Rubber Tree (*Hevea brasiliensis*)

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The taxonomic distribution of latex-producing plants is wide. Among the 12,500 latex species, which belong to 900 genera, 100 species distributed in 76 families produce rubber, or cispolyisoprene (Polhamus, 1962). Within the genus *Hevea*, which offers favourable traits for commercial exploitation, the species *H. brasiliensis* is almost exclusively exploited for natural rubber because of its productivity and the quality of its rubber.

Natural rubber, in the form of latex or coagulate, is collected and transported to a drying factory, which produces smoked sheets or dry granulate blocks of rubber. It is most often exported to places where it is transformed into consumer products. The world production has risen to nearly 7 million tonnes a year and comes from the tropical zone, where hevea is cultivated over around 8 million hectares. Almost 91% of the cultivation takes place in Southeast Asia and it is dominated by four countries: Thailand, Indonesia, India, and Malaysia (http://apps.fao.org). Africa contributes 6.5% of the production. In South America, cultivation is limited by a South American leaf disease caused by the fungus *Microcyclus ulei*.

Natural rubber represents a third of the total production of rubber. Compared to synthetic rubber made from petroleum, natural rubber has specific qualities, such as heat-resistance. The aviation tyre industry, for example, uses only natural rubber, and the tyre industry generally consumes 70% of the production, the remaining 30% being used for a variety of products.

BOTANY AND GENETIC RESOURCES

Botany and Taxonomy

Hevea is a tree with rhythmic growth and orthotropic branching. It may grow 30 m high and reach a circumference of 3 m in its natural environment,

the Amazon basin. In plantations, grafted trees do not reach such dimensions. It is a tropical species that requires insolation and humidity and does not tolerate altitudes higher than 600 m. The leaves have three leaflets of ovoid shape, arranged at the tip of a long petiole. They are renewed each year by a natural process of defoliation and refoliation during the dry season. Hevea is a monoecious plant that is preferentially allogamous, with unisexual yellow flowers of a few millimetres grouped in racemes. The fruit of hevea, the size of which ranges from 0.5 to 5 cm depending on the species, has a trilocular structure characteristic of Euphorbiaceae. It is made up of three carpels, each containing a seed.

Within the genus *Hevea* (Willd.), which is well-defined, the species are difficult to differentiate and have been the subject of much confusion, disagreement, and fluctuations between authors. Except for the species *H. brasiliensis*, the observations have been made in often difficult field conditions and are generally quite brief. Schultes (1990) presented the most recent synthesis and distinguished ten species, of which three are subdivided into four varieties. The natural hybridizations seem limited in an undisturbed forest environment, but several interspecific morphological variations are observed. We thus speak of a species complex, according to the definition of Pernes (1984). The nine related wild species are part of the primary gene pool of the cultivated species *H. brasiliensis*. They are thus of great use as genetic resources of cultivated hevea, in so far as they have useful characteristics such as genetic resistance to certain diseases (Schultes, 1977, 1990).

The Genome Structure

With the exception of a triploid clone of *H. guianensis* and the possible existence of a race of *H. pauciflora* with 18 chromosomes (Baldwin, 1947), all the species of the genus have a chromosome number of 2n = 36.

Strong similarities between different chromosome pairs suggest an ancient duplication of the chromosome stock. At meiosis, the formation of multivalents, especially of quadrivalents, is observed from time to time, which indicates some affinity between pairs of homologous chromosomes. Hevea was thus considered an amphidiploid genus, that is, a tetraploid that behaves like a diploid at meiosis, resulting from a crossing of two unidentified, diploid ancestral wild species (Bouharmont, 1960; Ong, 1985). The basic chromosome number would thus equal 9, which seems closest to the usual chromosome number of Euphorbiaceae, which ranges from 6 to 11 (Ong, 1985).

However, the analysis of segregations of isozymic or RFLP markers reveals a majority of non-duplicated loci (Seguin et al., 1996; Lespinasse et al., 2000b). Thus, hevea could be an amphidiploid in which the two homologous genomes have diverged considerably. It cannot be affirmed that the rare duplications observed in the hevea genome (Seguin et al., 1998)

indicate a polyploid origin. Such duplications could also come from chromosome remnants after polyploidization. The same situation is observed in cassava or manioc (*Manihot esculenta*), another Euphorbiacea with 2n = 36 chromosomes (Fregene et al., 1997).

A preliminary approach for global characterization of the nuclear genome of *H. brasiliensis* was proposed (Low and Bonner, 1985). The study of the kinetics of reassociation of short fragments of marked DNA (300 nucleotides) indicates the presence of 43% of single copy DNA and 32% of moderately repeated sequences (frequency of repetition around 1000); the remaining DNA are highly repeated or palindromic. From the kinetics of reassociation of single copy DNA, the overall genome size can be estimated at 6×10^8 base pairs. However, from an occasional study by flow cytometry, the size of the haploid genome of hevea has been estimated at 2×10^9 base pairs (2.1 pg/1C), identical in five species studied—*H. brasiliensis, H. benthamiana, H. guianensis, H. pauciflora,* and *H. spruceana*. This value has also been obtained by microdensitometry in *H. brasiliensis* and *H. camargoana* by Bennett and Leitch (1997).

GENETIC RESOURCES

Prospections, Introductions and Collections

In 1747, Fresneau gave the first description of the 'rubber' tree, which was later called hevea. First gathered by the *seringueros* in South America, natural rubber was produced and developed rapidly once its chewing and waterproofing qualities were discovered, the vulcanization process was developed in 1830, and plantations were established (Serier, 1993).

In 1876, on the insistence of Marckham, who had already successfully transferred cinchona from South America to Asia, Wickham, a British planter settled in Brazil, harvested 70,000 seeds close to Boim, at the mouth of the Amazon and Tapajos, upstream of Santarem (state of Pará, whence the initial name of the Para tree for the species *H. brasiliensis*). He shipped them to Kew Garden in the United Kingdom, where only 4% of the seeds germinated three weeks after they arrived. Several plantlets were then sent to Ceylon and Singapore, with varying success. The 22 trees planted in the botanical garden at Singapore in 1877 are thus considered the origin of almost all the hevea plantations in the world. The varieties (grafted clones) that resulted from selection in Asia from these plants introduced at the end of the 19th century were thus called Wickham clones. Even though it is probable that other genotypes were introduced subsequently, only the Wickham example has been recorded and the preponderant role of this material in the genetic composition of present cultivars has been acknowledged.

The Wickham clones proved to be very sensitive to *Microcyclus ulei* in South America. Important breeding efforts were made by the Ford and

Firestone companies in Brazil and Guatemala and subsequently by Brazilian research centres to obtain productive and resistant clones (clones F, FB, FX, FDR, MDF, MDX, IAN, CNSAM). The introgression of totally resistant genes from other species of the genus into *H. brasiliensis* was not successful, this type of resistance being easily overcome by the pathogen. The present strategy aims to associate several components of partial resistance through crosses to attain a sufficient level of long-lasting genetic resistance to the disease (Rivano, 1992; Lespinasse et al., 2000a).

The supposed narrowness of the genetic base of the Wickham material introduced and then selected in Asia and Africa, which is illustrated by the high general sensitivity of this material to *Microcyclus*, has led to the organization of prospecting efforts within the distribution area of *H. brasiliensis*.

