Genetic Resources

6

Genetic diversity plays a crucial role in the stability of our ecological system. Every species fulfils a role in the earth's biosphere and assures ecological survival. By this, biodiversity keeps the soil fertile, recycles all nutrients and cleans the air and water. The richer the genetic baggage, the higher shall be the capacity to fight different fungi, virus or bacteria. It is the diversity of genetic baggage that makes natural extinction so rare. Basically, biodiversity provides everything humans need to survive, like food, fresh air, clean water, clothing, medicine, wood and various raw materials for industrial uses. A rich ecological environment is indeed very complex and is impossible for humans to recreate. Genetic erosion is the loss of genetic diversity—often magnified or accelerated by human activities. Quite often, cultivation of a limited number of high-yielding genotypes can also lead to genetic erosion. Much of the diversity of the centre of origin of *Hevea* (Amazon basin, Brazil) is being lost due to extensive deforestation (see Chap. 2).

6.1 *Hevea* as a Species Complex

The genus *Hevea* includes ten species, which are inter-crossable (Clément-Demange et al. 2000). Schultes (1977a, b) and Wycherley (1992) refer readers to excellent reviews on the subject. The taxonomic considerations from 1874 to 1970 delineated the genus with several species on different occasions. Although the genus was considered to include 24 species in 1906, the species concept crystallized with nine species in 1970 (Schultes 1977a, b). The tenth species, *Hevea camargoana*, was added in 1971 (Schultes 1987). *Hevea paludosa* has been identified in Brazil and is often considered as the 11th species (Pires 1973; Goncalves et al. 1990). Three botanists are considered to be the principal workers on species delineation—Baldwin, Seibert and Schultes—who during their classical exploratory studies contributed significantly towards the botany of *Hevea*. A *Harvard University Gazette* (from the archive) says 'Schultes' field work, conducted mostly in the Colombian Amazon beginning in 1941, made him a leading voice in the field and one of the first in the 1960s to warn about destruction of the rainforests and disappearance of their native people' (Dijkman 1951).

The ten species recognized today as belonging to the genus *Hevea* are: *H. brasiliensis*, *H. guianensis*, *H. benthamiana*, *H. pauciflora*, *H. spruceana*, *H. microphylla*, *H. rigidifolia*, *H. nitida*, *H. camporum* and *H. camargoana* (Webster and Paardekooper 1989; Wycherley 1992; Schultes 1990a, b) (see Figs. 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7 and 6.8). Seven species are found in the upper Rio Negro region, considered to be the centre of origin of the genus. *H. brasiliensis* is found in southern areas outside this centre, in the upper Rio Madeira, where five other species are represented. It has generally been assumed that the species are freely inter-compatible (Baldwin 1947; Seibert 1947). Pires (1981)

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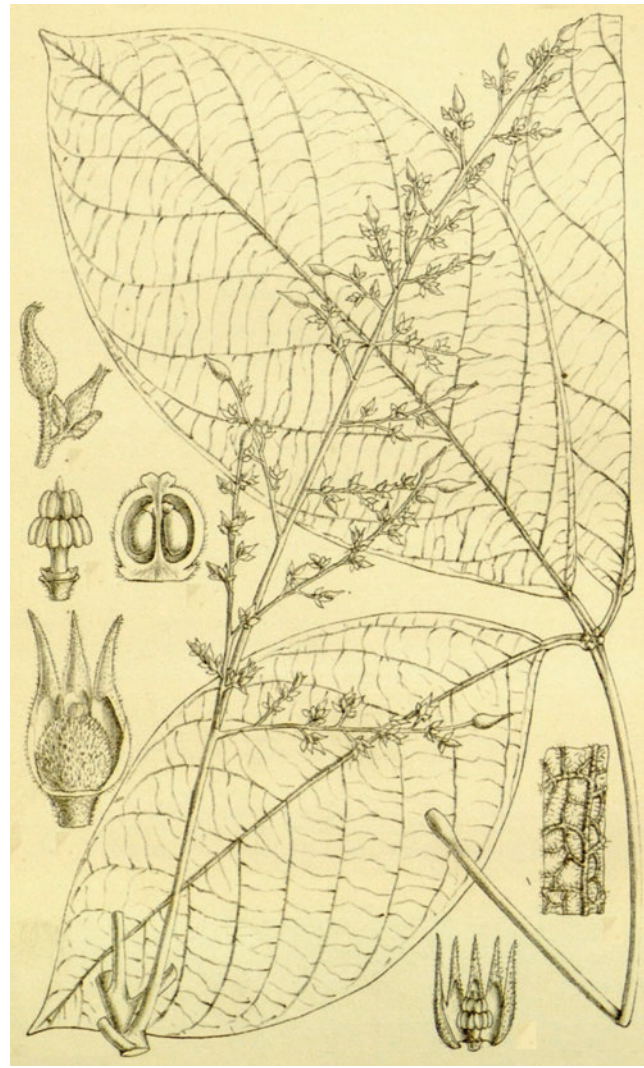

Fig. 6.1 *Hevea benthamiana* Müll. Arg

observed natural hybrids of *H. camargoana* × *H. brasiliensis*, and Gonçalves et al. (1982) analysed the progeny derived from hand pollination from this type of crossing. Consequently, *Hevea* species might be considered as a species complex due to the absence of a strict barrier to recombination between species. Many efforts have led to the identification of certain types which were formerly presented as other possible species. *H. paludosa* was identified in Brazil by Ule in 1905 and is often considered as an 11th species (Goncalves et al. 1990; Priyadarshan and Gonçalves 2003). An elaborate description of taxonomical and botanical aspects of *Hevea* has been reviewed by Schultes (1977a, b, 1987, 1990a, b) and Wycherley (1992). A summary of the salient features of different species of *Hevea* is presented in Table 6.1.

The species are inter-crossable (Clément-Demange et al. 2000). Schultes (1977a, b) and Wycherley (1992) refer the readers to excellent reviews on this subject. The taxonomic considerations from 1874 to 1970 delineated the genus with several species on different occasions. Even though 24 species were considered during 1906, the species concept crystallized with nine species in 1970 (Schultes 1977a, b). A tenth species, *H. camargoana*, was added during 1971 (Schultes

Fig. 6.2 *Hevea camporum* Ducke

1987). Brazil considers 11 species including *H. paludosa* (Pires 1973; Goncalves et al. 1990). Three botanists shall be considered principal workers on species delineation—Baldwin, Seibert and Schultes—who during their classical exploratory studies contributed significantly towards the botany of *Hevea*. The *Harvard University Gazette* (from the archives) says 'Schultes' field work, conducted mostly in the Colombian Amazon beginning in 1941, made him a leading voice in the field and one of the first in the 1960s to warn about destruction of the rainforests and disappearance of their native people' (see www.harvard.edu).

6.1.1 Distribution of Allied Species

The distribution of allied species of *Hevea* is wide among the countries of South America. *Hevea* species are indigenous to Bolivia, Brazil, Colombia, French Guiana, Guyana, Peru, Suriname and Venezuela. All species except *H. microphylla* occur in Brazil, the centre of origin (Goncalves et al. 1990). Four species have been found in Colombia and three occur in Venezuela. Two occur in Bolivia, French Guiana and Guyana (Fig. 6.9 a and b). *H. guianensis* is the most widely adapted species (Pushparajah 2001). Temperate type rubber thrives up to 2500–3000 m in the Andes Mountains (Senyuan 1990). These species of *Hevea* evolved in the Amazonian forests over 100,000 years ago (Clément-Demange et al. 2000). It is pertinent that species adaptation to a particular area is as per climatic and edaphic requirements. The centre of diversity lies within the constantly humid equatorial zone where the amount of precipitation is at least twice the evaporation losses on a yearly basis (Pushparajah 2001). Species like *H. camporum*, *H. paludosa* and *H. rigidifolia* show only limited adaptation. The specific adaptation needs to be closely studied, with reference to climatic and edaphic factors, when clones are to be developed for new environments, especially for marginal areas. It is worthwhile noting that except for *H. benthamiana*

Fig. 6.3 *Hevea guianensis* Aublet

(clones F 4512, F 4542) none of the other species have been utilized for the improvement of the rubber tree.

All *Hevea* species have $2n = 36$ chromosomes, with the exception of one triploid clone of *H. guianensis* ($2n = 54$) and the existence of one genotype of *H. pauciflora* with $2n = 18$ (Baldwin 1947; Majumder 1964). Although *Hevea* behaves as a diploid, it is believed to be an amphidiploid ($2n = 36$; $x = 9$) that stabilized during the course of evolution. This contention is supported by the observation of tetravalents during meiosis (Raemer 1935; Ong 1976; Wycherley 1976). In situ hybridization studies revealed two distinct 18S–25S rDNA loci and one 5S rDNA locus, suggesting a possible allotetraploid origin with the loss of 5S rDNA during the course of evolution (Leitch et al. 1998). But locus duplications are infrequent in the *Hevea* genome, and they could have occurred due to chromosomal modifications posterior to the polyploidization event (Seguin et al. 2003); consequently, the two unknown ancestral genomes of *Hevea* would have strongly diverged.

