTA71b) and to a 4.4 kb *Eco*RI–*Bam*HI fragment overlapping the intergenic spacer and the edges of the coding region (pTA71e–b) (provided by Quetier, Laboratoire de Biologie Moléculaire Végétale, Université Paris XI). The pRG3 probe covers almost the entire coding region of the 18S rDNA from radish (Grellet *et al.*, 1989).

RFLP procedures

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

Five micrograms of DNA were digested overnight by 3 UE/μg of the restriction endonucleases, *Dra*I and *Eco*RI. DNA from 12 clones was submitted to a double digestion using the same restriction endonucleases. Restriction fragments were separated in 0.7 per cent agarose gel with Tris acetate NaEDTA buffer for 16 h at 1.04 V cm^{-1}. Fragments were Southern blotted onto nylon Hybond N+ membranes in 0.4 N NaOH. Probes were labelled with ^{32}P by random priming. Pre-hybridization and hybridizations were performed at 68°C overnight in 6 × SSC, 5 × Denhardt, 0.5 per cent SDS, 25 μg ml^{-1} herring sperm DNA. Blots were washed at 68°C 30 min in 2 × SSC twice, 30 min in 2 × SSC, 0.1 per cent SDS and 30 min in 0.1 × SSC, 0.1 per cent SDS.

Results

EcoRI and DraI restriction map of rDNA unit

rDNA hybridization patterns obtained after digestion with *Eco*RI and *Dra*I, either individually or as double digestion, were compared for preliminary restriction mapping. All clones were characterized by a 3.6 kb *Eco*RI fragment (Fig. 1a) corresponding to the coding region (Fig. 2). One to three additional fragments varying from 4.7 to 6.4 kb in length hybridize to the pTA71 probe.

Digestion with *Dra*I gives similar results. All individuals were characterized by a 5 kb *Dra*I fragment and one to three variable bands ranging from 3.2 to 5 kb (Fig. 1b). The *Dra*I site in the coding region must be located in the middle of the *Eco*RI fragment since hybridization with the pTA71 probe on a double digested DNA led to two fragments of 1.8 kb instead of one of 3.6 kb (Fig. 3). The 5 kb *Dra*I fragment and the 3.2 kb fragment generated by double digestion hybridize both with probes corresponding to conserved, pTA71b, and variable regions, pTA71e–b (Fig. 3), and thus must overlap the coding region and the intergenic spacer. Furthermore, these fragments also hybridize with the 18S probe, pRG3, (Fig. 3) indicating that the involved coding sequence includes the 18S coding region (Fig. 2).

On the basis of this scheme, the variable bands of each clone were used to identify the different rDNA units. Variations are revealed between as well as within individuals. Indeed, some individuals bear more than one variable band (e.g. Por3, SIAL42 and SF23 on Fig. 1) which define more than one rDNA unit type. All individuals considered, 15 different rDNA repeat units were defined (Fig. 2). Regarding rDNA units 8, 9, 10,

Table 1 Constitution of the different rDNA groups. For each group, clones are grouped according to their presumed classification (C = Criollo, T = Trinitario, L = Lower Amazonian Forastero and U = Upper Amazonian Forastero) and their country of origin or selection. Clones deriving from open pollination are indicated by dl