In Malaysia, the Rubber Research Institute of Malaysia (RRIM) imported seedlings from wild trees of seven species of *Hevea* from Brazil in 1951-52 and in 1966. It also imported clones bred by Ford (F, FB, FX) and by the Instituto Agronomico do Norte (IAN) in Brazil (Ong, 1987).

In 1974, the Insitut de recherches sur le caoutchouc (IRCA, France), in co-operation with the Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA, Brazil), made a preliminary prospection in the Amazon region, in the states of Acre (22 clones) and Rondonia (20 clones), collecting graft wood in the forest from trees judged to be exceptional. To this study were added 18 clones from the Firestone prospection in the Peruvian Madre de Dios.

The international prospection organized in 1981 by the International Rubber Research and Development Board (IRRDB) for *H. brasiliensis*, in 16 districts and 60 localities of the Brazilian states of Acre (Lins et al., 1981), Rondonia (Gonçalves, 1981), and Mato Grosso (de Paiva, 1981), resulted in the collection of 9800 wild genotypes at the Asian centre of Malaysia (Ong et al., 1995) and 1500 genotypes at the African centre of Côte d'Ivoire (Chapuset et al., 1995). The geographic location of districts prospected is given in Table 1. This expedition collected essentially seeds, but also 130 genotypes from graft wood of trees judged to be exceptional (called ortet clones). Genetic material was exchanged between the two centres and distributed to other member countries of the IRRDB. Most of the genotypes were grafted and are conserved in the form of clones in living collection.

After 1945, Schultes put together, among others on the sites of Calima and Palmira, significant collections of material found in Colombia. In 1985, with the permission of the Colombian government, IRCA transferred 341 SCH genotypes from these two sites to Guadeloupe and then to Côte d'Ivoire (Nicolas, 1985).

The IRCA also received 23 CNSAM clones from the EMBRAPA at Manaus, comprising *H. pauciflora* genotypes and the only *H. brasiliensis* genotypes collected in the Brazilian state of the Amazonas, and introduced them in Côte d'Ivoire (Gonçalves et al., 1983).

Table 1. Code and origin of the major populations of *H. brasiliensis* in collection

Code	Collection	State and country of origin	Place of origin (districts)	River basin (tributaries of Amazon)
AC/T	IRRDB 1981	Acre, Brazil	Tarauaca	Jurua
AC/F	IRRDB 1981	Acre, Brazil	Feijo	Jurua
AC/S	IRRDB 1981	Acre, Brazil	Sena Madureira	Purus
AC/B	IRRDB 1981	Acre, Brazil	Basileia	Purus
AC/X	IRRDB 1981	Acre, Brazil	Xapuri	Purus
RO/A	IRRDB 1981	Rondonia, Brazil	Ariquemes	Madeira
RO/C	IRRDB 1981	Rondonia, Brazil	Calama	Madeira
RO/CM	IRRDB 1981	Rondonia, Brazil	Costa Marques	Madeira
RO/J	IRRDB 1981	Rondonia, Brazil	Jaru	Madeira
RO/JP	IRRDB 1981	Rondonia, Brazil	Jiparana	Madeira
RO/OP	IRRDB 1981	Rondonia, Brazil	Ouro Preto	Madeira
RO/PB	IRRDB 1981	Rondonia, Brazil	Pimenta Bueno	Madeira
MT/A	IRRDB 1981	Mato Grosso, Brazil	Aracatuba	Tapajos
MT/C	IRRDB 1981	Mato Grosso, Brazil	Juruena	Tapajos
MT/IT	IRRDB 1981	Mato Grosso, Brazil	Itauba	Tapajos
MT/VB	IRRDB 1981	Mato Grosso, Brazil	Vila Bella	Madeira
MDF	Firestone	Madre de Dios, Peru	?	Madeira
SCH	Schultes coll. Calima and Palmira	Colombia	?	?
W	Wickham	Para, Brazil	Santarem	Tapajos

In conclusion, *H. brasiliensis* can be considered to be well represented in the living collections, in Malaysia and Côte d'Ivoire, by several thousands of genotypes. However, the area of distribution of this species has been sampled in a highly heterogeneous fashion. The states of Amazonas and Para in Brazil, which are the major part of that area, were hardly prospected. It is also necessary to search for other species of *Hevea* in order to create a centre of conservation and study of the entire genus. The observations available on the Schultes and CNSAM collections show that there are genotypes of species other than *H. brasiliensis* in these collections.

Conservation, multiplication and exchange of accessions

In the natural state, hevea reproduces by seed. Once techniques of grafting were introduced by van Helten in 1918, clones could be bred and genotypes conserved in the form of living collections, which were durable and relatively inexpensive to create and maintain in gardens of grafted trees. Propagation was done in nurseries by grafting on stocks grown from seed.

The viability is one week for graft wood and three weeks for seeds (the seeds are recalcitrant: the duration of germination capacity is short). At the experimental stage, the pollen is conserved for no more than a month and the quantities that can be harvested are very small. Genetic transfer in this form is thus not considered feasible.

ORGANIZATION OF GENETIC DIVERSITY

This chapter summarizes the present understanding of the organization of diversity of hevea. It is based on the conclusions of earlier studies and presents complementary original results from isozymic analyses on a very large number of genotypes. These supplementary data allowed us to develop, on a sufficient number of genotypes, an original outline of comparison between morphoagronomic variability and genetic diversity of neutral markers, by means of a multiple factorial analysis.

Research on genetic diversity of hevea aims to better understand the extent and organization of the diversity and the relationship between agronomic variability and genetic diversity. These studies aim to respond to the problems of breeders, who wish to define an optimal strategy of genetic resource conservation, population sampling for agronomic evaluation, crossing scheme design, and progenitor choice.

Agromorphological Variability

Agronomic evaluation

Agronomic evaluation is done on prospected material in order to constitute two small collections before genetic recombination. One, called the working collection, is intended to quickly concentrate the alleles of agronomic interest in order to increase the possibilities of getting a usable clone, especially after crossing with the Wickham material. This objective could be met through simple agronomic evaluation, but genetic mapping allows us to assess the reduction of variability resulting from intensive and rigorous selection. The other collection must be constructed according to the concept of core collection, that is, in trying to maintain the widest possible diversity in a small sample. This task requires the use of all methods of evaluation: agronomic, morphological, isozymic, and molecular, for the nuclear and cytoplasmic genomes. Methodological research is in progress, especially on hevea, to optimize the selection of genotypes while integrating these different types of information (Hamon et al., 1998).

A preliminary assay on 2500 genotypes from the IRRDB prospection in the states of Acre (AC), Rondonia (RO), and Mato Grosso (MT) in Brazil, at the rate of one tree per genotype, allowed a preliminary overall evaluation of the production and growth of trees. The mean production of this wild material is very low, representing only 12% of that of the control Wickham clone, GT1. Similarly, its rate of growth is on average lower than that of GT1. The architectural aspect of the trees is characterized by a predominance of clones growing tall, with few or no branches; this character corresponds to a natural adaptation to the forest environment that allows young trees to quickly reach the light. However, there is a small proportion of clones that have

production close to that of GT1, good vigour, or abundant ramification (Chapuset et al., 1995; Clement-Demange et al., 1997).