Low and Bonner (1985b) characterized the *Hevea* nuclear genome as containing 48% of

Fig. 6.4 *Hevea microphylla* Ule

Fig. 6.5 *Hevea nitida* Muell-Arg

Fig. 6.6 *Hevea pauciflora* (Spr.ex Penth) Muell.-Arg. Var coriacea Ducke

Fig. 6.7 *Hevea rigidifolia* (Spruce ex. Benth)Muell.-Arg

Fig. 6.8 *Hevea spruceana* (Benth) Muell. Arg

slowly annealing DNA (putative single copy) and 32% middle repetitive sequences with the remaining DNA being highly repetitive or palindromic. The size of the whole nuclear genome was first estimated as 6×10^8 base pairs. An estimation of the size, using flux cytometry, demonstrated 1.9×10^9 base pairs for *H. brasiliensis*, *H. benthamiana*, *H. guianensis*, *H. pauciflora* and *H. spruceana* (Seguin et al. 2003). The evolution of the cytoplasmic genome was slower due to the lack of genetic recombination through meiosis. The estimated mean molecular size of chloroplast DNA (cpDNA) is 152 kb (Fong et al. 1994). Differentiation of the genus into species appears to be linked with the evolution of the Amazonian forest over the last 100,000 years. Alternations of humid and semi-arid periods responsible for the forest extension or fragmentation resulted in the formation of forest islets. These are assumed to have become zones of protection and differentiation under local selection pressures.

6.1.2 New Genetic Resources

The 'Wickham' population developed in Asia, issued from the collection of seeds in Brazil by Wickham in 1876, has been the basis for rubber domestication and was reputed to have a narrow genetic base. This justified the organization of other collections and transfers of wild germplasm from Amazonia to the main rubber-producing countries, mainly for *H. brasiliensis* but also for allied species. Moreover, the Ford Company and Firestone (companies owning rubber estates in Latin America) as well as Brazilian research contributed to the creation of a stock of selected 'Amazonian' and 'Wickham × Amazonian' germplasm (F, FX, MDF, FDR, IAN, IAC clones). From Brazil to Asia (Dean 1987; Baulkwill 1989), it is difficult to evaluate how narrow the genetic base initially was for what has now become the 'Wickham' domesticated population. Much importance was conferred to a small

Table 6.1 Allied species of the genus *Hevea* - occurrence and features

Species	Occurrence	Notable features[a]
H. benthamiana Muell.-Arg.	North and West of Amazon forest basin, upper Orinoco basin (Brazil)	Complete defoliation of leaves. Medium-size tree. Habitat: swamp forests
H. brasiliensis (Willd. ex. A. de. Juss.) Muell.-Arg.	South of Amazon River (Brazil, Bolivia, Ecuador, Peru)	Complete defoliation of leaves. From medium to big tree size. Habitat: well-drained soils
H. camargoana Pires	Restricted to Marajo island of Amazon River delta (Brazil)	Possibility of natural hybridization with *H. brasiliensis.* From 2to 25 m tree height. Habitat: seasonally flooded swamps.
H. camporum Ducke	South of Amazon between Marmelos and Manicoré rivers tributaries of Madeira river.	Retain old leaves until new leaves appear. Maximum 2 m tall. Habitat: dry savannahs.
H. guianensis Aublet	Throughout the geographic range of the genus (Brazil, Venezuela, Bolivia, French Guyana, Peru, Colombia, Surinam, Ecuador)	Retain old leaves until new leaves and inflorescences appear. Grows at higher altitudes (1100 m MSL) Medium-size tree. Habitat: well-drained soils.
H. microphylla Ule	Upper reaches of Negro River in Venezuela. It is not found in other region of geographic range of the genus	Complete defoliation of leaves. Small trees. They live on flooded area (igapós). Habitat: sandy or *lateritic* soils
H. nitida Mart. ex Muell.-Arg.	Between the rivers Uaupes and Icana tributaries of the upper Negro River (Brazil, Peru, Colombia).	Inflorescences appear when leaves are mature. Small- to medium-size trees (2 m). Habitat: *quartzitic* soils.
H. pauciflora (Spr.ex Bth.) Muell.-Arg.	North and West of Amazon River (Brazil, Guyana, Peru). Distribution discontinuous due to habitat preferences.	Retain old leaves until new leaves and inflorescences appear. No wintering. Small to big size trees. Habitat: well-drained soils, rocky hill sides.
H. rigidifolia (Spr. ex Bth.) Muell-Arg.	Among Negro River and its *affluents.* Uaupes and Içana Rivers (Brazil, Colombia and Venezuela)	Retain old leaves even after inflorescences appear. Small tree from savannahs. Sometime tall, with small crown on the top. Habitat: well-drained soils.
H. spruceana (Bth.) Muell.- Arg.	Banks of Amazon, Rio Negro and lower Madeira (Brazil)	Retain old leaves until new leaves and inflorescences appear. Flowers reddish purple. Medium-size tree. Habitat: muddy soils of islands
H. paludosa Ule[b]	Marshy areas of Iquitos, Peru	Small leaflets, narrow and thin in the fertile branches; up to 30 m. height. Habitat: marshy areas.

After Wycherley (1992), Schultes (1977a, b) Goncalves et al. (1990), Pires (1973) and Brazil (1971)

[a]Wintering characteristics mentioned here has a bearing on the incidence of fungal diseases especially secondary leaf fall (Oidium) since retention of older leaves may make the tree 'oidium escape'. Dwarf types are desirable of the possible wind fastness. All species are diploid ($2n = 36$) (Majumder 1964), and are crossable among themselves (Clément-Demange et al. 2000)

[b]Pires (1973) considered 11 species including H. paludosa; Brazil (1971) considers 11 species

number of 22 seedlings disseminated from Singapore to Malaysia after 1876, but a significant part of the Wickham seedlings which germinated in Kew Botanic Gardens was then sent to Ceylon (now Sri Lanka), raised and disseminated to different countries, especially India. However, it must be underlined that the original Wickham stock was collected in only one Brazilian site, Boïm, on the western banks of the Tapajós river, not far from Santarem. From then, directional selection applied to this population for more than one century and the limitation of the low fruit set

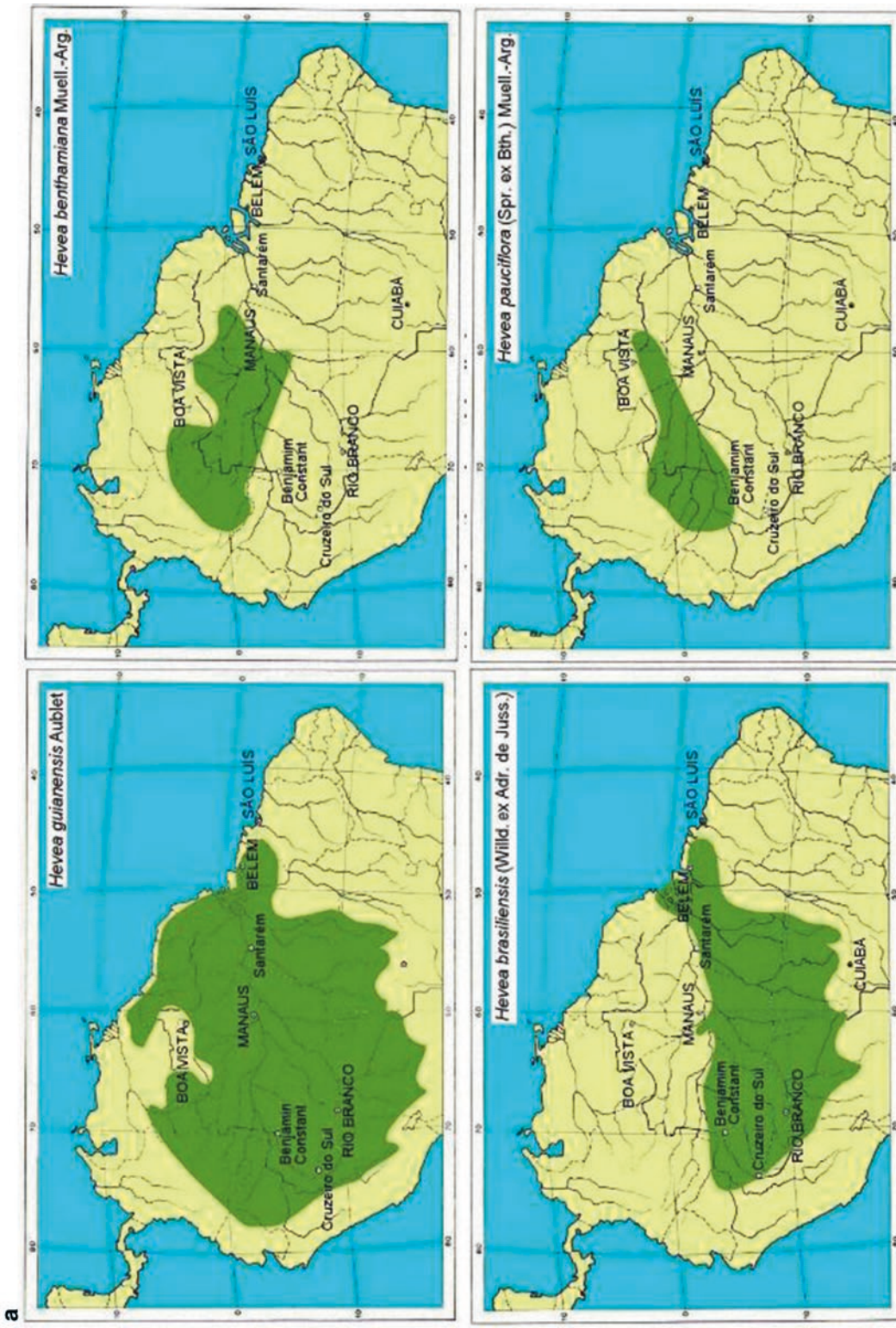

Fig. 6.9 (**a**) and (**b**) Distribution of *Hevea* species in the Amazon (Adopted from Brazil, 1971)

