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Mitochondrial DNA polymorphism and phylogenetic relationships in *Hevea brasiliensis*

Hong Luo [1], Benoît Van Coppenolle [1], Marc Seguin [2] and Marc Boutry [1,*]
[1] *Unité de Biochimie Physiologique, Université Catholique de Louvain, Place Croix du Sud 2–20, B-1348 Louvain-la-Neuve, Belgium (* author for correspondence; fax: 32 10 473872);* [2] *BIOTROP, CIRAD (Perennial Crops Department), B.P. 5035, F-34032 Montpellier, France*

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Abstract

Using fourteen random mitochondrial DNA probes, we have examined restriction fragment length polymorphism (RFLP) in wild and cultivated *Hevea brasiliensis*. A total of 395 accessions, including 345 from various prospectings collected in Brazil, Colombia and Peru and 50 cultivated clones, were analyzed. Two other species (*H. benthamiana* and *H. pauciflora*) were also included in the study for comparison. The high level of mitochondrial polymorphism allowed us to divide all the accessions analyzed into 212 distinct genotypes. The genetic variability of cultivated clones was limited to four genotypes forming two clusters. In contrast, considerable genetic variation was found in the wild collections. In almost all cases, accessions displaying the same RFLP profile were restricted to the same geographical area (same or neighbor administrative districts). In addition, accessions whose genetic closeness was predicted by RFLP profiles were also clustered according to geographical origin. In a few cases, however, similar RFLP profiles were found for accessions originating from geographically distant districts. This discrepancy can be explained either by seed dispersion (by river) or possibly by similar genetic events occurring independently in different geographical locations. Chloroplast DNA RFLP was also analyzed in 217 accessions, representative of 126 distinct mitochondrial genotypes. Very few differences were found, indicating that the chloroplast genome is more highly conserved than the mitochondrial genome.

Introduction

The genus *Hevea* is composed of nine species and occupies the Amazonian basin and parts of the adjacent uplands [40]. Because of its economic interest, the only *Hevea* species cultivated for rubber production, *Hevea brasiliensis*, was introduced at the end of the nineteenth century into the Far East and, more recently, into Africa. However, the genetic basis of cultivated rubber trees is very limited. All the Eastern clones originated from only a few seeds collected by Wickham (1876) near the Tapajoz river in Brazil [1, 31, 40]. Although the development of this small genetic source has led to about a ten-fold improvement in yield, the agronomic advance gained in the

early breeding phases has reached a plateau [37]. In order to broaden this narrow genetic basis to improve cultivated varieties, several prospectings were carried out in the Amazonian forests. The first, led by the botanist Schultes in Colombia (1940), established the first collection. In 1974, a French-Brazilian expedition created another seed collection, including about forty clones from the Brazilian states of Acre and Rondônia and some twenty from the Peruvian forest. In 1981, a large prospecting organized by the International Rubber Research and Development Board (IRRDB) in Brazil (states of Acre, Rondônia and Mato Grosso) produced a collection of thousands of seeds. The large number of genotypes collected makes their direct introduction into breeding programs difficult and thus necessitates a preliminary classification. Studies on agronomic variability and leaf morphology indicated differences between the 1981 germplasm and the original Wickham clones (cited in [6]). Isozyme polymorphism analysis distinguished four groups, the Wickham collection, the Schultes collection and, from the last prospecting, two groups, one from the states of Acre and Rondônia and the other from the state of Mato Grosso [6]. However, this analysis detected very few of the potential variants because of the limited number of loci which can be identified by this approach. The development of genomic DNA probes, which can reveal restriction fragment length polymorphism (RFLP), has made it possible to detect almost all existing DNA differences among various genotypes. RFLP analysis of nuclear DNA has been successfully applied to different plant genera and species to assess genetic variability [e.g. 2, 11, 12, 14, 15, 16, 35, 36, 38] and to unravel phylogenetic relationships [9, 10, 13, 21, 22, 39]. This method has also been applied to *Hevea*, resulting in the division of the wild accessions from the Amazonian forests into three genetic groups according to geographical origins [4]. The objective of the present study was to use a complementary approach to evaluate the genetic variability of *Hevea* genotypes by RFLP analyses of the mitochondrial and chloroplast genomes. Because of the uniparental inheritance, the mitochondrial and chloroplast RFLPs can reflect evolution occurring within a species without the inconvenience of the mixing and recombination of the two parental genomes that occur in the nuclear genome.

Materials and methods

Plant materials

Mitochondrial RFLP analysis was performed on a total of 395 *H. brasiliensis* accessions, including 345 from different prospectings collected in Brazil (states of Acre, Rondônia and Mato Grosso), Colombia and Peru, and 50 cultivated clones derived from the Wickham collection. In addition, two other species (*H. benthamiana* (F 4506) and *H. pauciflora* (P 9)) were included in the study for comparison. These various genotypes, provided by CIRAD (France) and IDEFDR (Ivory Coast), are listed in Table 1 with their state and district origin. Figure 1 illustrates the geographical localisation of the districts.

The seeds of broad bean (*Vicia faba* L. cv. Exelle), used to prepare chloroplast DNA (cpDNA), came from the Station d'Amélioration des Plantes (Gembloux, Belguim).

DNA extraction

Total DNA was extracted by a modification of the method of Rogers and Bendich [27]. Samples (0.15 g) of dried leaves were homogenized in liquid nitrogen. The fine powder produced was mixed with 1 × CTAB buffer (1% cetyltrimethylammonium bromide, 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.7 M NaCl, 0.5% polyvinylpyrrolidone) and incubated for 5 min at 65 °C. Cell wall debris, denatured proteins and most polysaccharides were removed by a chloroform/isoamyl alcohol (24:1) extraction. After addition of a 1/10 volume of 10% CTAB plus 0.7 M NaCl and incubation for 5 min at 65 °C, proteins were extracted by chloroform/isoamyl alcohol (24:1) and removed by centrifugation. A CTAB-nucleic acid precipitate was then obtained by the addition of

Table 1. Hevea accessions used for the mtDNA polymorphism investigation.