From the agronomic point of view, an east-west gradient appears in the area of distribution of the genotypes studied, as illustrated in the first plane of the principal components analysis (PCA) done on the mean values of production, growth, and architecture, by locality (Vi Cao, personal communication, Fig. 1). The genotypes of Mato Grosso are distinguished, especially from those of Acre, by a better aptitude for branching and greater production, but they seem more sensitive to *Colletotrichum*, a fungus responsible for a leaf disease.

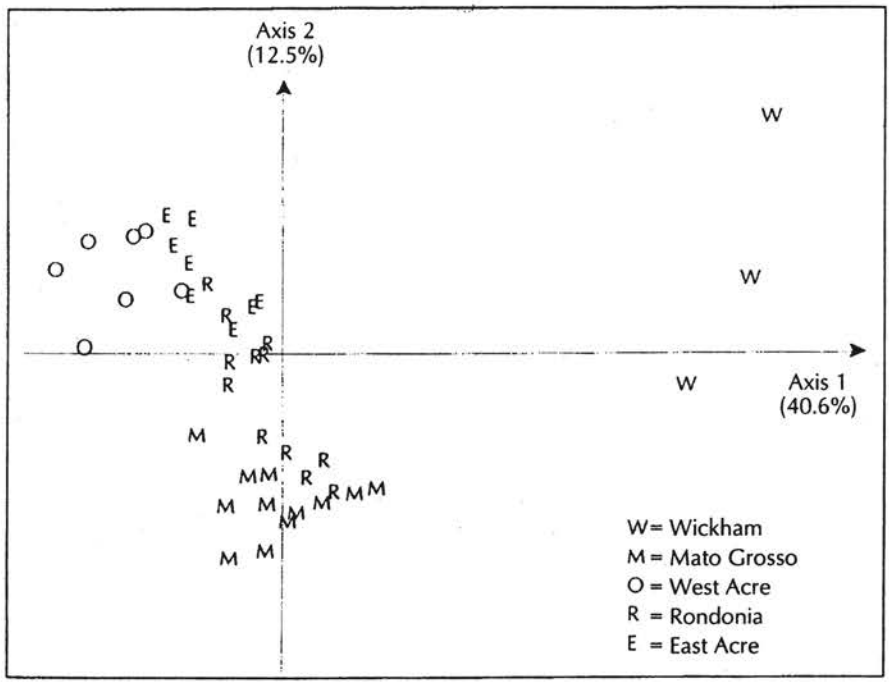

Fig. 1. Agronomic variability of Amazonian material and Wickham clones. Plane 1-2 of the PCA on mean values of three Wickham clones and 41 localities of the IRRDB collection, calculated on 2300 Amazonian clones.

Leaf morphology

A preliminary study on the genetic structuring of the IRRDB populations from the states of Acre, Rondonia, and Mato Grosso was done by biometric analysis of morphology of growth units and leaves. The observations are phenotypic and 34% of the variance is due to effects of the environment and to errors. Most of the genetic variability (42%) lies between the genotypes of a single prospecting site, while differences between states or between districts

seem small. Nevertheless, Acre stands out distinctly from the two other states (Nicolas et al., 1988).

Biochemical and Molecular Diversity

The genetic diversity of a sample from *H. brasiliensis* collections was characterized using biochemical and molecular markers. The numbers of accessions used in these studies are given in Table 2. The other species of the genus *Hevea* were nearly absent from the collections, and it was not possible to study their diversity. One or two genotypes of the four species *H. benthamiana, H. guianensis, H. pauciflora,* and *H. spruceana* could be characterized by isozymes or RFLPs, which allowed identification of the alleles missing in *H. brasiliensis.*

Table 2. Number of clones, per population and per type of marker, used in the studies of genetic diversity

Populations	Isozymes	RFLP	
		Nuclear	mtDNA
Wickham	203	73	28
Hybrids (Wickham × Amazonian)	81	0	21
Peru (MDF)	14	0	12
Colombia (SCH)	48	0	83
Brazil EMBRAPA-IRCA 1974	55	0	30
Brazil IRRDB 1981	486	92	220
Total	887	165	395

Isozymes

The isozymic analysis revealed 14 polymorphic loci, using 12 isozyme systems, in *H. brasiliensis* (Chevallier, 1988; Chevallier et al., 1988; Seguin et al., 1995a).

The primary level of analysis was done on intergenotype variability, that is, between the clones. The more precise analysis of diversity, in terms of population samples, was done on 486 clones of the IRRDB collection, which were taken from 16 districts of three states of Brazil. The genotypes were characterized for 8 polymorphic loci, without missing data, and 25 alleles were revealed.

The first axis of correspondence analysis (CA) done on allelic data represented 20% of the total variability (Fig. 2). It revealed a separation into two groups of clones. The first group comprises almost exclusively all the genotypes of four districts—three districts in Mato Grosso (MT/A, MT/I and MT/C) and one in Rondonia (RO/PB). The second group is made up of genotypes belonging to the 12 remaining districts—five in Rondonia, five in Acre, and the MT/VB district in Mato Grosso.

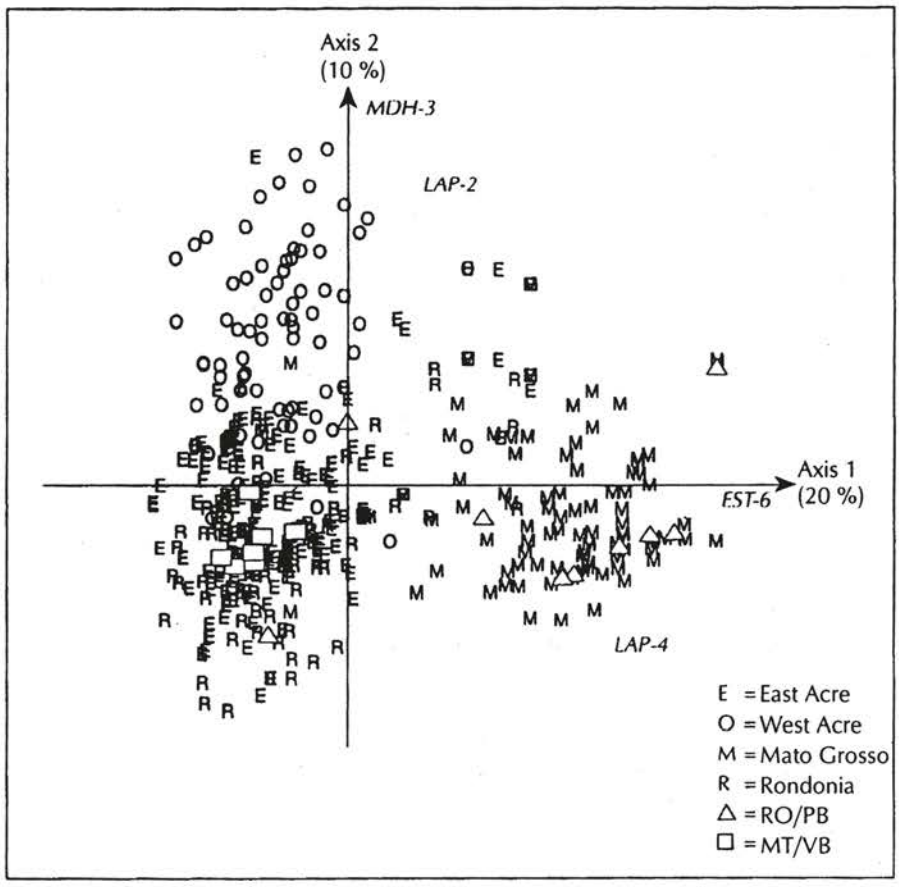

Fig. 2. Genetic diversity of the Amazonian material revealed by isozymes. Plane 1-2 of the CA on data of presence-absence of 25 alleles of 8 loci, in 486 clones of the IRRDB collection. MDH-3, LAP-2, LAP-4, EST-6: alleles contributing the most to the first two axes.