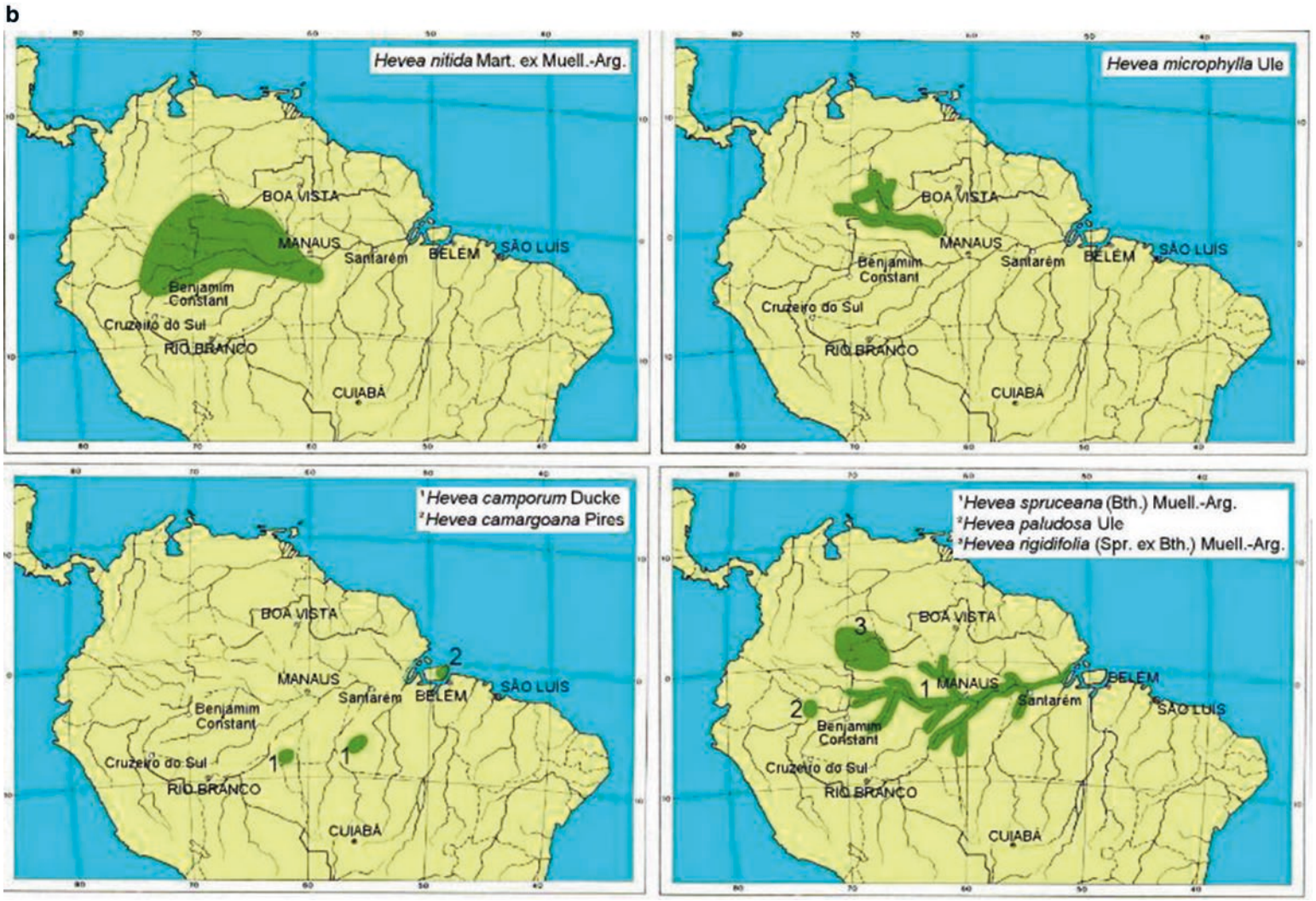

Fig. 6.9 (continued)

in *Hevea* probably further contributed to reducing the extension of this genetic base. Genetic diversity can now be compared with that of the available wild Amazonian populations by use of molecular genetic markers.

Many other introductions from Brazil to Asia and also Africa were carried out between 1896 and 1974, including some species that differed from *H. brasiliensis* (Dijkman 1951; Brookson 1956; Baptist 1961; Wycherley 1968; Hallé and Combe 1975; Nicolas 1976; Ong et al. 1983; Ong and Tan 1987; Tan 1987). All collections were quantitatively rather limited, especially for non-*brasiliensis* species.

In 1981, the IRRDB organized an international collection in Brazil predominantly of seeds, but also of budwood and seedlings (Nicolas 1981; Nouy 1982; Tan 1987; Simmonds 1989). This collection was carried out over three states (Acre, Rondonia and Mato Grosso) from 60 different locations spread over 16 districts. It resulted in the provision of around 10,000 new accessions for breeding. Of this, 37.5% of the seeds were sent to Malaysia and 12.5% to Côte d'Ivoire. Half of the collections were maintained in Brazil. The accessions from the budwood collection were brought to Malaysia and Côte d'Ivoire after a quarantine period of 1 year on the island of Guadalupe (as a protection from SALB disease). After the establishment of two IRRDB germplasm centres in Malaysia and Côte d'Ivoire, other IRRDB member countries were supplied with budwood from this material according to their request.

The field evaluation of this wild Amazonian germplasm showed that the latex yield was as low as about 10% of GT 1, one of the most cultivated clones. Attempts to improve it through Wickham × Amazonian crosses resulted in recombinants that still had a low yield, ranging between 30% and 50% of the level of GT 1, probably due to the important genetic gap lying between the two populations. Conversely, a wide variability was found within these crosses for growth, enabling the selection of very vigorous Wickham × Amazonian clones. A clear difference in branching habit could be observed between accessions from Acre and Rondonia, which more often have tall trunks with poor branching located at a high height, and those from Mato Grosso, which display abundant branching at a low height. Obviously, this wild Amazonian germplasm is bearing an important genetic burden in terms of unfavourable alleles. From the evaluation of IRRDB 1981 germplasm in Côte d'Ivoire, a working population of 287 accessions was selected, taking into account genetic diversity but mainly based on yield; the average yield level of this population is estimated at 36% of the level of GT 1 (Nicolas et al. 1988; Clément-Demange et al. 1998). Four genetic groups of this population could be the basis of pre-breeding work aimed at improving their yield level before testing them by crossing with the Wickham population. In 1995, an expedition was launched by the Rubber Research Institute of Malaysia (RRIM) to collect rubber seeds from Brazil. From this collection, about 50,231 seedlings were planted in Malaysia, including allied species (RRIM 1997; MRB 1999). In order to enlarge the genetic variability of *Hevea*, some research was carried out on mutation breeding (Ong and Subramaniam 1973; Markose et al. 1977) and on polyploidization of the $2n = 36$ *H. brasiliensis* species (Mendes and Mendes 1963; Shepherd 1969; Zheng et al. 1980, 1981). An artificial triploid has been produced by crossing a diploid and a tetraploid (Saraswathyamma et al. 1988). Naturally occurring triploids have also been reported (Nazeer and Saraswathyamma 1987). The existence of some putative genetically dwarf or semi-dwarf genotypes have been mentioned (Ong et al. 1983); *H. camargoana* would have a dwarf growth habit (Gonçalves et al. 1982). Some molecular genetic markers were associated with the dwarfing trait (Venkatachalam et al. 2004).

6.2 Molecular Diversity

The association between DNA sequence variation and heritable attributes has helped to define variations in plants at the molecular level. However, identification and utilization of recombinants with desirable traits is time con-

suming and laborious in rubber due to long generation time and larger size of the crop. With the advent of DNA markers, localization of desirable traits has become routine. The molecular marker systems can be broadly classified into three, viz., first-generation (restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD) and modifications); second-generation (simple sequence repeats (SSRs), amplified fragment length polymorphisms (AFLPs)) and third-generation markers (expressed sequence tags (ESTs), single nucleotide polymorphisms (SNPs)) (Gupta et al. 2001). Of these, SNPs are the new-generation markers used for marker-assisted selection (MAS). All marker systems, except SNPs, have been applied in *Hevea* to facilitate identification and characterization of genes (Saha and Priyadarshan 2012). Recently, a saturated linkage map of *H. brasiliensis* has been accomplished (Lespinasse et al. 2000a). Efforts were on for breeding *Hevea* at the molecular level ever since Low and Bonner (1985b) characterized nuclear genomes containing 48% of most slowly annealing DNA (putative single copy) and 32% middle repetitive sequences with remaining highly repetitive or palindromic ones. Also, the whole genome size was calculated as 6×10^8 base pairs.

