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A saturated genetic linkage map of rubber tree (*Hevea* spp.) based on RFLP, AFLP, microsatellite, and isozyme markers

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Abstract The first genetic map for *Hevea* spp. (2n=36) is presented here. It is based on a F_1 progeny of 106 individuals allowing the construction of a female, a male, and a synthetic map according to the pseudo-testcross strategy. Progeny were derived from an interspecific cross between PB260, a *H. brasiliensis* cultivated clone, and RO38, a *H. brasiliensis*×*H. benthamiana* interspecific hybrid clone. The disomic inheritance observed for all the codominant markers scattered on the 2n=36 chromosomes revealed that *Hevea* behaves as diploids. Homologous linkage groups between the two parental maps were merged using bridge loci. A total of 717 loci constituted the synthetic map, including 301 RFLPs, 388 AFLPs, 18 microsatellites, and 10 isozymes. The markers were assembled into 18 linkage groups, thus reflecting the basic chromosome number, and covered a total distance of 2144 cM. Nine markers were found to be unlinked. Segregation distortion was rare (1.4%). Average marker density was 1 per 3 cM. Comparison of the distance between loci in the parental maps revealed significantly less meiotic recombination in the interspecific hybrid male parent than in the female parent. *Hevea* origin and genome organisation are discussed.

Key words *Hevea* · Genetic map · F_1 cross · Double pseudo-testcross · Consensus map

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Introduction

Rubber tree is a perennial crop which belongs to the genus *Hevea* and to the botanical family Euphorbiaceae. The genus *Hevea* encompasses ten species, all originating from Amazonia (Schultes 1990). They are all strongly outcrossing and monoecious. Only one species, *H. brasiliensis*, is cultivated for latex. Among the other nine species, only *H. benthamiana* produces a latex of reasonable quality, but this species has rarely been used in breeding programmes. Cultivated varieties are clones propagated vegetatively by grafting. The crop is located essentially in Asia and Africa but not in Latin America because of South American Leaf Blight (SALB) disease, caused by the pathogen *Microcyclus ulei*.

The genetics of rubber tree has been poorly investigated. This lack of knowledge is due to the heterozygous nature of the crop, its long growing cycle that includes 5 years before latex collection, and its low seed yield per pollination (an average of ten seeds obtained for 100 flowers pollinated). As a rule, rubber tree displays inbreeding depression, making it difficult to develop appropriate progeny for classical genetic studies.

Isozymes, minisatellites, and restriction fragment length polymorphisms (RFLPs) have already been applied to rubber tree to investigate the polymorphism between clones. A simple method based on 13 isozymes has been set up for clonal identification (Chevallier 1988; Leconte et al. 1994). This method is very useful because of the lack of morphological characters that would normally allow for distinction between clones. Other efficient methods of identification have been set up with minisatellites and nuclear RFLPs (Besse et al. 1993a). Genetic diversity of the *Hevea* germplasm, including wild and cultivated materials, has been studied with isozymes and nuclear RFLPs (Besse et al. 1994; Chevallier 1988; Seguin et al. 1995; Seguin et al. 1996).

The level of ploidy in the *Hevea* genus remains doubtful. The high number of chromosomes, 2n=36, compared to the majority of the Euphorbiaceae species which have 2n=14 to 22, favours a tetraploid origin for

Hevea spp. Cytogenetical studies have revealed that mostly bivalents and few tetravalents are formed during meiosis (Bouharmont 1960; Ong 1975). *In situ* hybridisation revealed two distinct rDNA loci (Leitch et al. 1998). Though some observations may suggest an allotetraploid origin with two ancestral species with 2n=18 each, no diploid ancestor could be found. Moreover, isozyme studies have shown that 11 of the 13 enzymes are encoded at a single locus with a diploid-like inheritance having at the most, two alleles per individual. The other 2 enzymes revealed 2 possible loci with a disomic inheritance (Chevallier 1988).

Genetic mapping for rubber tree would be useful in order to gain a better knowledge of the genome organisation, to enable genome comparison with other genera of the Euphorbiaceae family such as cassava (Fregene et al. 1997), to depict the genetic basis of key quantitative traits such as resistance to SALB (Lespinasse et al. 1997), and to develop marker-assisted selection for these traits.