The second axis, which represents 10% of the variability, shows an intra-state structuring with the differentiation of a third group made up of two districts farthest west of Acre, AC/T and AC/F (Fig. 2). As for the morphoagronomic data, the genetic variability is low between the groups or origins and high within the groups. Despite this relatively low part of intergroup diversity—30% of the total inertia on the first CA plane—there appears a very clear genetic differentiation into three groups.

This structuring into three groups has also been indicated in a study on a smaller sample of genotypes, but for 11 isozymic loci (Besse et al., 1994; Seguin et al., 1996). This study does not include the RO/PB district, but includes the Wickham population. The Wickham population proved to be genetically close to the Mato Grosso group. It has a high level of heterozygosity and polymorphism, which is however much lower than that of the Amazonian

populations, which have a greater allelic richness. No allele is specific to the Wickham collection, nor to any of the three states in the Amazonian prospection. The genetic diversity of the Wickham clones has moreover made it possible to perfect an isozymic method of varietal identification (Leconte et al., 1994).

The second level of analysis pertains to the diversity between populations of genotypes, the populations being defined as a function of their location. The level of subdivision depends on the refinement of analysis desired, but above all on the number of genotypes studied per population.

Interpopulation diversity was characterized using the Nei distance (1978) on allelic frequencies of 8 isozymic loci. Figure 3 shows the UPGMA tree constructed for 16 Brazil districts, one population from Peru (MDF), two collections from Colombia (Schultes populations), and the Wickham population, or 590 genotypes in all. For the Brazilian populations, this analysis

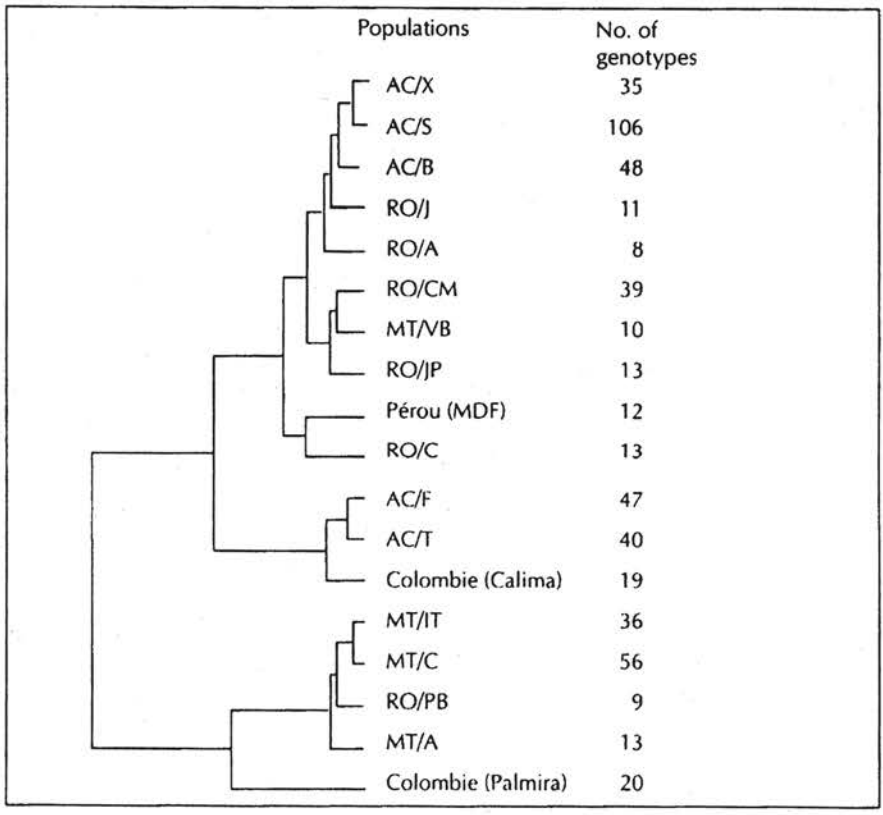

Fig. 3. Genetic distances between populations of Amazonian clones. UPGMA tree drawn from the Nei distance (1978) calculated on allelic frequencies per population, for 8 enzymatic loci.

by population leads to the same conclusions as the CA on the genotypes taken individually. The districts are grouped into three subgroups: West Acre; East Acre, Rondonia and the MT/VB district; and Mato Grosso and RO/PB.

The Peruvian population is grouped first with a Rondonia district (RO/C). The two Colombian populations (SCH) seem genetically very distant from each other. The first, Calima, approaches the two districts of West Acre; the second, Palmira, is closer to the Mato Grosso group than to other populations, but has specific alleles characteristic of species other than *H. brasiliensis*, which make this Palmira population clearly distinct from others.

The isozymic study was done by identifying the alleles at each locus using the genetic analysis of zymograms. This allowed analysis of the genetic diversity of hevea using population genetics. The calculation of *F* statistics of Wright thus showed that 75% of the diversity of the Brazilian material is located at the intrapopulation level, and thus that 25% of the variability corresponds to the differentiation between districts. When the three genetic groups identified on the CA are considered as population units, the proportion of intragroup diversity reaches 80%.

Moreover, the populations made up by the prospected districts all show a departure from panmixia with a deficit of heterozygotes, the values of the fixation index F_{IS} ranging from 0.10 to 0.60. Taking into account the geographic extent of districts, it is probable that this deficit is due not to an intradistrict differentiation into subpopulations, but rather to preferential crosses between closely linked trees with a low rate of dissemination of pollen and seeds. Since the passport data is available for the Amazonian collection of IRRDB at the scale of the prospecting locality in each district, it is possible to tackle this question, subject to analysis of a larger number of genotypes.

Nuclear RFLPs

The RFLP study was done on a smaller number of genotypes: 73 Wickham and 92 Amazonian from 15 districts, the RO/PB district not being represented. On the other hand, it was done on a larger number of loci than for isozymes. As the genetic determinism of RFLP patterns is not established, the exact number of polymorphic loci was not known. A single probe may in fact reveal two loci or more. However, as 25 probes have been used, the number of polymorphic loci must not be far from 25. The gene mapping of hevea has shown that generally a single locus has been revealed with this type of genomic probe (Seguin et al., 1996, 1998; Lespinasse et al., 2000b).

The results obtained using RFLPs emphasize the genetic enrichment contributed by the Amazonian populations and the existence of a high genetic structuring depending on the origin of prospected genotypes. Moreover, in relation to isozymes, in allowing access to a larger part of the genome, nuclear RFLPs contribute a specific information: they reveal a marked differentiation of the five districts of Acre from the other ones, which form a distinct genetic

group. On the other hand, the RFLPs do not reveal the individualization of the western Acre group, which is revealed by isozymes.

Overall, the nuclear RFLP study confirms and reinforces the observations made using isozymes. In particular, the same partition of the intra- and intergroup diversity is found, with also 30% of the variability on the primary plane of the CA corresponding to the differentiation of the group of three districts of Mato Grosso, this group appearing to be the closest to the Wickham population.