group A (unit)		
T	Trinidad	ACT2-11, ICS6
	Costa Rica	CC39
	Ecuador	EQX(27, 94, 100), MOQ(413, 663)
	Honduras	dlMT1, TJ1
	Mexico	dlRIM105
	Colombia	SC6, SPEC54-2
	Ivory Coast	IFC(6, 7, 11, 19, 413, 420, 422)
	Ghana	IFC304, W41
	Cameroon	SNK(12, 109)
	Nigeria	N38
	Indonesia	WA40
C	Trinidad	ICS84
	Costa Rica	LAF3
	Venezuela	IS201, dlOC61, POB, PV2
	Mexico	dl La Esmida
L	Brazil	Comun, ERJOH(1, 2, 3, 5, 8, 9, 15) SIAL (70, 325), SIC864, dlPara, IFC361
	Costa Rica	MAT1-6, MAT1-9
	Ivory Coast	IFC(1, 2, 4, 5, 15, 303, 307, 414)
U	Ecuador	SCA12
	Colombia	EBC(5, 6, 10)
	Peru	IMC(5, 31, 67, 78), MO98
	Ivory Coast	UPA(401, 402, 413, 603)
	Ghana	T(60/887, 79/501)
group C (unit 4)		
T	Trinidad	ICS75
	Ecuador	MOQ122
C	Venezuela	BOC210, CATA211, CHO31, CHUAO49, Hernandez212, JS210, MTC201, CUM209 OC(63, 73), Por(210, 211, rojo), dlPV(4, 6), POC, ZEA(1, 206), Pro.201, POR
	Trinidad	ICS100
	Costa Rica	LAF1, LAF2
	Ghana	Q7
	Indonesia	G8
L	Brazil	ERJOH12
	Venezuela	VEN(1, 5, 11)
U	Ecuador	LCT-EEN(325, 326)
group F (units 1 and 8 or 8 only)		
L	Brazil	ERJOH(4, 7)
U	Ecuador	LCT-EEN295
group H (units 1, 9 and 12)		
L	Guyana	GU(144, 154, 349)
group M (units 1 and 15)		
L	Ecuador	Nacional
group O (units 14 and 15)		
U	Ecuador	LCT-EEN(84, 127, 371)
group Q (unit 13)		
U	Ecuador	LCT-EEN67
group B (units 1 and 4)		
T	Trinidad	ICS(46, 53, 89, 95, 98)
	Costa Rica	CC10, UF(10, 221, 667)
	Ecuador	MOQ(216, 647)
	Venezuela	dlCHUAO(24, 120), CNS(22, 23)
	Mexico	dlRIM(8, 15, 19, 76, 113)
	Grenada	GS(29, 36)
	Colombia	SC5, SPEC160-9
	Panama	UF168
	Ghana	K5, ACU85
	Indonesia	DR1, dlG23
	Samoa	LAFI-7
C	Venezuela	BO204, CATA201, CHUAO211, OC77, CUM214, JS(202, 206)
	Nicaragua	ICS(39, 40, 48, 60)
	Colombia	SPEC185-4
L	Brazil	ERJOH(11, 14), SIAL42
	Ecuador	dlECNR, dlEET59
U	Ecuador	LCT-EEN(202, 355)
	Colombia	SPA5
	Ghana	T79/416
group D (units 5 and 3)		
T	Colombia	SPEC138-8
	Ecuador	EQX107
C	Venezuela	dlPor3
	Nigeria	CD8/6
U	Peru	MO81, PA4
group E (units 2 and 6)		
U	Peru	NA32, P(1, 2, 16, 32A)
	Colombia	SPA(11, 17)
	Ghana	T63/967
	Ivory Coast	UPA608
group J (units 10 and 5)		
L	Venezuela	VENC4
U	Peru	MO9, PA120
group K (unit 5)		
L	Venezuela	VEN(15, 20, 31)
group N (units 7 and 2)		
U	Ecuador	SCA(6, 9)
group P (unit 11)		
U	Peru	PA13
group R (units 4 and 5)		
L	Ivory Coast	SF23