Mapping in outbreeding heterozygous perennial crops is not as advanced as in annual crops. They require more time and more space, given the long growing cycle and large crop size. Except for particular situations (Lanaud et al. 1995; Plomion et al. 1995; Tulsieram et al. 1992) only progeny issued from the cross between two heterozygous parents (F_1 cross) are usually available. In this case, up to four alleles per locus may segregate. Data can be analysed as a double pseudo-testcross (Grattapaglia and Sederoff 1994) and a map constructed separately for each parent. New algorithms for recombination rate estimates can also be used, taking advantages of meioses occurring in both parents (Maliepaard et al. 1997; Ritter et al. 1990; Ritter and Salamini 1996; Stam 1993; Stam and van Ooijen 1995). In these F_1 crosses, marker phase (coupling or repulsion) can not always be deduced from parent and grandparent banding patterns (Carlson et al. 1991), which adds one source of complexity.

We report here the construction of a genetic map for rubber tree based on a F_1 cross and a series of several molecular marker types. Three separate linkage maps were produced. First, a pseudo-testcross map was established for each parent. Then a consensus map between the two parental maps was established using homologous markers segregating in both parents.

Materials and methods

Plant material

Segregation data used to construct the map were obtained from a three-generation pedigree population (Fig. 1). Despite the general low fructification rate associated with artificial pollination (Legnate and Clément-Demange 1991), a population of 214 individuals was obtained at the IDEFOR-dpl, Ivory Coast, from a cross between PB260, a *H. brasiliensis* widely grown clone, and RO38 (alias FX3899), an interspecific hybrid *H. brasiliensis*×*H. benthamiana*. RO38 shows some resistance to SALB, whereas PB260, like all the high-yielding cultivars is highly sensitive. Seeds and budwood of progeny individuals were sent to Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Kourou, French Guyana. Each progeny was clonally propagated by grafting and conserved in a greenhouse. Genetic mapping was performed on a subsample of 106 progenies. Leaf samples of each individual progeny, both parents, and the maternal grandparents were sent to laboratories at CIRAD, Montpellier, France, for molecular analysis. Plant material of the two paternal grandparents was not available for this study.

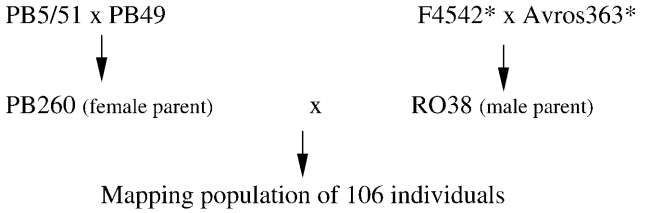


Fig. 1 Three-generation pedigree population used for *Hevea* genetic mapping. Individuals denoted with * were unavailable in this study

RFLPs

Total DNA was extracted as previously described (Besse et al. 1994) from fresh or freeze-dried leaves. DNA samples (7 µg) were restricted with *Eco*RI, *Eco*RV, and *Xba*I, using 3 U of enzyme per microgram of DNA, and DNA fragments were then separated on 0.8% TAE agarose gels for 16 h at 1.5 V cm^{-1}. A total of 557 RFLP probes were obtained from several sources. Five hundred and forty-two of them (gHbCIR) were part of a *Pst*I genomic library (Besse et al. 1994). 10 probes (RGA) were obtained by polymerase chain reaction (PCR) amplification utilising consensus primers (C. Kaye, personal communication) designed to amplify the R genes of the NBS (consensus primers generous gift of A.F. Adam-Blondon) and Pto families (Vallad et al. 1998), essentially as described in Leister (1996), Kanazin (1996) and Yu (1996); 2 of them (RGAptoRO2 and RGAptoRO3) were homologous to the *Pto* gene (Martin et al. 1993), and the other 8 probes (RGAnbs) were homologous to genes of the NBS family (Staskawicz et al. 1995). Five gene probes related to rubber biosynthesis, Glutamin Synthetase (GS) (Pujade-Renaud et al. 1997), Rubber Elongation Factor (REF) (Attanyaka et al. 1991; Goyvaert et al. 1991), Hevein (Broekaert et al. 1990), Hevein Receptor (RecHevein), and Glutamin Synthetase 2 (GS2) were kindly provided by Dr V. Pujade-Renaud. One rDNA probe, subclone from the wheat pTA71 probe (Gerlach and Bedbrook 1979), representing the *Eco*RI-*Bam*HI fragment covering all the IGS and parts of 18 S and 25 S genes was also used (Besse et al. 1993b). Blotting and hybridisations were performed as described by (Besse et al. 1993a, 1994).