Cytoplasmic RFLPs

The cytoplasmic genomes most often have a single-parent heredity (Reboud and Zeyl, 1994; Mogensen, 1996), which prohibits genetic recombination, and a rate of evolution particular to each genome (Wolfe et al., 1987; Palmer and Herbon, 1988). The study of mitochondrial and chloroplastic diversity is thus complementary to that of the nuclear genome.

The study of mitochondrial genome (mtDNA) has been done on 395 genotypes of hevea. A strong polymorphism has been revealed by RFLP (Luo and Boutry, 1995; Luo et al., 1995). As with the nuclear scale, a divergence of populations is found in Acre, as well as a separation between the genotypes of western Acre (AC/T and AC/F districts) in relation to those of the east (AC/B, AC/S, and AC/X) as with the isozymes. The Peru genotypes (MDF) are grouped preferentially with the genotypes of the RO/C district of Rondonia, as with the isozymes.

On the other hand, the populations of Mato Grosso and Rondonia are heterogeneous and are not grouped according to their origin. Similarly, the Colombian populations (SCH) appear highly polymorphic and are not individualized. Moreover, unlike the isozymes and nuclear RFLPs, the mtDNA appeared nearly monomorphic within the Wickham clones, all of which, except the GT1 clone, have the same specific mitochondrial type. The cultivated clone GT1, male-sterile, has a unique mitochondrial type that is very different from the dominant Wickham type.

The diversity of the chloroplastic genome (cpDNA) has also been studied using RFLPs, with the cpDNA probes of broad bean, on 217 out of the 395 preceding genotypes. As for many other species, the cpDNA shows, in hevea, a very low RFLP polymorphism. Only two chloroplastic types have been found, against 126 mitochondrial types detected on the same sample of clones. The minority type, present in 37 genotypes, is characteristic of three districts of eastern Acre, to which are added three out of six genotypes of the RO/C district present in the study (Luo et al., 1995).

The organization of biochemical and molecular diversity

It thus seems clear that molecular markers have made a considerable contribution to the characterization of genetic resources of hevea. They have

revealed the extent of diversity of *H. brasiliensis*. The various types of marker used have given consistent results overall on the organization of this diversity, which is structured according to the geographic origin of genotypes. From this set of studies, it is possible to draw the following conclusions.

On the basis of genetic similarities between the genotypes or populations, six genetic groups have been identified within the material of *H. brasiliensis* studied (Seguin et al., 1996).

- group 1 comprises two districts in western Acre and the Calima population of Colombia;
- group 2 corresponds to three districts in eastern Acre;
- group 3 comprises six districts of Rondonia, the MT/VB district of Mato Grosso, and the MDF population of Peru;
- group 4 comprises three districts of Mato Grosso and the RO/PB district of Rondonia;
- group 5 is made up of the Palmira collection of Colombia;
- group 6 corresponds to the Wickham population.

The geographic location of populations and genetic groups is shown in Fig. 4.

These genetic groups include geographically closed populations. Moreover, the relationship between the geographic distance and the genetic distance is found not only within a single group, but also between groups. Thus, the molecular differentiation of group 6 (Wickham) essentially relies on the existence of a particular mitochondrial type, but at the nuclear level this group seems closer to group 4, geographically the closest. The place of Schultes populations of Colombia is more difficult to discuss without precise passport data.

This organization of the diversity of *H. brasiliensis* seems very clearly linked to the hydrographic network of the Amazonian basin (Table 1, Fig. 4), which determines the gene flow and thus the genetic similarity between populations or groups of populations. For the Brazilian populations, the passport data for which are available and precise, each group can be associated with a river basin (Fig. 4): the Jurua river with group 1; the Purus with group 2, the Madeira and its branches—the Guapore river in Brazil and the Madre de Dios river in Peru—with group 3; and the Tapajos with group 4.

Similarly, certain similarities between the groups can be explained by gene flows following the course of rivers or streams. For example, the proximity between the RO/C population and group 2 is linked to the Abuna river, a tributary of the Madeira river, and that of groups 4 and 6 to the Tapajos river.

This situation would justify designating the genetic groups of hevea by the name of the corresponding river. However, to simplify the presentation, we have designated the groups by their numbers 1 to 6. The RO/PB district is a small exception: located on a tributary of the Madeira river, it is associated

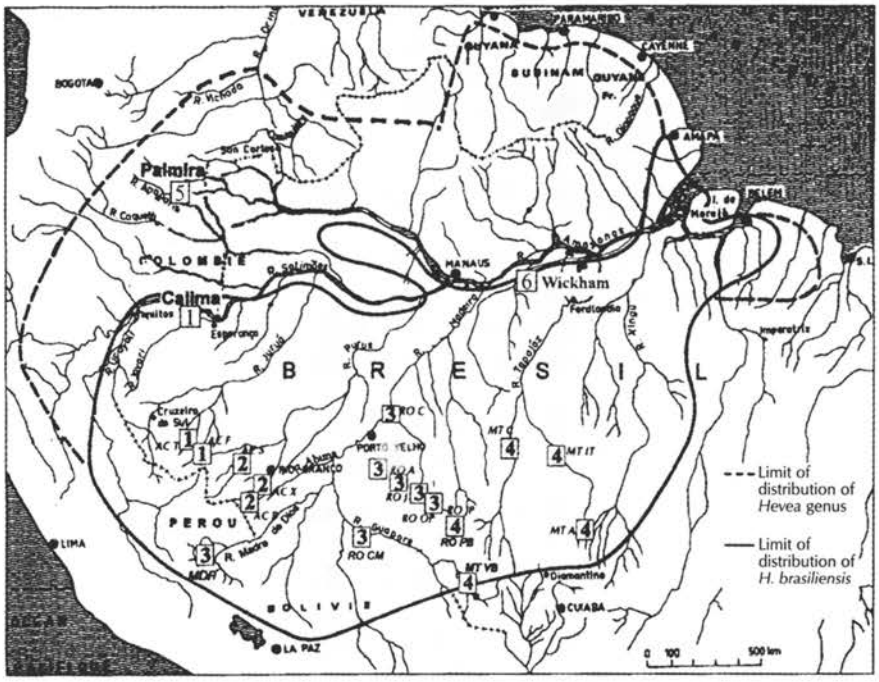

Fig. 4. Correspondence between geographic origin and genetic similarity in *Hevea brasiliensis*. The numbers 1 to 6 correspond to the genetic groups identified by molecular markers (see text). The exact site of collection is not known for the clones prospected by E. Schultes in Colombia (Calima and Palmira collections).

with group 4, of the Tapajos river, and not with group 3 (Figs. 2 and 3). The area prospected in this district is in fact close to the area between the two river basins (Fig. 4) and it can be stated that there is a significant dissemination via the basin of the Tapajos river. The diversity of the cultivated Wickham clones is less than that of the Amazonian genetic resources, especially at the cytoplasmic level. Still, the nuclear diversity of the Wickham group seems greater than might have been foreseen from the history of hevea domestication in Asia.

These conclusions are drawn from all the diversity studies using molecular genetic markers, which is not to say that all the types of markers have given exactly the same results. Each study has actually contributed specific information. There are several factors that explain the differences observed with the different types of markers.