Low and Bonner (1985b) characterized *Hevea* nuclear genome as containing 48% of slowly annealing DNA (putative single copy) and 32% middle repetitive sequences with remaining highly repetitive or palindromic DNA. The whole nuclear genome size was first estimated as 6 × 108 base pairs. Estimation with flux cytometry demonstrated 1.9 × 109 base pairs for *H. brasiliensis*, *H. benthamiana*, *H. guianensis, H. pauciflora, and H. spruceana* (Seguin et al. 2003). The evolution of cytoplasmic genome was slower, due to the lack of genetic recombination through meiosis. The estimated mean molecular size of chloroplast DNA (ct DNA) is 152 kb (Fong et al. 1994). Differentiation of the genus into species appears to be linked with the evolution of the Amazonian forest over the last one hundred thousand years. Alternations of humid and semi-arid periods responsible for the forest extension or fragmentation resulted in the formation of forest islets. These are assumed to have become zones of protection and differentiation under local selection pressures.

Seguin et al. (2003) proposed a general organization of *H. brasiliensis* germplasm with 6 genetic groups: *group 1* made up with the two districts AC/T (Tarauaca) and AC/F (Feijo) in the western part of Acre, and with the Calima component of the Schultes' collection; *group 2* made up with the three districts AC/B (Brasileia), AC/S (Sena Madureira), and AC/X (Xapuri) in the eastern part of Acre; *group 3* made up with the six following districts of Rondonia: RO/A (Ariquemenes), RO/C (Calama), RO/CM (Costa Marques), RO/J (Jaru), RO/JP (Jiparana), RO/OP (Ouro Preto), the district MT/VB (Vila Bella) of Mato Grosso, and accessions MDF (Madre de Dios Firestone) from the Firestone collection in Peru; *group 4* made up with three districts MT/A (Aracatuba), MT/C (Juruena), and MT/IT (Itauba) of Mato Grosso, and the district RO/PB (Pimenta Bueno) of Rondonia; *group 5* made up with the Palmira component of the Schultes collection and *group 6* made up with the domesticated Wickham population (Fig. 6.10). Even if no prediction can be made about the progenies of crosses between these groups, they can be used as a base for managing the genetic variability in the long term and organizing the recombination process. Methodological researches have been carried out in order to select the genotypes for making up a collection of reduced size of the Amazonian germplasm, representative of the predominant part of the total variability of this germplasm, according to the concept of 'core collection' (Hamon et al. 1998). The germplasm characterization and diversity analysis studies coordinated by Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD) were funded by the European Union from 1985 to 1997. On the contrary, Lekawipat et al. (2003) used twelve microsatellite markers to detect DNA polymorphism among 108 accessions of *H. brasiliensis* including 40 Wickham clones and 68 wild accessions (1981 Amazonian accessions). Genetic similarity values between genotypes calculated from all the

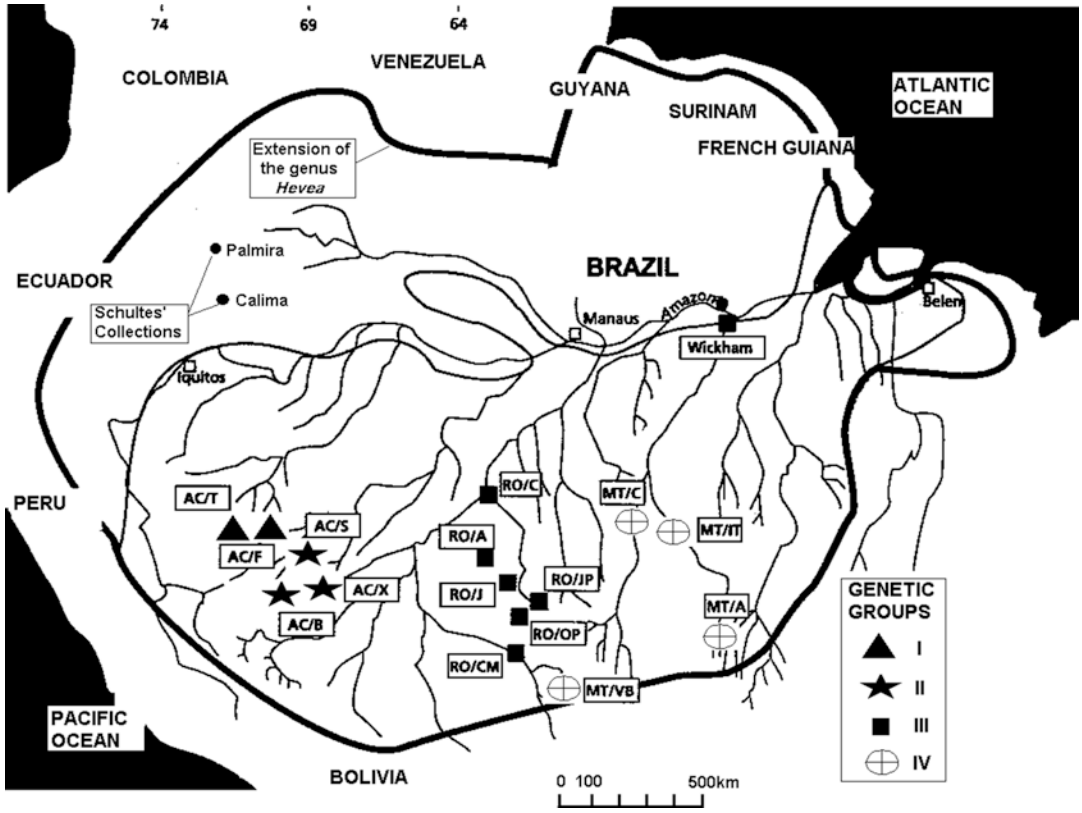

Fig. 6.10 Geographical origin of *Hevea* clone analysed with isozymes or RFLP markers for genetic diversity assessment

microsatellite markers were used to produce a dendrogram of the relationship among accessions, using the unweighted pair-groups method with arithmetic average. A total of 170 alleles were detected. The number of alleles ranged from 5 to 21, with an average of 14 alleles per marker. The results clearly demonstrated that wild accessions are more polymorphic than cultivated Wickham clones and could be divided into three clusters, depending on the geographical origin of collection areas such as Acre, Rondonia and Mato Grosso state. Despite the narrow genetic basis of Wickham clones, their high level of polymorphism could be detected. García et al. (2011) designated primers from sequences reported in GenBank using Primer3, PrimerQuest and OlgoPerfect software for PCR amplification of microsatellites. The primers so obtained were thermodynamically analysed by Oligo Analyzer 3.1 software and experimentally evaluated on 12 *Hevea* clones (GT1, PB 235, PB 260, RRIM 600, IAN 710, IAN 713, IAN 873, FX 3864, FX 3899, GU 198, AVROS 1581 and AVROS 2037). Four microsatellite markers were seen to be sufficient for discriminating 10 of the 12 clones. Clustering analysis showed narrow genetic base of Brazilian clones compared to Asiatic ones.

6.3 Gene Flow and Paternity Identification

Pre-breeding of the Amazonian genetic groups was considered based on recombination through seed gardens. For methodological purposes, one seed garden made up with 50 Amazonian genotypes and GT1 clone, planted at CNRA (Côte d'Ivoire), was subjected to the analysis of gene flux and paternity identification with isozymes and microsatellites (Blanc et al. 2001; Lidah

2005). Paternity identification with microsatellites was carried out with the Cervus software (Marshall et al. 1998). A high level of confidence was found for paternity identification carried out with 8 microsatellite markers. The distribution of the contribution of the different genotypes to pollination was found to be highly unequal, with 4 genotypes accounting for 40%, 14 genotypes accounting for 80% and 25 genotypes accounting for 95% of the total fertilization of the seed garden. The variation of selfing rate was assessed among the genotypes with an average of 5%, and no selfing was found on GT1 as expected for a male sterile clone. The isolation of the seed garden was confirmed since no allele other than those belonging to the parental population was found. The efficiency in paternity identification which is made possible by microsatellites suggests the new possibility to exercise selection on seedlings raised from natural pollination and to identify paternity a posteriori only on the best trees. Here, male sterile clones GT 1 and BPM 24 can be used to ensure cross pollination (see Sect. 7.5.2).

6.4 Genetic Mapping

The availability of numerous molecular genetic markers (MGMs) led to the development of genetic linkage mapping based on the analysis of the percentage of crossing over between the loci of two markers during meiosis (a genetic and not a physical distance) and the ranking of the different loci on the different chromosomes of one species. Due to the heterozygous nature of rubber clones, the construction of genetic linkage maps in *Hevea* requires specific methodology. Unlike annual crops, a cross between two heterozygous parents in *Hevea* can yield information up to four alleles, which are segregated further. The first comprehensive genetic linkage map of *H. brasiliensis* has been built recently, mainly by use of RFLP markers but also AFLPs, microsatellites and isozymes (Lespinasse et al. 2000a). This was accomplished through a double pseudo-test cross as per the methodology of Grattapaglia and Sederoff (1994), and a map was constituted separately for each parent. Further, homologous markers segregating in both parents were ascertained and consensus map prepared. The parents used were PB260 and RO38. The F_1 synthetic map of 717 markers was distributed in 18 linkage groups corresponding to the 18 chromosomes. This comprised of 301 RFLP, 388 AFLP, 18 microsatellite and 10 isozyme markers. The genetic length of the 18 chromosomes was fairly homogeneous, with an average map length per chromosome of 120 cM. Many AFLP markers were seen in clusters, which were attributed as reduced recombination frequency regions. Though the RFLP markers were well distributed all over the 18 linkage groups, these were insufficient to saturate the map. AFLPs and a few microsatellites together contributed to saturating the map. A partially non-random arrangement of duplicate loci observed in RFLP profiles indicate that they have homology descending from a common ancestor (Lespinasse et al. 2000a). The origin of such duplications is still unknown and *H. brasiliensis* continues to behave as a diploid.