Geographical origin [1]	mt genotype code [2]	Number of accessions
AC/B	AB 1–14	17
AC/F	AF 1–13	17
AC/S	AS 1–27	30
AC/T	AT 1–12	17
AC/X	AX 1–16	17
AC/B,S	ABS 1–2	5
AC/B,X	ABX1	4
AC/S,X	ASX1	2
AC	A 1–3	17
RO/C	RC 1–7	11
RO/CM	RM 1–12	16
RO/J	RJ 1–7	9
RO/JP	RJP 1–4	5
RO/OP	ROP1	2
RO/A	RA 1–3	5
RO/PB	RPB1	1
RO	R 1–5	14
MT/A,C; RO/PB	MR 1–2	10
MT/VB; RO/CM	MVRC	7
MT/A	MA 1–3	3
MT/C	MC 1–18	20
MT/IT	MI 1–6	8
MT/VB	1	1
MT/C,IT	MCI 1–2	10
Peru	MDF 1–4	10
IAN [3]	IAN	2
Peru; AC	MDFA	3
IAN [3]; GU [3]	IAGU	6
GU [3]	GU	1
SCH	S 1–38	83
F 4506	F	1
P9	P	1
GT1 [3]	GT1	1
GU [3], IAN [3], IRCA [3], IR [3] Pb [3], PR [3], RRIC [3], RRIM [3], AF [3], AVROS [3], NAB [3]	W	41

[1] AC, state of Acre, including the following districts: T, Tarauaca; F, Feijo; S, Sena Madureira; B, Brasiléia; X, Xapuri; RO, state of Rondônia, including the following districts: C, Calama; A, Ariquemes; J, Jaru; OP, Ouro Preto; JP, Jiparana; CM, Costa Marques; PB, Pimenta Bueno; MT, state of Mato Grosso, including the following districts: C, Juruena; IT, Itauba; A, Aracutaba; VB, Vila Bela; MDF, Peruvian clones (1974); GU, IAN, IRCA, IR, Pb, PR, RRIC, RRIM, AF, AVROS, NAB, GT1, Wickham clones or/and their derivatives; SCH, prospection of Schultes in Colombia (1940); F4506, clone *H. benthamiana*; P9, clone *H. pauciflora*.
[2] Obtained from the assessment of mtDNA RFLP profile.
[3] Cultivars derived from the Wickham clones.

an equal volume of 1% CTAB, 50 mM Tris-HCl pH 8.0, 10 mM EDTA. After solubilization in 10 mM Tris-HCl pH 7.5, 1 mM EDTA, total DNA was precipitated by ethanol before Southern hybridization of mitochondrial DNA (mtDNA).

cpDNA from broad beans was prepared as follows. Two-week-old light-grown leaves (about 300 g) were homogenized (4 °C) in a blender for 7 s in homogenization buffer (5:1, v/w), consisting of 0.35 M sorbitol, 2 mM EDTA, 50 mM Hepes (pH 7.5, KOH), 2 mM $MgCl_2$ and 5 mM sodium ascorbate. The homogenate was filtered through two layers of Miracloth (Calbiochem) and centrifuged for 10 s at 5000 rpm in a Beckman JS13 rotor. The pellet from each 250 ml of homogenate was resuspended in 5 ml of homogenization buffer and loaded on top of a discontinuous gradient, composed of 15 ml of 40% Percoll and 15 ml of 90% Percoll both containing 3% (w/v) polyethyleneglycol 400, 1% (w/v) BSA, 1% (w/v) Ficoll 400, 2 mM EDTA pH 8.0, 1.2 mM $MgCl_2$, 0.07 mM Hepes pH 8.0, 0.34 M sorbitol, 5 mM sodium ascorbate and 0.65 mM glutathione (reduced type). After centrifugation for 12 min at 7000 rpm in a Beckman JS13 rotor, the intact chloroplast fraction was collected from the interface of the two Percoll layers, diluted with 4 volumes of homogenization buffer, and centrifuged for 5 min at 6000 rpm in a Beckman JS13 rotor. The chloroplasts were then lyzed by treatment with sarkosyl and proteinase K, and nucleic acids were purified by isopycnic (cesium chloride) centrifugation, as previously described [5], except that the refractive index for the DNA-cesium chloride solution was adjusted to 1.3883.

RFLP probes

DNA samples from fourteen randomly chosen clones (Table 2) from a broad bean mtDNA library (*Hin*d III fragments cloned in an *E. coli* plasmid, [30]) were pooled as five mixtures (Table 3) and radioactively labelled with ^{32}P α-dCTP (Amersham) using nick translation or random priming kits from BRL and Amersham,