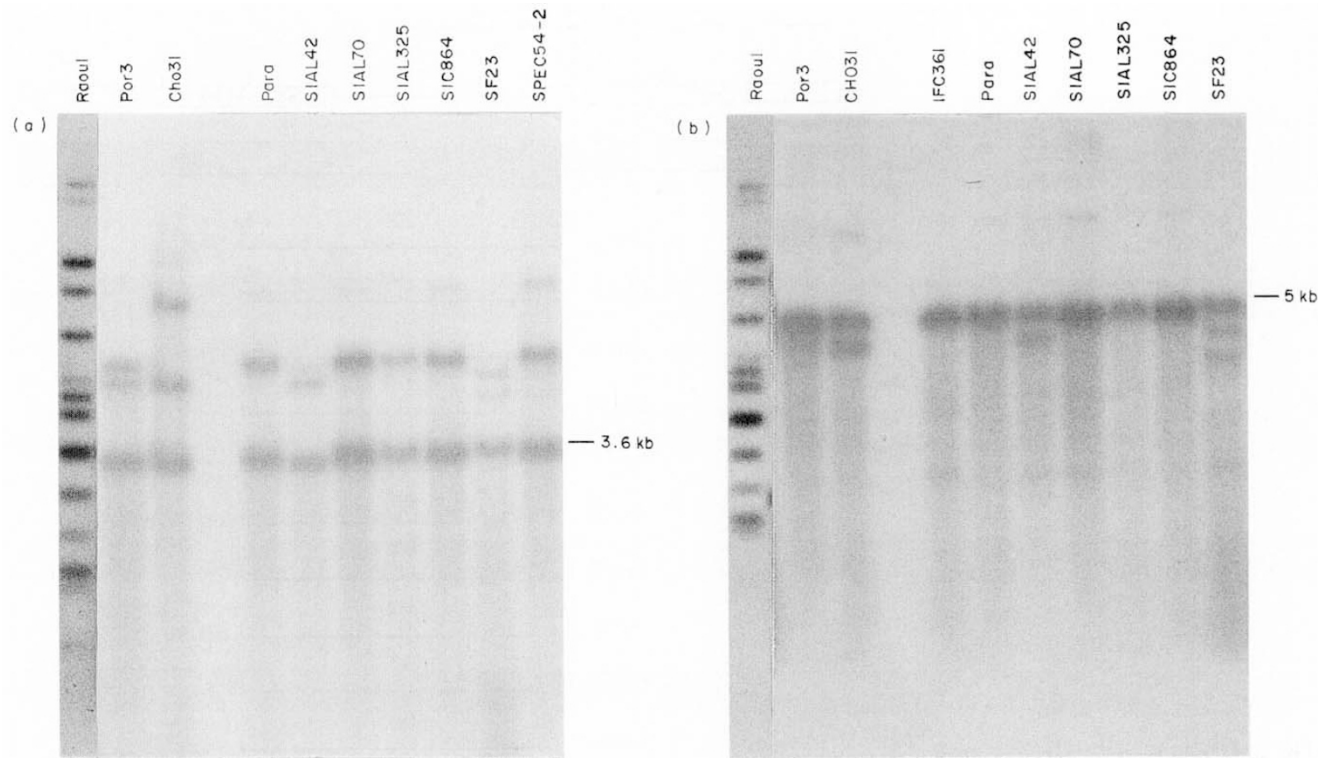

Fig. 1 Example of rDNA polymorphism between different cocoa genotypes revealed by the pTA71 probe on *Eco*RI (a) and *Dra*I (b) digests. The size of the conserved fragments is indicated on the right. The faint bands larger than 5 kb were not taken into account in the interpretation since they are suspected to derive from partial digestion. Raoul sizes are 18.5, 14.9, 9, 7.4, 5.6, 4.4, 3.9, 3.5, 2.9, 2.3, 1.8 and 1.4 kb.

11, 12 and 13, the difference between the total length of the *Dra*I fragments and that of the *Eco*RI fragments could be due to the presence of an additional *Dra*I site (D) in the intergenic spacer. The non-hybridization of the small *Dra*I fragment of the intergenic spacer with the wheat and radish probes is probably a consequence of the incomplete homology of this region between species. For rDNA units 14 and 15, the total length of the *Eco*RI fragments is lower than the total length of the *Dra*I fragments. This could indicate the presence of an additional *Eco*RI site in the 18S coding region at 200 bp upstream from the E1 site in rDNA unit 14, whereas in rDNA unit 15, an additional *Eco*RI site could be located in the 25S coding region 300 bp downstream from the E2 site (Fig. 2). This hypothesis is based on the assumption that the fragments of 200 bp and 300 bp are not seen on hybridization because of their small size.

Variability of the rDNA within cocoa species

Among the 192 clones tested, the clones sharing identical rDNA units were grouped. Fifteen different groups were identified (Table 1) which are most of the time defined by one or two rDNA units. Only one out of the 15 groups is characterized by three rDNA unit types which correspond to the presence of three variable bands in the individuals of group H.

Eighty per cent of the clones studied fall into three major groups (A, B and C), the other 12 groups consist of one to nine clones each. Group A is mainly composed of Forastero and African Trinitario. Group B contains mostly American Trinitario along with few Criollo whereas group C is made up mainly by Criollo. These three groups are based on only two different rDNA units. Group A and C are characterized by rDNA unit 1 and 4 respectively, whereas group B is defined by both rDNA units 1 and 4.

The other 12 groups contain mainly Forastero clones. They are defined by the 15 different rDNA units. Eight very uncommon rDNA units (8 to 15), bearing additional restriction sites, allow seven groups (F, H, J, M, O, P and Q) to be clearly identified. These groups are essentially composed of Upper Amazon clones, with only few clones from Lower Amazon (VEN, GUI).