In yet another study, Souza et al. (2011) found 603 microsatellite markers, with 309 of them (51%) showing polymorphism. Chi-square test carried out on the genotyping polymorphic loci showed that 110 loci followed a segregation ratio of 1:1, 28 followed a ratio of 1:2:1 and 87 (38.7%) followed a ratio of 1:1:1:1. The map consists of 225 markers, distributed in 23 linkage groups (LG) and 2471.2 cM in length with an average genetic distance of 11 cM between adjacent markers. The largest group has 215.9 cM (18 markers) and the smallest has 2.71 cM (2 markers). This reflects a real polymorphism in a full-sib cross.

Genetic linkage maps associated with phenotyping studies (field evaluation of the genotypes) can generate phenotypic comparisons between a huge number of classes of alleles and lead to the identification of quantitative trait loci (QTL). The research developed on the cross PB260 × RO38 was targeted to understanding the genetic determinism of the resistance of this cross to SALB, first with manual infection at the laboratory level (Lespinasse et al. 2000b). Eight QTLs, with one predominant on linkage group

g13, were identified for resistance in RO38 map through Kruskal-Wallis marker-by-marker test and interval mapping method (Lander and Botstein 1989; van Oojen et al. 1992). The F_1 consensus map confirmed results obtained in parental maps. It was further rationalized that the resistance alleles of RO 38 have inherited from its wild grandparent (*H. benthamiana*) and no favourable allele came from AVROS 363, the Wickham parent. Eight different QTLs for five strains of fungus were found available in RO38, with specificity of resistance to different strains. Field evaluation against the pool of *Microcyclus* strains available in French Guyana was carried out under the real infestation conditions, and it confirmed the presence of the predominant QTL in g13 previously found under controlled infestation (Le Guen et al. 2003). Then it was shown that this major QTL was no more efficient against two widely virulent and highly aggressive strains; for one of them, another QTL located on the linkage group g12 was able to reduce the aggressiveness. This genetic mapping and QTL approach is currently being continued with other crosses for analysing the genetic determinism to different sources of South American Leaf Blight (SALB) resistance. Research for identifying and cloning the real genes responsible for this QTL in linkage group g13 is undertaken at CIRAD in the framework of the building of a bacterial artificial chromosome (BAC) bank and of a physical map of the rubber tree genome based on the clone RO38 that inherited the resistance trait from F4542. Among other applications, this will make possible the search for the DNA fragments bearing the QTL g13 and the development of the 'chromosome walking' technique towards genes associated with QTL g13 on these fragments. This physical map with a high density of MGMs (fine mapping) will also allow one to assess the stability of linkage between the neighbouring genetic markers.

Studies on microsatellite markers and their transferability allied species is an upcoming area of research. Mantello et al. (2012) constructed di- and trinucleotide-enriched libraries. From these two libraries, 153 primer pairs were designed and initially evaluated using 9 genotypes of *H. brasiliensis.* A total of 119 primer pairs had a good amplification product, 90 of which were polymorphic. A total of 46 polymorphic markers were characterized in 36 genotypes of *H. brasiliensis*. The expected and observed heterozygosities ranged from 0.1387 to 0.8629 and 0.0909 to 0.9167, respectively. The polymorphism information content (PIC) values ranged from 0.097 to 0.8339, and the mean number of alleles was 6.4 (2–17). The microsatellites were also tested in 6 other *Hevea* species. The percentage of transferability ranged from 82% to 87%. Locus duplication was found in *H. brasiliensis* and also in 5 of other species in which transferability was tested. Six other species (*H. guianensis, H. rigidifolia, H. nitida, H. pauciflora, H. benthamiana and H. camargoana*) were used to evaluate the transferability of the markers. All loci were tested under the same PCR conditions used for *H. brasiliensis*. Of the 46 loci tested, 40 (87%) were amplified for *H. guianensis* and *H. pauciflora*, 39 (85%) were amplified for *H. camargoana, H. nitida and H. pauciflora* and 38 (82%) were amplified for *H. benthamiana*. This high percentage of transferability may be useful in the evaluations of genetic variability and to monitor introgression of genetic variability from different *Hevea* species into breeding programmes. Earlier, cross-species amplification of the SSR markers developed for *H. brasiliensis* was successful in the wild *Hevea* species *H. guianensis, H. rigidifolia, H. nitida, H. pauciflora, H. benthamiana and H. camargoana* (Souza et al. 2009). The data indicated a high degree of sequence homology in the microsatellite flanking regions of these species.

The main ex situ collections of South America including Amazonian populations that have never been previously described have been subjected to genetic diversity studies (de Souza et al. 2015) (Fig. 6.11). Genetic data were analysed to determine the genetic structure of the wild populations, quantify the allelic diversity and suggest the composition of a core collection to capture the maximum genetic diversity within a minimal sample size. A total of 1117 accessions were genotyped with 13 microsatellite markers. A total of 408 alleles, 319 of which were shared between

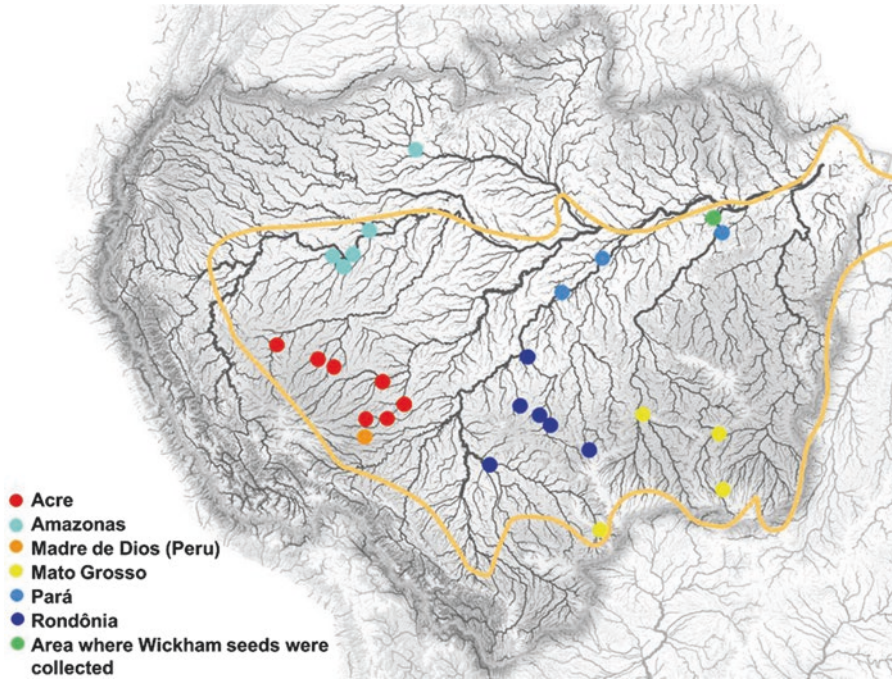

Fig. 6.11 The locations of the sampled sites within Brazil (de Souza et al. 2015)

groups and 89 that were private in different groups of accessions were identified. Principal component analysis revealed primary division into the following two subgroups: cluster 1, which consisted of varieties from the advanced breeding germplasm that originated from the Wickham and Mato Grosso accessions; and cluster 2, which consisted of the wild germplasm from the Acre, Amazonas, Pará and Rondônia populations and *Hevea* spp. These analyses revealed a high frequency of gene flow between the groups, with the genetic differentiation coefficient (GST) estimated to be 0.018. Additionally, no distinct separation among the *H. brasiliensis* accessions and the other species from Amazonas was observed. A core collection of 99 accessions was identified that captured the maximum genetic diversity. Such a core collection could provide resources for forming an association panel to evaluate traits with agronomic and commercial importance and to have genomic breeding (please see Chap. 13 for further details). Phumichai et al. (2015) investigated the genetic diversity and population structure of eight populations of *Hevea* rubber genotypes from Malaysia, India, Sri Lanka, Indonesia, France, Thailand, Brazil and China, in addition to individual primary clones, using 10 nuclear and 11 polymorphic novel chloroplast (cp) microsatellite markers. The Brazilian clones exhibited the greatest genetic diversity. Polymorphisms among different cpSSRs allowed delineation of chlorotypes. Such results provide valuable data for in situ or ex situ conservation and utilization of germplasm collections for breeding programmes.