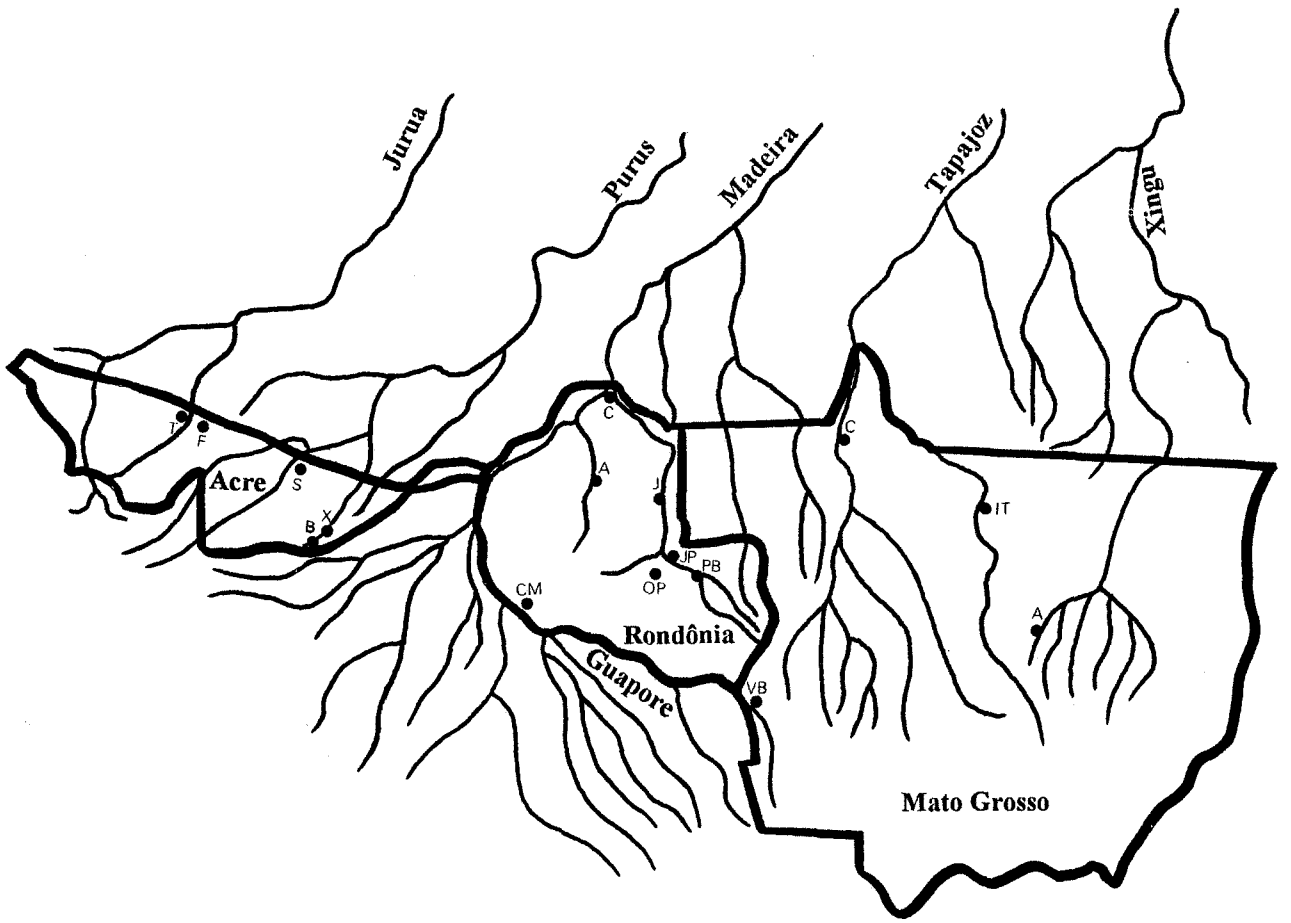

Fig. 1. Geographical localization of the various states and districts prospected. The name of each state and district is abbreviated as described in Table 1.

respectively. Some probes were included into two different pools but, in that case, different restriction enzymes were used. About 250 bp were sequenced at both insert ends of the fourteen clones. Six of the clones contained genes identified by sequence comparison with data bases (see Table 2).

Southern hybridization

Total *Hevea* DNA (5 μg) was digested with *Eco* RI or *Hind* III, following the supplier's instructions (BRL). Fragments were size-separated through a 0.9% agarose gel and blotted onto a Hybond-N$^+$ (Amersham) membrane. Hybridization was performed for 20 h at 42 °C in a buffer containing 6 × SSC (1 × SSC is 0.15 M sodium chloride, 0.015 M sodium citrate pH 7.0 (NaOH)), 50% formamide, 1% sodium dodecylsulfate (SDS), 2 × Denhardt's solution (1 × Denhardt's solution is 0.2 mg/ml bovine serum albumin, 0.2 mg/ml Ficoll (M 400000), 0.2 mg/ml polyvinylpyrrolidone), 0.02% denatured herring sperm DNA and 10^6 cpm/ml of DNA probes. The membranes were washed twice for 5 min in 2 × SSC and 0.1% SDS at room temperature, once for 15 min at 50 °C in the same medium, and twice for 15 min at 50 °C in 1 × SSC and 0.1% SDS. The blots were then exposed for 72 h at −70 °C to Kodak XAR X-ray films, using Du Pont Lightning Plus intensifying screens. Membranes were rehybridized after stripping with 0.4 M NaOH for 30 min at 45 °C followed by a

Table 2. mtDNA clones of broad bean used for RFLP analysis of *Hevea*.

Clone	Insert length (bp)[1]	Encoded mitochondrial gene[2]
25	1980	*nad4* (NADH dehydrogenase subunit 4)
48	3600	–
61	1920	26S rRNA (26S ribosomal RNA)
69	3250	*nad5* (exons 1 and 2 of NADH dehydrogenase subunit 5)
106	2250	–
110	6500	*nad5* (exons 4 and 5 of NADH dehydrogenase subunit 5)
113	1560	–
120	4300	–
121	2400	–
125	1610	*trnH* (histidine transfer RNA)
129	1700	–
147	1900	–
153	3500	*nad1* (NADH dehydrogenase subunit 1)
161	1600	–

[1] Based on electrophoretic mobility.
[2] Identified as explained in Materials and methods.

wash for 30 min at 45 °C in 0.1 × SSC, 0.1% SDS, 0.2 M Tris-HCl pH 7.5.