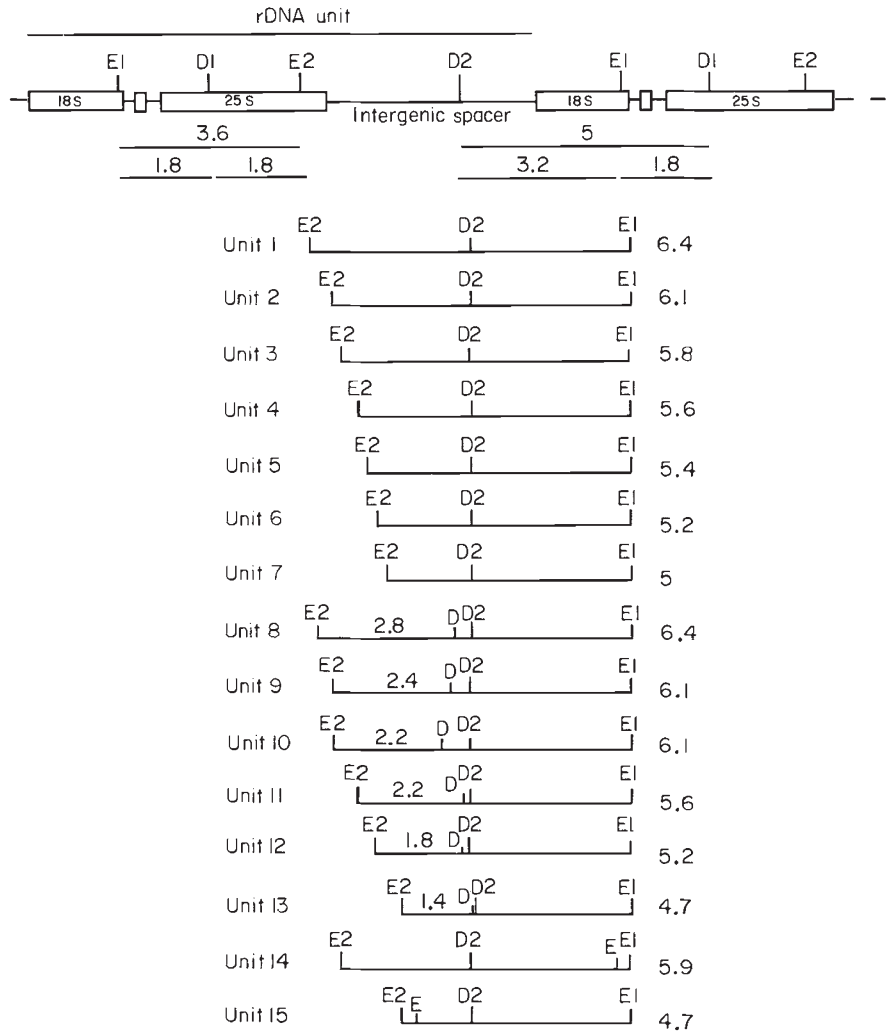

Fig. 2 Diversity of the rDNA repeat units within *Theobroma cacao* species. The general organization of rRNA genes is described with the position of the *Eco*RI (E1, E2) and *Dra*I (D1, D2) conserved restriction sites and the length in kb of the conserved fragments. For each unit, the length of the fragment E1E2 (kb) is reported with the position of an additional site.

Discussion

The results presented in this paper yield original information on the general organization of nuclear rRNA genes of cocoa and on the variability assessment that can be drawn from it.

The general organization of cocoa rRNA genes, based on a number of observations, is very consistent with that of other species. The length of the entire cocoa rDNA unit ranges from 8.3 to 10 kb which is within the size range of a number of other plant rDNA repeat units (Hemleben *et al.*, 1988). The size of the coding region (6 kb) and organization of the 18S, 5.8S and 25S genes were inferred from other species since the size and the organization of the coding region are very commonly found (Delseny, personal communication). Indeed, the two conserved *Eco*RI sites (E1 and E2) of the cocoa rDNA coding region have been reported for several plant species, including pea (Jorgensen *et al.*, 1987), carrot (Taira *et al.*, 1988), radish (Delcasso-Tremoussaygue *et al.*, 1988), several crucifers (Delseny *et al.*, 1990) and rubber tree (Besse, personal communication). Although *Dra*I has been reported to cut only once in the intergenic spacer of some plants (Delseny, personal communication; Levesque *et al.*, 1990), the combined presence of a conserved *Dra*I fragment of 4.9 kb with additional variable bands shown in onion (Harvey, 1991) is similar to the 5 kb conserved fragment and one to three variable bands of cocoa.