It is not surprising that genetic markers of different kinds contribute different, although not contradictory information. Cytoplasmic markers, because of single-parent heredity (Reboud and Zeyl, 1994; Mogensen, 1996),

allow better detection of migratory flows, the traces of which disappear rapidly at the nuclear level following genetic recombination between indigenous and immigrant populations (Palmer, 1987; Crozier, 1990). For hevea, for example, the similarity between the RO/C district and the genetic group 2 is revealed specifically by chloroplastic markers. Thus, there are gene flows, probably linked to the flotation of grains along the course of the Apuna river, from the districts AC/X and AC/S of group 2 to the north of Rondonia, but these migrations are less intensive and the genetic divergence between the two groups is maintained.

The speed of evolution varies a great deal according to the genome or type of sequence considered (Wolfe et al., 1987; Palmer and Herbon, 1988). With a very slow evolution it is less probable that a mutation would appear associated with a recent differentiation in subpopulations. Inversely, a very rapid molecular evolution leads to the phenomenon of homoplasy, or evolutionary convergence: a particular character, a particular form of marker, has a high probability of appearing independently in several isolated populations and is thus not characteristic of a population. That depends also on the time of divergence involved in the differentiation of units studied, populations or species. For hevea, this phenomenon could explain the complex organization of mitochondrial types between groups 3 and 4, which are well differentiated at the nuclear level. However, the situation could also indicate the existence of migratory flows, slight but diverse, between these two groups and it is not possible to draw any conclusion. A too rapid evolution would also explain why no structuring was observed within the IRRDB collection during a study on the variations of length of gene coding for ribosomal RNA (Besse, 1993; Besse et al., 1993).

Finally, and undoubtedly a determining factor in a number of molecular diversity studies, the quality of results depends on the level of diversity sampling. The differences in results obtained with isozymes or RFLPs in hevea does not arise from a difference in nature. It can be said that the two types of markers are neutral with respect to selection, that they correspond to the same type of nuclear sequence, and that they have a similar rate of evolution on average. On the other hand, the differences could be explained by the mode of sampling of populations—the number and representativity of genotypes studied—or of the genome, that is, the number of loci studied and their distribution over the entire genome. The fact that groups 1 and 2 are not distinguished in the RFLP study is probably due to a quite low number of original genotypes of Acre, as suggested by the PCA done on the isozymes on the same sample (Fig. 5). In parallel, the absence of observed divergence between groups 2 and 3 with the isozymes would come from what has not been detected from the locus or marker allele of this differentiation, the few isozyme markers obtained in hevea not sufficiently covering the entire genome.

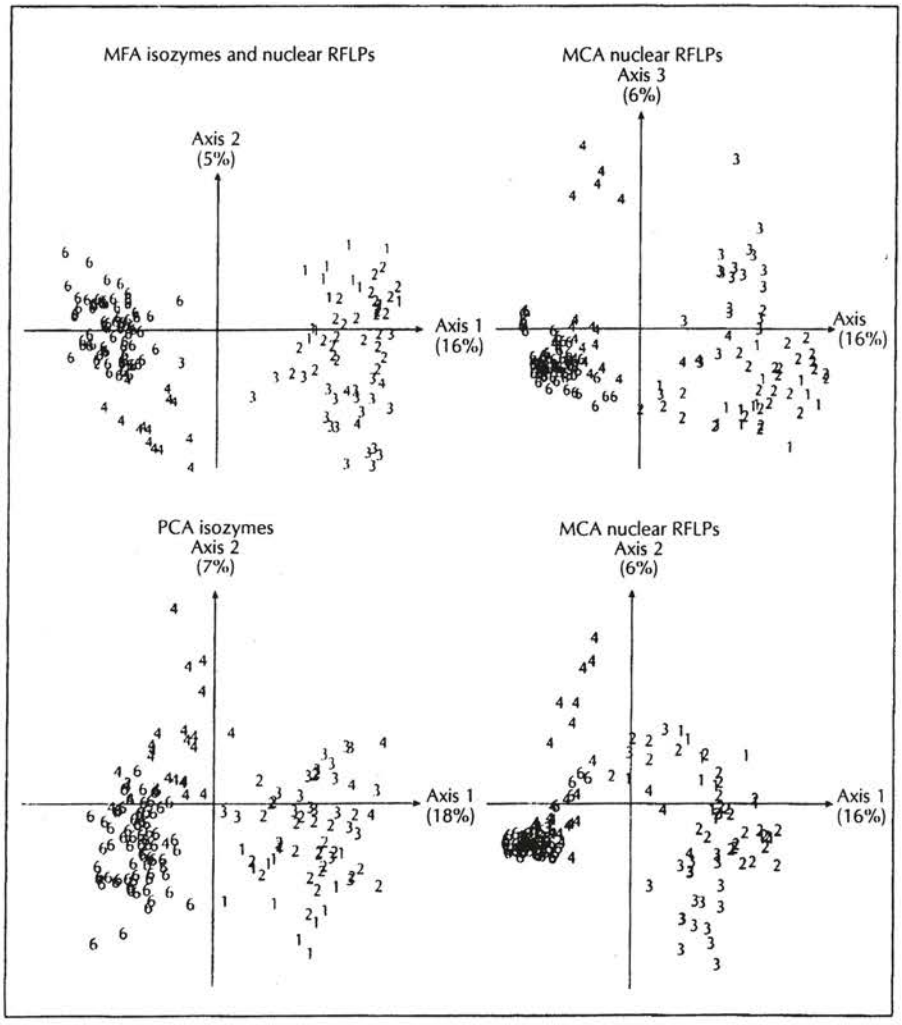

Fig. 5. Comparison by MFA of the diversity revealed by isozymes and by nuclear RFLPs. Analysis of 72 Wickham clones and 89 Amazonian clones of the IRRDB collection. Graphic representation of the primary axes of MFA and of partial analyses (PCA isozymes and MCA RFLPs). The numbers 1 to 6 correspond to the genetic groups identified by molecular markers (see text).

Molecular Diversity and Morphoagronomic Variability: Comparison by Multiple Factorial Analysis

Multiple factorial analysis (MFA) has been developed to analyse simultaneously and compare qualitative or quantitative variables organized in

groups of the same kind. This method is well adapted to the simultaneous analysis of molecular, qualitative data of different types (isozymes, RFLPs) or agronomic, quantitative data. The principle of MFA is presented in this work (Perrier et al., 2002) and in greater detail in Escofier and Pages (1988).

Comparison between isozymes and nuclear RFLPs

We have used MFA, in the first place, to obtain a measurement of the relationship between the various molecular markers used in hevea. A preliminary MFA was done on 161 genotypes (89 Brazilian clones from the IRRDB collection and 72 Wickham clones) characterized by two groups of molecular variables: the isozymes (44 alleles for 11 loci) and nuclear RFLPs (113 bands for 25 genomic probes).

The results of the MFA are given in Table 3 and Fig. 5. The correlation values correspond to correlations between the coordinates of points (hevea genotypes) on the axes of factorial analyses. The number of axes to be taken into account depends partly on their eigenvalue. For MFA, an eigenvalue of the primary axis higher than 1 indicates that more than one group of variables contribute to it or, in other terms, that there exists an axis of inertia common to groups of variables (Escofier and Pages, 1988). On the other hand, for the subsequent axes of MFA, the eigenvalue is not a sufficient criterion and the choice of retaining an axis or not retaining it depends, as for any PCA, on the biological interpretation that one can make (Escofier and Pages, 1988). The quality of the representation and the contribution of a partial axis to an axis of MFA are also parameters to be taken into account, as for the interpretation of a classic PCA.