Data analysis

The mtDNA types were determined by analyzing the mtDNA RFLP data as follows. Each fragment detected by Southern analysis was treated as a unit character. All the RFLP profiles revealed by different probe/enzyme combinations were processed in such a way that the presence or absence of each hybridization band was designated 1 or 0, respectively, to produce a binary matrix. The common fragments among various genotypes were assumed to be homologous. The genetic similarity for a given pair of mtDNA types was represented by the proportion of common fragments and was measured using the formula $F = 2N_{xy}/N_x + N_y$, where N_x and N_y are the number of fragments identified in the x and y mtDNA types, respectively, and N_{xy} the number of shared fragments [23]. From this value, the genetic distance, an estimate of nucleotide base change per base position between different pair of mtDNA types, was obtained, using the method of Nei [24]. The phylogenetic trees based on genetic distances were constructed by the unweighted pair-group method with the arithmetic average (UPGMA, [34]) and the Neighbor-joining method [28, 29] using PHYLIP (Phylogenetic Inference Package)

Table 3. Different mixtures of mtDNA clones of broad bean used for RFLP analysis of *Hevea*.

Mixture	Clone	Restriction endonuclease
M1	25	*Eco* RI
	61	
	113	
	125	
M2	120	*Eco* RI
	129	
	147	
M3	48	*Eco* RI
	69	or
	110	*Hind* III
	153	
M4	25	*Hind* III
	113	
	129	
	147	
M5	61	*Hind* III
	106	
	120	
	121	
	125	
	161	

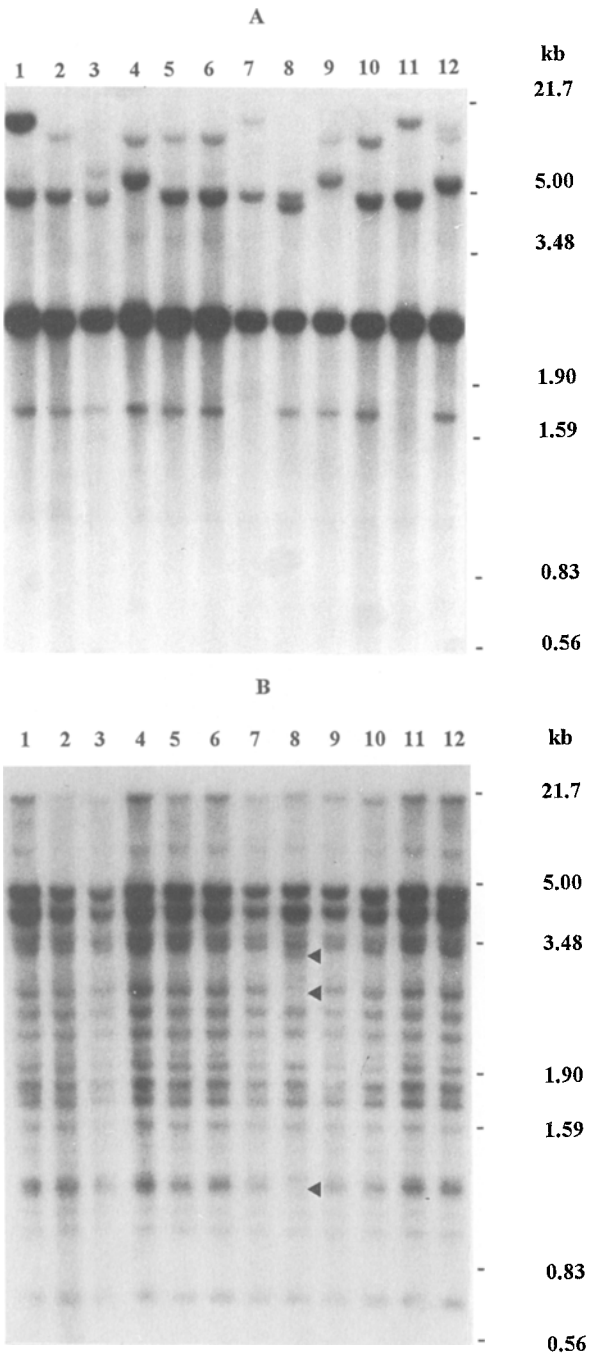

Fig. 2. Examples of mtDNA and cpDNA RFLPs of *Hevea*. Total DNAs of 12 genotypes (1, MT.C.05.11; 2, RO.CM.10.89; 3, RO.C.08.46; 4, RO.J.05.08; 5, RO.CM.12.19; 6, RO.CM.10.35; 7, RO.JP.03.26; 8, RO.C.09.34; 9, RO.J.05.04; 10, RO.C.09.04; 11, RO.JP.03.26; 12, RO.J.06.08) were digested with *Eco* RI, electrophoresed, transferred to a nylon membrane and probed with mixture 2 (3 mtDNA fragments, Table 3) (A) or total broad bean cpDNA (B). The molecular size in kilobase pairs is shown on the right. In (B), the arrowheads indicate the bands specific to or absent from the genotype displayed in lane 8.

software (Felsenstein, Dept. of Genetics, University of Washington, Seattle, WA, version 3.4, 1991). Factorial analysis of correspondences (FAC, [3]) based on the 1-0 binary matrix was performed using the ANCORR program from ADDAD (Association pour le Développement et la Diffusion de l'Analyse des Données, Paris, 1983). All the programs were run on a Sun Sparc 10/30 station under SunOS release 4.1.3.

Results

mtDNA polymorphism

To detect mtDNA polymorphism in dried *Hevea* samples available in small quantities, we chose to hybridize the total DNA prepared from these samples with mtDNA fragments of broad bean cloned in a bacterial plasmid. Among a variety of randomly chosen clones, 14 were selected for their strong hybridization signals with *Hevea* DNA, indicating a high sequence conservation between these two species. These RFLP probes represent a total length of 38 kb (Table 2). Partial sequencing of the inserts (results not shown) indicated that at least six bear known mitochondrial genes (Table 2).