6.5 Nuclear Vs. Cytoplasmic Diversity

Accessions of Amazonia could be categorized into genetic groups according to their geographic origin (Acre, Rondonia, Mato Grosso). This was revealed with a RFLP analysis of 92 clones of Amazonian and 73 of Wickham origin (Besse et al. 1994). On the other hand, cultivated clones conserved relatively high level of polymorphism, despite narrow genetic base and continuous assor-

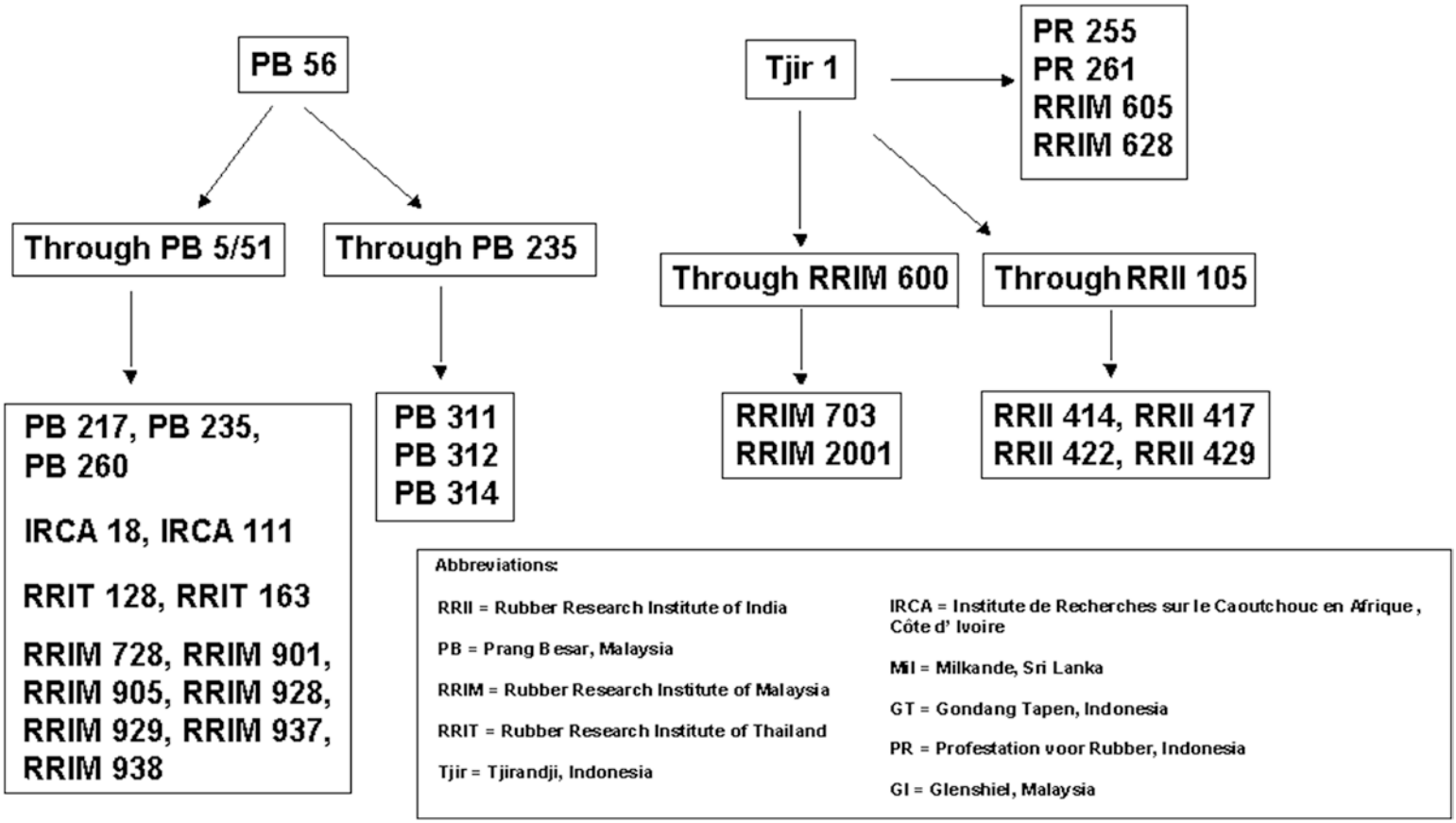

Fig. 6.12 Transmission of cytoplasm of PB 56 and Tjir 1 in the derivation of modern clones

tative mating and selection. As expected, polymorphism is very prudent among allied species of *Hevea*. A comparison of isozyme analysis (Lebrun and Chevallier 1990) with that of DNA markers showed much similarity (Besse et al. 1994). Identification of all Wickham clones could be done with 13 probes associated with restriction enzyme *Eco RI* (Besse et al. 1993a, b). The cultivated clones are genetically close to the Mato Grosso genotypes. Rondonia and Mato Grosso clones are more polymorphic as per RFLP data (Besse et al. 1994). A Rondonia clone (RO/C/8/9) showed eight specific restriction fragments and a unique malate dehydrogenase (MDH) allele, indicating this clone is of inter-specific origin. Such molecular markers are useful in rubber tree breeding since no distinct morphological traits exist. Mitochondrial DNA (mt DNA) polymorphism was analysed in 345 Amazonian accessions, 50 Wickham clones and two allied species (*H. benthamiana, H. pauciflora*) (Luo et al., 1995). While the variation in wild accessions was considerable, the cultivated clones formed only two clusters.

6.5.1 Potentiality of mtDNA

The aforesaid observations amply indicate that the selection was indirectly towards nuclear DNA polymorphism, while evolving modern clones. Luo et al. (1995) argue that the geographic specificity towards nuclear and mtDNA polymorphisms are due to great level of genetic structuring among natural populations in the Amazon forests in relation to hydrographic network. In wild accessions, seed dispersal and selection are as per the environmental conditions. If this is true, we observe that much of the variations produced in the natural habitat are being lost due to selection pressure of environmental factors. This is a matter of concern since the wild accessions have not rendered much contribution in evolving high-yielding clones so far, after introduction to other parts of the globe. On the other hand, Wickham clones exhibited high nuclear DNA polymorphism, perhaps due to breeding under different climates. It is presumable that the nuclear genome has been forced to enhance variation to suit the diverse hydrothermal situations of newly introduced areas, resulting in selection of rightly adapted clones under a given environment. The mtDNA of Wickham clones has lesser variation because their female progenitors are all primary clones, naturally bred under the similar environmental conditions of Malaysia and Indonesia. These clones were introduced later into India and Sri Lanka for further breeding programmes. Moreover, cytoplasmic donors for most of the improved clones are either PB 56 or Tjir 1 (Fig. 6.12). While the cytoplasm of PB 56 is transferred through PB 5/51, the cytoplasm of Tjir 1 was through RRII 105, RRIM 600 and RRIM 605. In conventional breeding systems followed in rubber, the best parents of one generation are used as parents for the next cycle of breeding (Simmonds 1989). Obviously, this is the reason for the mtDNA profile showing only two clusters. A possible explanation for greater polymorphism in mtDNA in wild accessions is that they must have been evolved through inter-specific hybridization. mtDNA polymorphism in wild accessions needs to be exploited fully. A molecular survey of available Amazon accessions and isolation of competent molecular variants in their progeny are the possible exercises that would give meaningful results.

Plant mitochondrial genomes encode tRNAs, rRNAs, proteins and ribosomal proteins and range in size from 200 Kb in *Brassica hirta* (Palmer and Herbon 1987) to 2.74 Mb in *Cucumis melo* (Rodríguez-Moreno et al. 2011). Mitochondrial genome expansion in land plants is primarily due to large intergenic regions, repeated segments, intron expansion and incorporation of foreign DNA such as plastid and nuclear DNA (Turmel et al. 2003; Bullerwell and Gray 2004). Accumulation of repetitive sequences in plant mitochondrial genomes cause frequent recombination events and dynamic genome rearrangements within a species (Chang et al. 2011; Allen et al. 2007). Several mutations by gene rearrangement of the mitochondrial genes were found associated with cytoplasmic male sterility (CMS) such as the T-urf13 gene in maize (Dewey et al. 1986), pcf gene (a fusion of atp9 and cox2 portions) in petunia (Young and Hanson 1987), cox1 in rice