The first two axes of MFA indicate the close similarity between the genetic structuring revealed by the two types of markers, while it is essentially the RFLPs that contribute to the third axis of MFA (Table 3). The primary axis of MFA corresponds to the opposition between groups 4 and 6 (Tapajos river), on the one hand, and groups 1, 2 and 3, on the other. This opposition is shown in the second row for the isozymes and in the third for RFLPs (Table 3b; Fig. 5). It is less marked with RFLPs, as indicated by the values of correlation, quality of representation, and contribution (Table 3b). Even though there is an eigenvalue lower than 1, this axis of MFA, which corresponds to an axis of inertia common to two types of markers, should be retained. The third axis of MFA corresponds to the differentiation between groups 3 and 4. This opposition is clearly indicated by RFLPs (second axis of the multiple correspondences analysis or MCA), but does not appear clearly on the third axis of the PCA of isozymes, which present a lower correlation and contribution (Table 3b).

These elements of structuring are found on the first plane of partial analyses—separated PCA isozyme and MCA RFLP—but in a less marked fashion. Moreover, the MFA shows that the correlation between the two types

Table 3. MFA on isozymes and nuclear RFLPs for 161 clones of hevea. a: eigenvalues and part of inertia of primary axes of factorial analyses; b: correlation, quality of representation and contribution of axes of partial analyses (PCA and MCA)

a

Factorial analysis	Factors (axis)	Proper value	Inertia (%)
MFA	1	1.9	16
	2	0.6	5
	3	0.5	4
PCA isozymes	1	8.1	18
	2	3.2	7
	3	2.5	6
MCA RFLP	1	20	16
	2	7.4	6
	3	7.2	6

b

		Axis 1 of MFA		
Factorial analysis	Factors (axis)	correlation	quality	contribution
PCA isozymes	1	0.97	0.94	0.50
	2	0.04	0	0
	3	0.03	0	0
MCA RFLP	1	0.97	0.93	0.50
	2	0.01	0	0
	3	0	0	0

Axis 2 of MFA			Axis 3 of MFA		
correlation	quality	contribution	correlation	quality	contribution
0.05	0	0	0.08	0	0.01
0.87	0.75	0.53	0.08	0	0
0.14	0.02	0	0.58	0.34	0.22
0.04	0	0	0.09	0	0.02
0.17	0.03	0.02	0.80	0.64	0.49
0.73	0.53	0.34	0.07	0	0

of marker is very high for the most significant and most structured part of their variability.

Comparison between isozymes and morphoagronomic characters

The MFA was subsequently extended to the comparison between molecular diversity and phenotypic variability. The analysis was done on 171 Amazonian genotypes of the IRRDB collection for 51 isozymic alleles (13 loci) and 26 morphoagronomic variables (production, growth, architecture), without missing data.

The results are given in Table 4 and Fig. 6. The first axis of the MFA has an eigenvalue of 1.2—higher than 1, but still low—which means that the correlation between the two groups of variables is low. However, it is not nil because of the particular characters of the genotypes of group 4, very clearly differentiated by the isozymes (axis 1 of Fig. 6) and with production values tending to be higher than average. The second axis of the MFA must not be taken into account in this analysis because it does not allow us to draw a clear interpretation of the relation between the two groups of variables.

The results of the MFA thus suggest that there is a relationship between the diversity of neutral markers and the phenotypic variability in hevea. This relationship may seem to be in contradiction with the low correlation value between the primary axis of two partial analyses (Fig. 6). In fact, considering the sample size, this correlation of 0.13 is significant at 10%. Thus, the MFA

Table 4. MFA on isozymic and morphoagronomic data for 171 clones of hevea. a: eigenvalue and part of inertia of primary axes of factorial analysis; b: correlation, quality of representation, and contribution of axes of partial analysis (PCA) to the primary axis of MFA

a

Factorial analysis	Factors (axis)	Eigenvalue	Inertia (%)
MFA	1	1.2	10.5
	2	0.9	8.3
PCA isozymes	1	5.6	11
	2	3.9	7.6
Morphoagronomic PCA	1	12.7	49
	2	2.2	8.5

b

	Factors		Axis 1 of MFA	
Factorial analysis	Axis	Correlation	Quality	Contribution
PCA isozymes	1	0.67	0.45	0.56
	2	0	0.01	0.01
Morphoagronomic PCA	1	0.81	0.67	0.57
	2	0.03	0.00	0

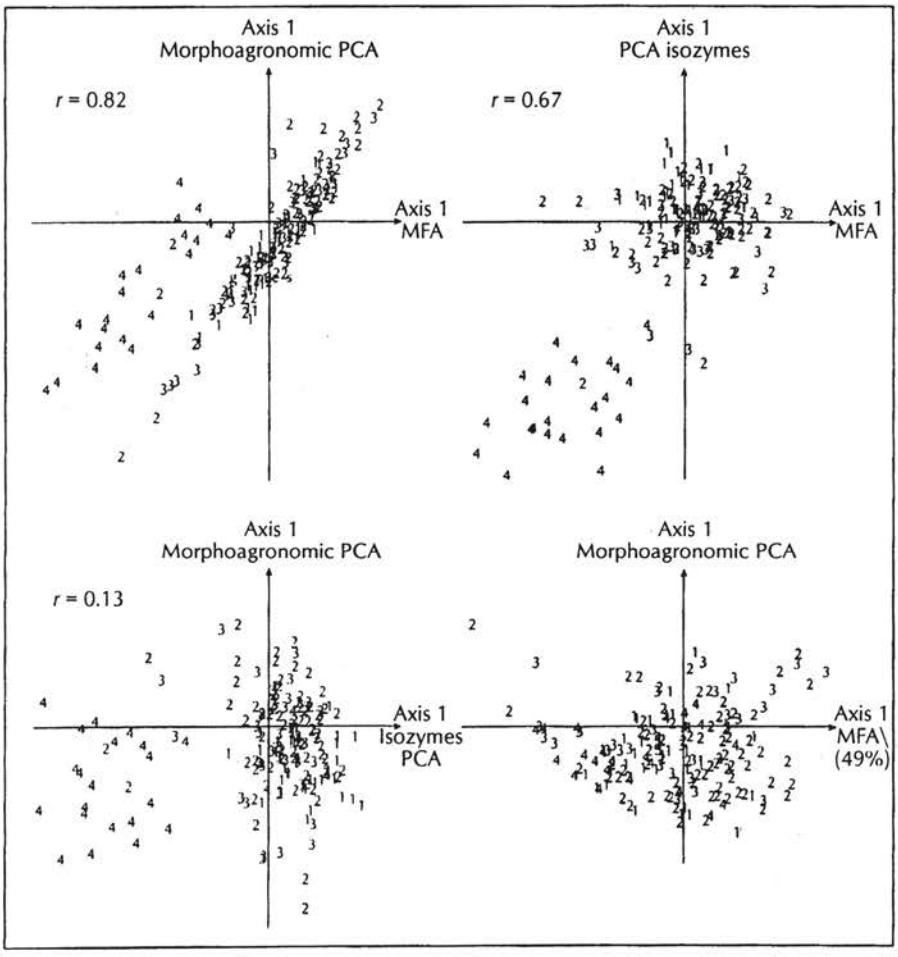

Fig. 6. Comparison by MFA of the diversity of isozymes and of the morphoagronomic variability in 183 Amazonian clones of the IRRDB collection. Graphic representation of the primary axes of MFA and of partial analyses (PCA). The numbers 1 to 6 correspond to the genetic groups identified by molecular markers (see text).

reveals a relationship that would be difficult to identify from separate analyses. The morphoagronomic data are imprecise because they are from single-tree measurements, without repetitions. That is why a differentiation does not appear with the data except when one works on larger numbers and average values, on the population scale (Fig. 1). The relationship between molecular diversity and phenotypic variability would appear undoubtedly more clearly if one uses a better evaluation of morphoagronomic characters. A close examination of the morphoagronomic PCA (Fig. 6) shows that the clones of

group 4 are, among the genotypes of other groups, situated on the same side of the primary axis; such a distribution cannot be retained as significant from this single partial analysis.