Most of the clones show only one or two types of rDNA units indicating that the rRNA genes are most likely located at only one locus. The presence of only one rDNA unit per individual would reflect homozygosity at the rDNA locus whereas the presence of two rDNA units would reflect heterozygosity. This hypothesis is supported by the observation of only one nucleolus per cocoa cell that could reflect the existence of a unique nucleolar organizer in cocoa (Glicenstein & Fritz, 1989). The presence of three different rDNA

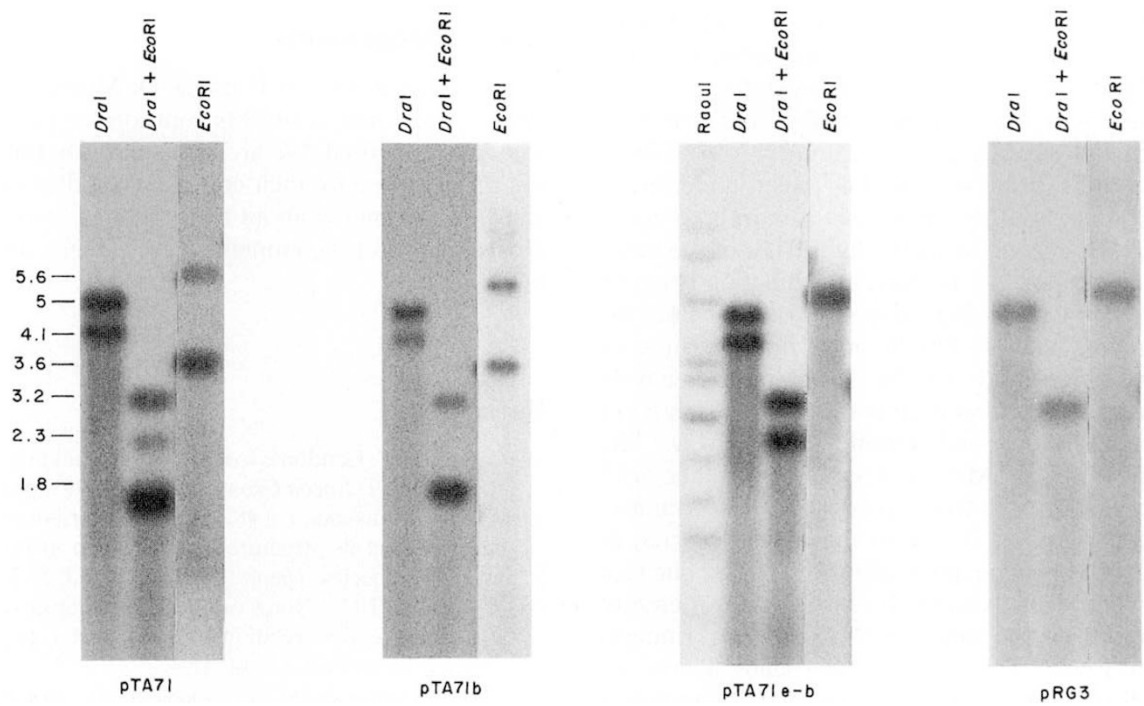

Fig. 3 Hybridization patterns obtained for a clone (CHO31) of group C with the four different heterologous probes. The sizes of the hybridized fragments (kb) are indicated on the left. Raoul sizes are 9, 7.4, 5.6, 3.9, 3.5, 2.9, 2.3, 1.8 and 1.4 kb.

units in clones of group H could therefore be explained by the fact that one of the rDNA gene clusters is in a homogenization process. Indeed two of the three bands give a weak hybridization signal and define two rDNA units (9 and 12) that share an additional *Dra*I site. The rDNA 9 and 12 would thus derive from the same chromosome, whereas the rDNA unit 1 would be located on its homologue.