The mtDNA RFLPs of 397 wild and cultivated accessions were analyzed using these probes and two different restriction endonucleases, *Eco* RI and *Hin*d III (25 probe/enzyme combinations). A total of 183 mitochondrial restriction fragments were detected (see Fig. 2A for some examples). Forty (22%) of them were common to all genotypes and thus yielded no phylogenetic information. However, 38 (21%) fragments were unique to a single genotype and 105 (57%) fragments were shared by two or more genotypes. On average, 7.3 unique fragments could be detected for one probe/enzyme combination. This high level of mitochondrial DNA polymorphism allowed us to divide the analyzed clones into 212 distinct

genotypes (Table 1), which were named according to the state and the district of origin.

To evaluate the genetic diversity of the chloroplast genome, we also examined the cpDNA RFLP of a large number of accessions. We therefore probed total DNA from 217 accessions, representing 126 distinct mitochondrial genotypes, with cpDNA purified from broad bean. The RFLP profiles are shown in Fig. 2B for the 12 genotypes whose mtDNA RFLP are analyzed in Fig. 2A. While seven different RFLP profiles could be detected with a mixture of 3 mtDNA probes (M2 in Table 3) totaling a length of 8 kb, the cpDNA RFLP profiles were identical with the exception of a distinct profile found in 30 mitochondrial genotypes (37 accessions) from three districts of the state of Acre: Brasiléia (AC/B), Sena Madureira (AC/S), Xapuri (AC/X), and from the Calama district of the state of Rondônia (RO/C) (an example is shown in Fig. 2B, lane 8). Although we did not extend this analysis to all *H. brasiliensis* accessions, it is quite clear that the chloroplast genome shows much less polymorphism than does the mitochondrial one. These results also indicate that the polymorphism-detecting mtDNA probes do not contain any cpDNA sequences which have possibly moved to the broad bean mitochondrial genome, as shown for other species [18, 19, 25].

Genetic relationships among genotypes

A genetic distance matrix was generated with each mtDNA genotype being treated as an operational taxonomic unit (OTU). The phylogenetic trees predicting the genetic relationships among all the genotypes analyzed were then constructed by the UPGMA (Fig. 3) and Neighbor-joining (Fig. 4) methods.

A sample of 261 genotypes derived from two prospectings in Amazonian forests were studied for their mtDNA variability, 218 accessions being prospected in 1981 (109 from Acre, 54 from Rondônia, 55 from Mato Grosso) and 43 prospected in 1974) (13 from Rondônia, 18 from Acre, 12 from Peru). *H. benthamiana* (F 4506) and *H. pauciflora* (P 9) were also included for comparison. The classifications obtained by both methods (UPGMA, Fig. 3 and Neighbor-joining, Fig. 4) showed similar results, with a few exceptions which will be discussed later. To facilitate the analysis, we have numbered various clusters, which bring together genotypes derived from one or several close districts within a state.

In the state of Acre (AC), samples were collected from five different districts: Brasiléia (B), Xapuri (X), Feijo (F), Tarauaca (T) and Sena Madureira (S). Almost all the accessions from AC/B,X and a large number from AC/S are clustered together (Figs. 3 and 4, cluster 1). These three districts are contiguous (Fig. 1). Most clones from the Calama district of Rondônia (RO/C), all the Peruvian clones (MDF) and eight Acre clones prospected in 1974 define another cluster (2) close to cluster 1. In addition, all the genotypes bearing a distinct cpDNA RFLP profile fall into these two clusters (data not shown). This genotypic neighborhood, defined by the mtDNA and cpDNA RFLPs, and also seen on nuclear RFLP analysis [4], may reflect the fact that the Calama district (Rondônia) is in the Rio Madeira basin downstream of the districts of Xapuri and Brasiléia (Acre), which are split between two basins, that of Rio Madeira and that of Rio Purus. Eight genotypes from Acre/Sena Madureira (AC/S) form an independent cluster (3). Going further west in the state of Acre, we find cluster 4 coinciding with the districts of Feijo (AF) and Tarauaca (AT). One exception, cluster 5 of 4 AF and AT genotypes, is separated from the preceding cluster and resembles accessions from Mato Grosso and Rondônia.

In the states of Rondônia and Mato Grosso, eleven distinct districts were prospected (Fig. 1). Firstly, two distantly located Rondônia districts, Costa Marques (RO/CM) and Ariquemes (RO/A) overlap (cluster 6). Within this cluster, we find clones from an adjacent Mato Grosso District (Vila Bela), belonging to the same basin (Rio Guapore) as the district of Costa Marques (Rondônia). This observation is consistent with the results obtained by isozyme and nuclear RFLP analyses [4]. All the accessions from two other adjacent Rondônia districts, Jiparana (JP)