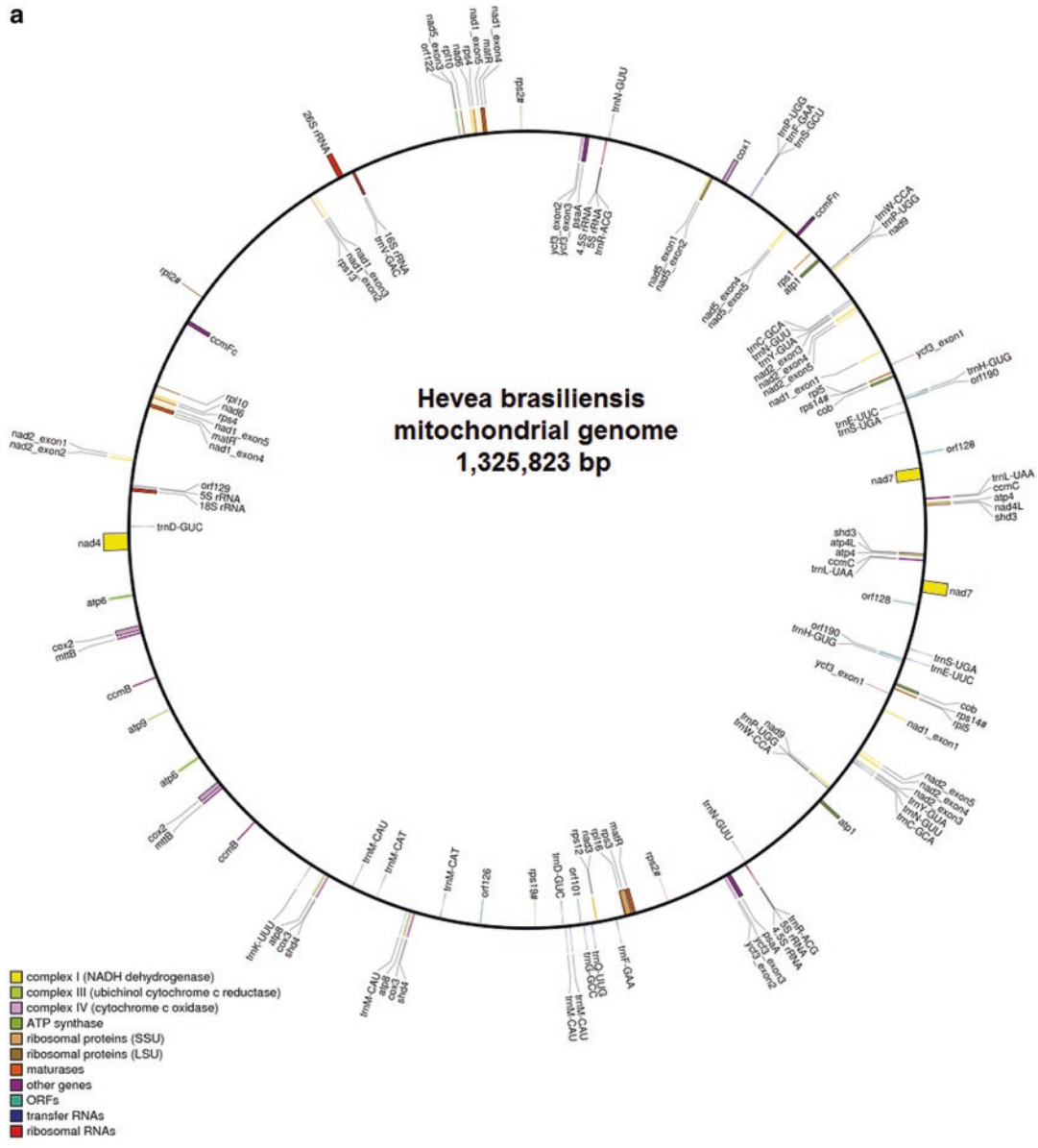

Fig. 6.13 (**a**) Annotated representation of the rubber tree mitochondrial genome. (**b**) *Hevea* mitochondrial genome: *Grey arches* indicate the mapping of each pair of the Illumina paired-end sequence data. Direct repeats are shown as *blue arches* and inverted repeats as *orange arches* (After Shearman et al. 2014)

(Wang et al. 2006) and mutations in ATPase subunits in sunflower (Laver et al. 1991) and Brassica (Landgren et al. 1996). RNA processing also plays an important role in controlling CMS as evidenced in *orf355/orf77* (*atp9*) and *T-urf13* in maize (Gallagher et al. 2002; Dill et al. 1997). With the development of next generation sequencing (NGS) technologies, new strategies have been used to obtain plant mitochondrial genomes. A combination approach of shotgun and paired-end NGS sequencing from non-enriched whole genome DNA libraries have been successfully used to obtain the mitochondrial genomes. Clone BPM 24 exhibits cytoplasmic male sterility, inherited

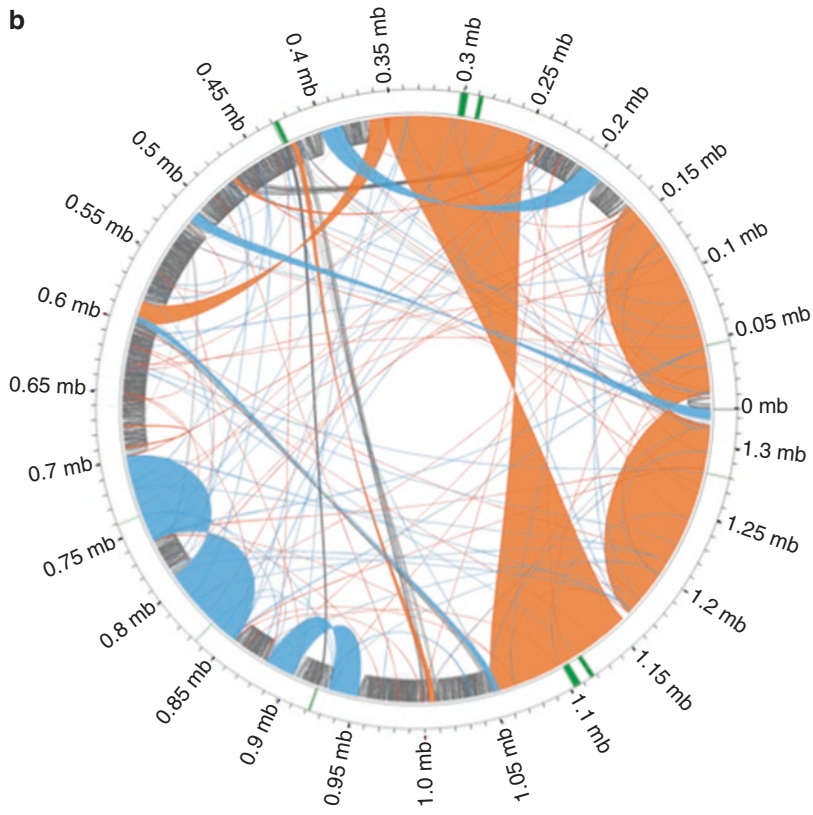

Fig. 6.13 (continued)

from the variety GT 1. Shearman et al. (2014) constructed the rubber tree mitochondrial genome of a cytoplasmic male sterile variety, BPM 24, using 454 sequencing, including 8 kb paired-end libraries, plus Illumina paired-end sequencing. They further annotated this mitochondrial genome with the aid of Illumina RNA-seq data and performed comparative analysis. Shearman et al. (2014) then compared the sequence of BPM 24 to the contigs of the published rubber tree, variety RRIM 600, and identified a rearrangement that is unique to BPM 24, resulting in a novel transcript containing a portion of atp9 (Fig. 6.13 a, b). The novel transcript is consistent with changes that cause cytoplasmic male sterility through a slight reduction to ATP production efficiency. The exhaustive nature of the search rules out alternative causes and supports previous findings of novel transcripts causing cytoplasmic male sterility.

6.5.2 Potentiality of cpDNA

Chloroplast genomes are sufficiently large and complex to include structural and point mutations that are useful for evolutionary studies from intra-specific to inter-specific levels (Neale et al. 1988; McCauley 1992; Graham and Olmstead 2000; Provan et al. 2001). Since the first complete chloroplast (cp) genome sequence of liverwort (*Marchantia polymorpha*) was reported in 1986 (Ohyama et al. 1986), more than 150 chloroplast genomes have been sequenced and characterized thus disclosing an enormous amount of evolutionary and functional information of chloroplasts. In chloroplasts, transcripts undergo a series of RNA processing steps such as intron splicing, polycistronic cleavage and RNA editing. RNA editing is a mechanism to change genetic information at the transcript level by nucleotide insertion, deletion or conversion

(Bock 2000; Knoop 2010). The chemical composition of natural rubber is cis-polyisoprene, a high-molecular weight polymer formed from sequential condensation of isopentenyl diphosphate (IDP) units catalysed by the action of rubber transferase (Cornish 2001). IDP is also an important intermediate for biosynthesis of essential oils, abscisic acid, cytokinin, phytoalexin, sterols, chlorophyll, carotenoids and gibberellins (Chappell 1995a; McGarvey and Croteau 1995; Lichtenthaler et al. 1997; Cornish 2001). There are two IDP biosynthesis pathways: the mevalonate (MVA) pathway which occurs in cytosol (Chappell 1995b); and the 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-Derythritol 4-phosphate (MEP) pathway which occurs in plastids (Lichtenthaler 1999; Ko et al. 2003). One approach to improving rubber production in *H. brasiliensis* would be to engineer chloroplasts and modify metabolic flux to produce more biosynthetic intermediates. The availability of the complete chloroplast genome sequence should also facilitate the chloroplast transformation technique. The improved transformation efficiency and foreign gene expression can be achieved through utilization of endogenous flanking sequences and regulatory elements (Birch-Machin et al. 2004; Maliga 2004; Tangphatsornruang et al. 2010). Transformation of chloroplast genome offers a number of advantages over nuclear transformation including a high level of transgene expression, polycistronic transcription, lack of gene silencing or positional effect and transgene containment (Daniell et al. 2002; Maliga 2002, 2004; Bock 2007). Tangphatsornruang et al. (2011a, b) reported the complete chloroplast genome sequence of rubber tree as being 161,191 bp in length including a pair of inverted repeats of 26,810 bp separated by a small single-copy region of 18,362 bp and a large single-copy region of 89,209 bp (Fig. 6.14). The chloroplast genome contains 112 unique genes, 16 of which are duplicated in the inverted repeat. Of the 112 unique genes, 78 are predicted protein-coding genes, 4 are ribosomal RNA genes and 30 are tRNA genes. Relative to other plant chloroplast genomes, Tangphatsornruang et al. (2011a, b) observed a unique rearrangement in the rubber tree chloroplast genome: a 30-kb inversion between the *trnE(UUC)-trnS(GCU)* and the *trnT(GGU)-trnR(UCU)*. A comparison between the rubber tree chloroplast genes and cDNA sequences revealed 51 RNA editing sites in which most (48 sites) were located in 26 protein-coding genes and the other 3 sites were in introns. Phylogenetic analysis based on chloroplast genes demonstrated a close relationship between *Hevea* and *Manihot* in Euphorbiaceae.