CONCLUSION AND PROSPECTS

Refined analysis of genetic diversity of hevea using molecular as well as phenotypic traits has greatly improved our understanding of the genetic organization of this plant. The input of molecular markers was critical. The results obtained independently by different techniques converged towards a clear picture of the genetic structure, which can be considered nearly definitive, at least for the Brazilian populations most extensively studied.

A high correlation has been found between the various molecular tools. For the Amazonian populations, the relation established between nuclear and mitochondrial diversity is particularly interesting. It proves that the differentiation between populations, though not very high, is quite real and that the gene flows between populations exist but remain of low intensity. The Amazonian basin does not present a geographic barrier to migration, but the vastness of the area considered and the limited dispersal of seeds helped maintain the inherited genetic structuring from the fragmentation of the forest at the Pleistocene, according to the model of forest refuge (Haffer, 1982; Prance, 1987).

The genetic similarity relationships between populations of hevea revealed by cytoplasmic and nuclear markers show that gene flows are determined mainly by the hydrographic network. Hevea seeds are dispersed along the water courses, either through human action (Seibert, 1948) or by natural flotation, as has been found with other Amazonian tree species (Goulding, 1993). A similar structuring of diversity, depending on the hydrographic network, has been found in another perennial Amazonian species, the American oil palm *Elaeis oleifera* (Barcelos, 1998; Barcelos et al., 1999).

The different types of molecular markers have given results that are overall in accordance, which confirms the MFA, but each contributes specific information. The maternal heredity of cytoplasmic markers allows a better appreciation of gene flows between populations—between group 2 and the RO/C district, for example—or the foundation effects, as for the Wickham populations, which are nearly monomorphic at the mitochondrial level.

Certain differentiations between groups were clearly revealed only by isozymes or RFLPs. In nuclear terms, that can be attributed to differences in the sampling of populations (number of genotypes analysed) or of the genome (number of markers used). It would thus be useful to characterize the diversity of genetic resources of hevea using markers that are mapped and distributed throughout the genome. The development of microsatellite markers

(Lespinasse, 1993; Seguin et al., 1997) and the publication of a dense molecular genetic map of hevea allows us to consider such a study on a sufficient number of genotypes (Lespinasse et al., 2000b). Thanks to PCR-based markers such as microsatellites, it will be possible to use high output genotyping techniques for diversity studies in tropical crops such as rubber tree. This will allow a more powerful analysis of population genetic organization and history, through the assessment of linkage disequilibrium between markers or genes.

It is interesting to note the existence of a relationship between molecular diversity and agronomic variability in hevea. The correlation is however much lower than between the different molecular markers themselves and the MFA has proved useful to make that apparent on a subsample of genotypes. The relationship between the two levels of variability—genetic and phenotypic—needs to be studied from more precise phenotypic data, collected from a larger sample of genotypes. It would also be useful to apply MFA using principal coordinates analysis (PCoA), which would provide a better basis for choices of indexes of distance, since the χ^2 distances (in CA or MCA) and Euclidean distances (in PCA) are not necessarily the most suitable from a biological point of view.

Our results indicate that differentiation in hevea populations is great enough to affect all the genomes and concerns a large number of genes, that is neutral markers as well as genes that determine phenotypic characters. In consequence, in the present state of our understanding, structuring of *Hevea brasiliensis* diversity in six genetic groups will be very useful in defining a sampling strategy for genetic diversity studies of this species.

APPENDIX

Plant Material

The genotypes retained in this study are hevea clones from the international collection of CNRA (Centre National de la Recherche Argonomique) in Côte d'Ivoire. The geographic origin of populations of clones is specified in Table 1. All the clones were evaluated in the field for the characters of latex production, growth in thickness, and architecture, but in different assays (Chapuset et al., 1995). The most important assay comprised 2500 clones of the IRRDB expedition of 1981, without repetition (1 tree per clone). Molecular markers were applied to samples of the collection, of variable size and coverage. The numbers used in these different studies are summarized in Table 2.

Methods of Detection of Diversity

The protocols used for the study of genetic and agronomic diversity are described by Chapuset et al. (1995) for morphoagronomic evaluation on 26 variables, by Lebrun and Chevalier (1990) for isozymes, by Besse (1993) and Besse et al. (1994) for nuclear RFLP with 25 genomic probes, and by Luo et al. (1995) for cytoplasmic RFLPs, mtDNA, and cpDNA.

The technique of isozymes uses 12 enzymatic systems, which enable the detection of up to 14 polymorphic loci in the species *H. brasiliensis* (Chevallier, 1988; Chevallier et al., 1988; Seguin et al., 1995a, b). Several hundreds of genotypes have been characterized by the isozymes (Table 2) using these 12 systems, but with a variable number of missing data according to the genotypes or the series of manipulations. This is why the data were analysed for only 8 of 13 isozyme loci depending on the experiment. To have a sufficient number of genotypes, it was often necessary to reduce the number of loci to limit or eliminate the missing data.

For nuclear RFLPs, 25 homologous probes corresponding to single sequences or nearly unrepeated sequences in the genome of hevea (genome bank *Pst*1) were used. These probes were coupled with two restriction enzymes, *Eco*R1 and *Sst*1 (Besse et al., 1994; Seguin et al., 1995a). For cytoplasmic RFLP, the probes come from banks of broad bean (Luo et al., 1995).

Data Analysis

Data were treated mainly by factorial analysis, the use of which is discussed in this work (Perrier et al., 2002). Moreover, interpretation of results is easier with factorial analysis than with the tree methods when one works on a large number of individuals.

The agronomic, quantitative data were treated by PCA. The data of presence/absence of RFLP bands were treated by CA on a binary table, which in turn is based on an MCA. The data of isozymic alleles were coded, 0 for absence, 1 for presence at the heterozygous state and 2 for presence in the homozygous state, for each genotype. These data were treated by CA or by PCA in the MFA (Perrier et al., 2002). For the CA, only bands or alleles of frequency between 2 and 98% in the total sample were retained as variables. In the MFA, all the alleles were retained, no matter what their frequency.

Diversity has also been analysed at the population level by analysis in hierarchical clustering using, for the isozymes, the Nei distance (1978) on the allelic frequencies and, for the agronomic, quantitative data, the city block distance on the means per population. The trees were constructed by the UPGMA method. The organization of genetic material in subpopulations was studied by calculations of *F* statistics, by which departure from panmixia was evaluated and the ratio of intra- and interpopulation diversities was quantified.

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