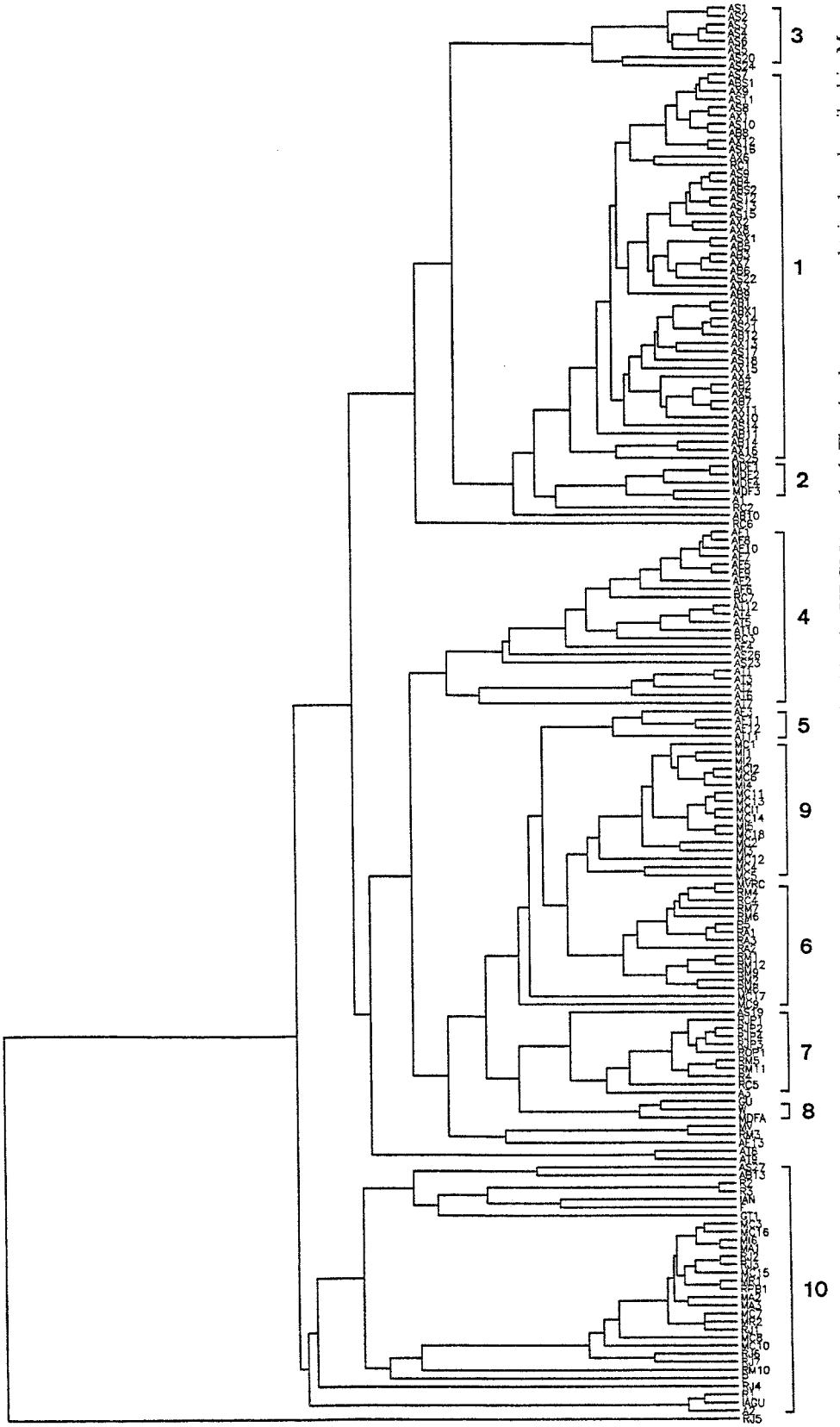

Fig. 3. Predicted phylogenetic relationships of 212 distinct groups of *Hevea* determined by the UPGMA method. The dendrogram was obtained as described in Materials and methods from the mtDNA RFLP data shown in Table 1.