Shotgun genome sequencing of *H. brasiliensis* using pyrosequencing technology revealed the complete chloroplast genome sequence (Tangphatsornruang et al. 2011a, b). Gene content and structural organization of the rubber tree chloroplast genome is similar to that of *M. esculenta*, with an exception of the 30-kb-fragment rearrangement. By comparing the rubber tree chloroplast genes and the cDNA sequences, the distribution and the location of RNA editing sites in the chloroplast genome could be determined (Tangphatsornruang et al. 2011a, b). The phylogenetic relationships among angiosperms, based on chloroplast DNA sequences including those of the rubber tree chloroplast DNA provided a strong support for a monophyletic group of the eurosid I and demonstrated a close relationship between *Hevea, Manihot, Jatropha* and *Populus* in *Malpighiales*.

As a synthesis of these diversity studies on DNAs (nuclear, mt and cp), good relationships were found between the results issued from the different genetic markers. Even if the contribution of isozymes is important by itself, molecular markers provided important clarifications for the distinction of different groups. There would be no barrier to migration of *Hevea* genes within the Amazonian basin. However, the wideness of the area and the limited dispersion of *Hevea* seeds allowed the preservation of the current structure, which is assumed to have initially resulted from the fragmentation of the Amazonian forest during the pleistocene period, according to the refuge theory presented by Haffer (1982). Moreover, the *Hevea* germplasm genetic structure clearly appears as geographically structured in relationship with the hydrographic network of the Amazonian forest, which confirms the role of riv-

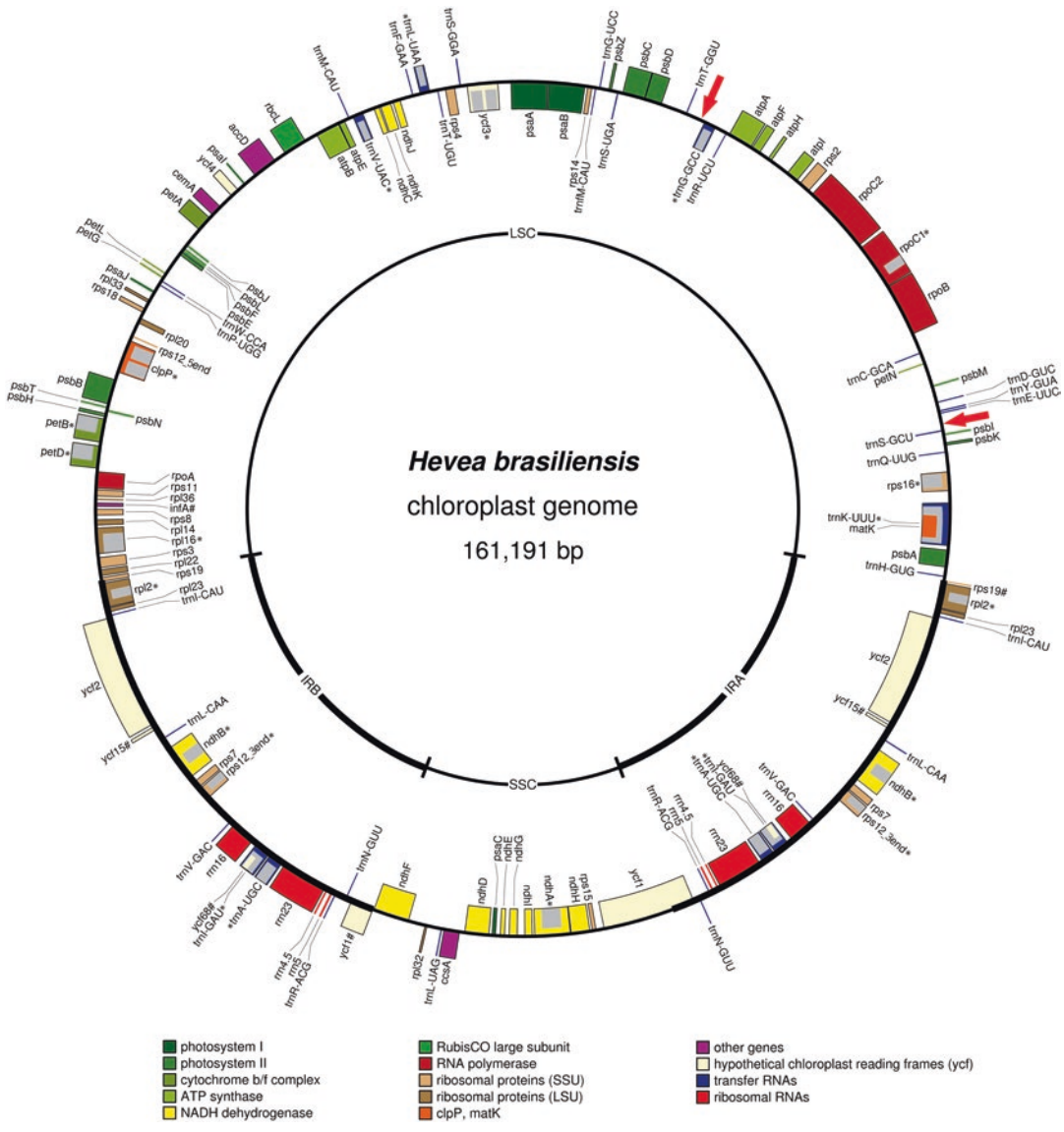

Fig. 6.14 Map of *H. brasiliensis* chloroplast genome. The *thick lines* indicate the extent of the inverted repeats (IRa and IRb) which separate the genome into small and large single-copy regions. Genes on the outside of the map are transcribed clockwise and those on the inside of the map are transcribed counter clockwise. Genes containing introns and pseudogenes are marked with * and #, respectively. *Arrows* indicate the positions of a 30-kb-unique rearrangement in relative to the cassava chloroplast genome (After Tangphatsornruang et al. 2011a, b) (Photo courtesy: Sithichoke Tangphatsornruang, National Center for Genetic Engineering and Biotechnology, Bangkok)

ers and inundated zones in the transport of seeds and dissemination of the species (Besse et al. 1993a; Luo et al. 1995; Seguin et al. 1996). Also, differentiation among populations could be jointly explained by both geographical location within the hydrographical Amazon network and by isolation by distance among populations belonging to distinct catchments (Le Guen et al. 2009). Mato Grosso (Brazil) populations were seen to be genetically more distant from all other populations. The mt DNA of Wickham population has lesser variation since their female progenitors

are restricted to a very small set of primary clones. Cytoplasm donors for most of the improved clones are either PB56 or Tjir1. Obviously, this is the reason for the mt DNA profile showing only two clusters (Priyadarshan and Gonçalves 2003). A possible explanation for greater polymorphism in mtDNA of wild accessions is that many might have been evolved through inter-specific hybridization.

6.6 Impact of Genetic Erosion

As said earlier, genetic erosion can result from a narrow genetic base in the original collections or by practices that reduce genetic diversity. That the original 22 seedlings of Wickham collection, as it is believed till date, is the base population from which the day-to-day *Hevea* clones were evolved had been genetically narrow to enrich the *Hevea* gene pool. In addition, these populations were subjected to several rounds of controlled crossing that further narrowed the diversity. Moreover, the strategy followed by the breeders to select only the desirable genotypes and to reject the unwanted ones (without assessing the utility other than yield) is the main reason that reduces diversity. Concerted efforts to infuse the Amazonian germplasm through controlled crossings never met with enriching the diversity as desired as expected. This is because selection was, and has always been in favour of higher yield only. Preserving other genotypes/entries cannot be accomplished due to space constraints unlike annual species. This drawback needs to be addressed resolutely if the diversity of *Hevea* rubber is to be increased. Genetic diversity not produced or preserved is equivalent to genetic diversity lost. The total number of clones is not more than hundred that are being cultivated worldwide for natural rubber production.

Molecular characterization of *Hevea* has not been done systemically. Only molecular diversity of Amazonian accessions and a few clones had been studied to an extent. A very systematic study of all *Hevea* clones at molecular level is appreciable, since the wisdom of understanding differences in morphological and molecular diversity has accumulated of late. QTL mapping is yet another area that needs to be undertaken with international co-ordination. As mentioned in this article, much work at the molecular level had been carried out like for Tapping Panel Dryness, latex production, defence genes and alike. Only growth related traits have been attempted for QTL mapping (Souza et al. 2013). But a sincere and systematic effort to tie up this variation with QTLs for yield had not been done so far. This exercise is difficult but not impossible. This systematic exercise can only elucidate the intricacies underlying diversity of *Hevea* rubber. Such an exercise can lead to setting up of a molecular library for *Hevea* and scientists working worldwide can contribute to this molecular library. The deposition of microsatellites, SSRs and ESTs is not enough, but a library that includes genes for QTLs is most warranted. The contribution of Rahman et al. (2013) on gene sequencing of *Hevea* is a sincere and systematic step towards this. Nevertheless, the attempts of Salgado et al. (2014) on de novo transcriptome analysis in *Hevea* stems promise. This tempo needs to be accelerated further, should there be a comprehensive gene library for *Hevea* rubber.

One of the early contributors to the science of plant genetic resources, Harlan (1970) remarked: 'The varietal wealth of the plants that feed and clothe the world is slipping away before our eyes, and the human race simply cannot afford to lose it', and he also predicted a 'genetic wipe out of centres of diversity' (Harlan 1975). Genetic wipe out has not really happened but the modern varieties have replaced traditional varieties or land races. One of the primary duties of a Plant Breeder is to evolve, document and manage genetic diversity. As such, there are no land races in *Hevea* rubber, but only modern clones. In this context, how much genetic diversity is getting conserved, catalogued and utilized and how much genetic erosion happens are the options left to one's own wisdom.