Variability was found both in the length of the intergenic spacer and in the presence of restriction enzyme sites in the coding region and in the intergenic spacer. Variable restriction sites and spacer length variability are commonly found in the intergenic spacer (Zimmer *et al.*, 1988; Glaszmann *et al.*, 1990) while polymorphism of restriction site in the coding region is more unusual although it has been reported for several crucifers (Delseny *et al.*, 1990). Such an extended polymorphism for both restriction site and spacer length has however been reported for cucumber (Kavanagh & Timmis, 1986) and pea (Jorgensen *et al.*, 1987).

The rDNA gene analysis indicates similarities with the organization of the cocoa species based on morphological characters, isozyme markers and geographical origins.

rRNA genes, as well as morphological characters (Pound, 1938; Allen, 1987), and isozymic markers (Lanaud, 1987), indicate the presence of the greatest variability among clones originated from the Upper Amazon region, supporting the hypothesis that this region is the primary centre of diversity. Lower Amazon Forastero clones constitute a rather homogeneous group with rDNA units bearing no additional restriction site. However, some wild Guyanese clones (GU) have rDNA units with an additional *Dra*I site, as seen in some Upper Amazon clones. Similarly, their isozymic patterns are closer to the LCT-EEN Upper Amazon ones than to the isozymic patterns of other Lower Amazons (Lanaud, 1987). These clones could arise from a diversification scheme different from the one that gave rise to the majority of the Lower Amazon clones.

In some other cases, rDNA genotypes grouped together clones with the same morphological characteristics. Such a correlation is illustrated by the clustering of some Forastero clones (LCT-EEN325, 326 and VEN1, 5 and 11) which have Criollo-like large, white, rosy or light purple beans (Allen, 1987; Lanaud *et al.*, 1986), with the Criollo group (group C). Another example is found in the classification of some clones derived from the Nacional variety (Lower Amazon Forastero). The clones ECNR and EET59 (group B) are classified as Nacional genotypes although they are suspected to derive from a hybridization with a Venezuelan Trinitario type (Bartley, personal communication). The rRNA gene analysis also suggests their close relationship to American Trinitario.

rDNA restriction patterns are often highly homogeneous within a population and, considering the relatively rapid rate of concerted evolution of the ribosomal gene sequences, exhibit characteristic heterogeneity between groups (Zimmer *et al.*, 1988). Indeed, results presented in this paper underline a strong structuring of the rRNA genes into three major groups (A, B, C), one being the hybrid (B) of the other two (A, C) supporting the current distinction between Forastero and Criollo. Indeed in spite of difficulties for discriminating between Criollo and Trinitario, most of the clones reported to be Trinitario effectively appeared as hybrids between two homogeneous types made of Forastero and Criollo, respectively. The grouping of some African Trinitario together with Forastero could result from crosses between Trinitario and Lower Amazon Forastero that have occurred at the early time of cocoa introduction to Africa. The lack of rDNA unit 4 in African Trinitario might therefore be the result of the dilution of American Trinitario genes into Lower Amazon Forastero genes. Moreover, almost all the Criollo are characterized by the unit 4 and only few Criollo bear only the rDNA unit 1. As little is known about the origin of Criollo clones, these clones may have been considered as Criollo because of their morphological characteristics, but could in fact result from some ancient crossing with Forastero followed by fixation of morphological Criollo characters and Forastero rDNA units. It can be therefore concluded that the presence of a rDNA unit 4 is likely to be characteristic of Criollo types which makes rRNA gene analysis a powerful tool for discriminating Forastero and Criollo populations.

No relation with geographical conditions can be demonstrated as was done for rice (Sano & Sano, 1990) and for *Triticum dicoccoides* (Flavell *et al.*, 1986). Indeed, several clones from Costa Rica (MAT), which are supposed to originate from an introduction of the Brazilian Comun type (Soria, 1970), are grouped together with the Comun clone showing that no differentiation due to geographical changes has occurred.

The complexity of the evolution of rRNA genes leads to a very particular representation of the variability of the species. Considering all plant species, relations can be shown with ecological, morphological or isozyme characters either individually or jointly. It is, therefore, necessary to gather information from different kinds of markers (morphological characters, isozymic markers, RFLP, RAPD) to obtain a global view of species diversity.

The present results highlight the importance of RFLP markers to assess genetic variability in cocoa. Further studies using nuclear and cytoplasmic probes will be carried out to yield a more accurate description.

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