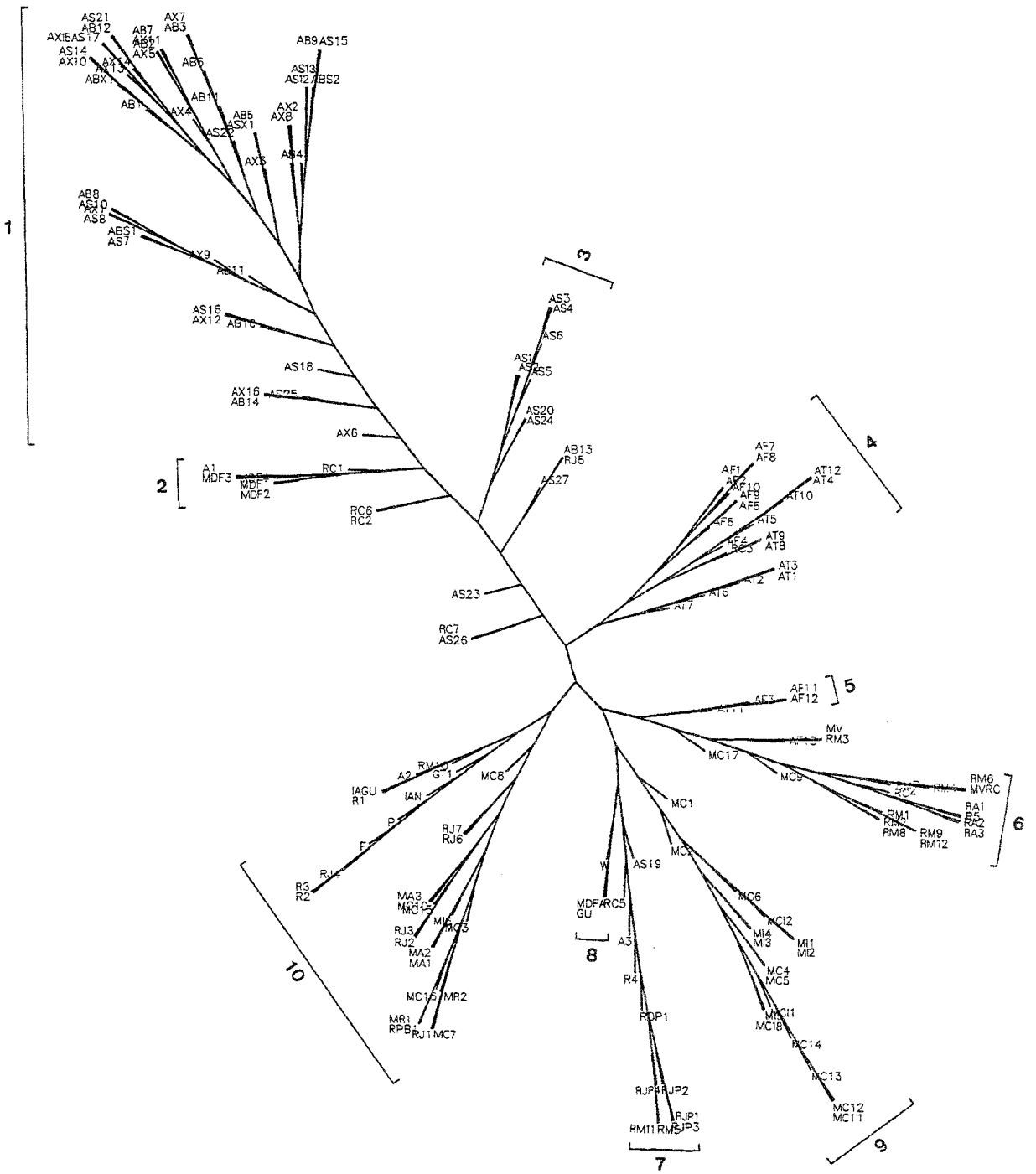

Fig. 4. Predicted phylogenetic relationships of 212 distinct groups of *Hevea* determined by the neighbor joining method. The dendrogram was obtained as described in Materials and methods from the mtDNA RFLP data shown in Table 1.

and Ouro Preto (OP), as well as one accession from Calama (RO/C) and two from Costa Marques (RO/CM), form another cluster (7). At its border is group 8, consisting of cultivated genotypes derived from the Wickham collection. Most of these possess the same mtDNA geno-

types (W). This is not surprising considering that they originate from a small number of seeds. In addition, free and controlled crosses, which have taken place since breeding started, may have led to a decrease in cytoplasmic source diversity. This contrasts with the nuclear DNA data, which reveal a larger polymorphism [4]. This discrepancy will be discussed later.

Two Mato Grosso districts, Juruena (MT/C) and Itauba (IT), each located within the tributaries of the Rio Tapajoz, were clustered together (9). The final cluster (10) is rather heterogeneous, since it contains genotypes from 3 districts of Rondônia and 3 districts of Mato Grosso. It also includes four accessions, RO38 (R1), AC58 (A2), RO52 (R2) and RO53 (R3), prospected in 1974. The first two of these have been considered as natural interspecific hybrids between *H. brasiliensis* and *H. benthamiana* [8] and form an isolated branch within cluster 10, together with a group of derivatives of cultivated hybrids between an original Wickham clone and *H. benthamiana* (IAGU). They probably all have a similar cytoplasm source. In the same cluster, we also found two other species, *H. benthamiana* (F) and *H. pauciflora* (P), again reflecting the large diversity of this cluster. Finally, the only Wickham clone characterized by a male sterile phenotype (GT1) was isolated within cluster 10. It thus contains a cytoplasmic genome distinct from that of the other cultivated lines. This raises the possibility of male sterility linked to a defective nuclear-cytoplasmic interaction.

A factorial analysis of correspondences (FAC) was performed on the same data (Fig. 5) and the results, in general, agree with those of the phylogenetic trees. AC/B,X,S (closed stars) types are well separated from those of AC/F,T (open stars), Rondônia (squares) and Mato Grosso (diamonds). Although some Rondônia genotypes (squares) are dispersed in the plane, most are formed into two groups also containing those from Mato Grosso (diamonds). These observations thus confirm the clustering seen in Figs. 3 and 4. The diversity of the Schultes collection (triangles) partially overlaps with that of the Amazonian forests, but its extent is limited to the center of the plane.

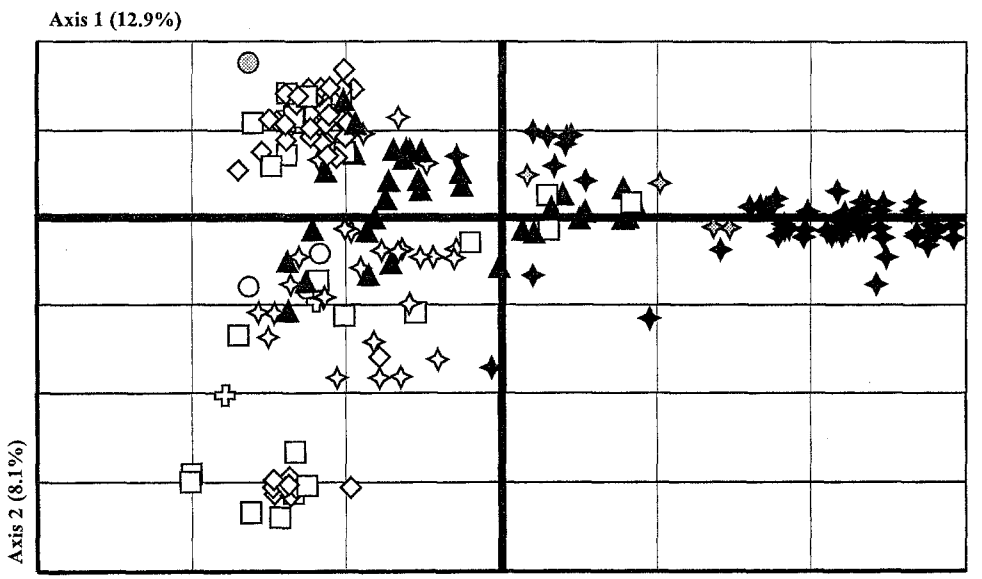

Fig. 5. Factorial analysis of correspondences (FAC) of the 212 distinct groups of *Hevea* based on mtDNA RFLP data. The first and second axes represent 12.9% and 8.1%, respectively, of the total genetic variation. Closed stars: districts of Brasiléia, Xapuri and Sena Madureira in the state of Acre; hatched stars: Peruvian clones (MDF); squares: state of Rondônia; diamonds: state of Mato Grosso; triangles: Schultes clones; hatched circles: cultivated clones (group W); circles: putative interspecific hybrid derivatives; cross: clones of *H. benthamiana* (F) and *H. pauciflora* (P).

The Schultes genotypes are not well represented on the first plane (first two axes) of the FAC analysis. The genetic diversity of the Schultes collection appears clearly on the third axis of the FAC which represents 5% of the total variability (data not shown), indicating that this population from Colombia is quite divergent from the Brazilian ones.

Discussion

Using fourteen mitochondrial DNA probes, we have examined the mtDNA polymorphism and predicted the phylogenetic relationships of wild and cultivated *H. brasiliensis*. A high degree of polymorphism was found, with 212 different types being found among the 397 accessions analyzed. This contrasts with the very limited chloroplast variability seen in the 217 genotypes we analyzed. This difference reflects the higher stability of the chloroplast genome during evolution, as already reported [7, 26]. Similar results (high mitochondrial and low chloroplast polymorphism) were also obtained for *Theobroma cacao* [17].

Although the highest number of probe/enzyme combinations used in the RFLP analysis should, theoretically, give the most accurate phylogenetic tree, studies have shown that fewer probes are sufficient if a great diversity exists among the taxa analyzed [22, 35]. In addition, since restriction polymorphism of the mitochondrial genome in most plants is due, in part, to DNA rearrangements, the use of several enzymes in RFLP analysis may result in the detection of several RFLPs by a single probe. Some of the detected mutational events are thus not independent and the reliability and accuracy of phylogenetic studies based on these data may be affected. Nevertheless, Miller and Tanksley [21] have shown that dendrograms produced from a single enzyme resulted in the same overall groupings as those based on the entire data set obtained from five enzymes together. In the present study, phylogenetic trees constructed using two independent sets of 11 (*Eco* RI) or 14 (*Hind* III) probes also showed an overall grouping similar to the results obtained with 25 probe/enzyme combinations (data not shown). However, it would be interesting to compare the results obtained here with the dendrogram that could be drawn directly from nucleotide substitutions. This would require sequencing the same regions in clones representative of the major clusters.

In general, we obtained similar results with the UPGMA and Neighbor-joining methods and factorial analysis of correspondences. It should be noted that the rooted tree (Fig. 3) may be misleading in putting two genotypes, belonging to branches separated by several nodes, adjacent to one another. Caution should also be applied when successive nodes are very close since, in this situation, branch hierarchy depends on very small number of differences. We found a major discrepancy with the single clone RJ5, whose dramatic isolation in the rooted tree (Fig. 3) is not found in the unrooted tree (Fig. 4). In fact, RJ5 was also found to differ from other Rondônia samples and appeared much closer to Mato Grosso group in both isozyme and nuclear RFLP analyses [4]. In our study, of the 38 unique mtDNA fragments detected, nine were restricted to RJ5. This great variability could stem from the presence of a cytoplasmic genome introduced by a natural interspecific cross or a high frequency of mtDNA rearrangement in this accession. RJ5 deserves a more thorough examination for its potential application in introducing new cytoplasms into cultivated *Hevea*.

Like in a previous report analyzing nuclear RFLP [4], we were able to distinguish most accessions according to their geographical distributions and the hydrographical network. However there were exceptions. For instance, the association between the district RO/J (Rondônia) and those of MT/C, MT/IT, MT/A (Mato Grosso) cannot be explained geographically. One possible explanation would be independent, but convergent, evolution. This would be very unlikely if several independent mutations were involved. However, the existence in plant mitochondria of sublimons (DNA molecules in sub-stoichiometric amounts) [33] suggests the possibility of the independent amplification of sublimons in two dis-

tinct genotypes, leading to the appearance of several new hybridization bands.

Interestingly, the mtDNA types of *H. benthamiana* and *H. pauciflora* do not form branches clearly separated from the others within cluster 10. This is not really surprising if we remember that interspecific hybrids seem to occur in nature, indicating the low genetic distance between those species. Some of the samples included in cluster 10 are thought to be hybrids between different species and could thus contain a cytoplasm close to these species.

The cultivated Wickham clones originated from a few seeds collected on the Rio Tapajoz river bank. Isozyme [6] and nuclear RFLP [4] analyses have shown considerable variability, despite their narrow genetic basis and their high level of inbreeding, whereas the present study failed to reveal great variability at the mtDNA level. Indeed, these cytoplasms are found mainly at two positions on the dendrogram, next to the Rondônia or Mato Grosso types. This genetic uniformity may be explained as follows. The majority of the cultivated clones originated from a few prominent parents because of the conventional breeding system in which the best genotypes in one generation are used as parents (especially as female) for the next cycle of breeding [32]. Thus, breeding could have led to a decrease in cytoplasmic information while preserving nuclear variability.

In contrast to previous nuclear RFLP results which classified Wickham clones with the Mato Grosso group (cluster 9) [4], at the mitochondrial level we found that most of the Wickham clones were closer to RO/JP,OP,CM clones (cluster 7) (Figs. 3 and 4). In fact, the MT/C and MT/IT genotypes are within the area of the Rio Tapajoz river from which the original Wickham clones originated, whereas Rondônia is around the tributaries of Rio Madeira. However, this apparent discrepancy is minimized by the observation that cluster 8 (to which the Wickham clones belong) is not far from the Mato Grosso clones belonging to cluster 9 (Fig. 4). The wild genotypes show a much greater mtDNA polymorphism than the Wickham clones. This observation should be taken into account by geneticists in the choice of new cytoplasms for breeding programs.

It should be noted that the mtDNA of *Hevea* appears to be maternally inherited as is that of most plant species, since in the present study, all the IAN or GU clones derived from crosses with a Wickham clone as female parent, share the mitochondrial genotype of the latter, whereas all IAN clones sharing the IAGU mitochondrial type have the same female parent or grand-parent, F4542 which is a *H. benthamiana* clone (not included in this study).

A more general conclusion concerning the rubber tree genetic resources is the existence of a great level of genetic structuring among natural populations in the Amazonian forests in relation to the hydrographic network. This is in accordance with a pattern of *Hevea* dispersion following the river stream.

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