Isozymes

The 13 isozymes used were malate dehydrogenase (MDH), phosphoglucose isomerase (PGI), alanyl aminopeptidase (AAP), leucine aminopeptidase (LAP), esterase (EST), acid phosphatase (AP), alcohol dehydrogenase (ADH), diaphorase (DIA), isocitrate dehydrogenase (ICD), phosphogluconate mutase (PGM), phosphogluconate dehydrogenase (PGD), glutamate oxaloacetate transaminase (GOT), and shikimate dehydrogenase (SDH). The protocoles were as defined by Chevallier (1988) and Lebrun and Chevallier (1990).

Microsatellite procedures

Microsatellites were obtained from the hybridization of a *H. brasiliensis Pst*I genomic library with $(AC)_n$ or $(TC)_n$ microsatellite

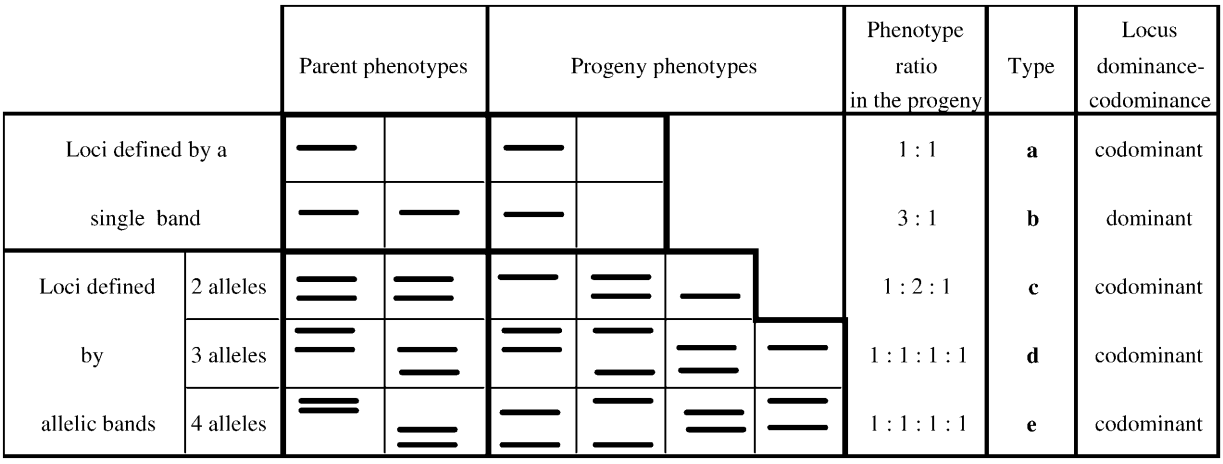


Fig. 2 Informative patterns for mapping as defined by Ritter et al. (1990). The five configurations presented correspond to segregating loci in the progeny. All the segregating loci configurations found in a F_1 progeny can refer to one of these five configurations. Two kinds of loci are presented: loci defined by a single band, for which only one segregating allele is observed; and loci defined by allelic bands, for which the different segregating alleles are observed. All loci for which one segregating allele in each parent and no segregating allele common to both parents were observable were referred to as configuration type-e loci

oligonucleotide probes (Seguin et al. in preparation). PCR primers were also defined for two rubber biosynthesis genes containing a microsatellite: *hmg1* (Chye et al. 1991) and *MnSOD* (Miao and Gaynor 1993). PCR reactions to search for microsatellite polymorphism between the parents and to score microsatellite loci in the progeny were carried out in a 25-µl volume containing 100 ng of genomic DNA, 0.1 µ*M* of each primer, 200 µ*M* of each dNTP, and 0.75 U of BioTaq^R DNA polymerase (Eurobio), 20 m*M* TRIS HCl, 100 m*M* KCl, 1.5 m*M* $MgCl_2$. One denaturation cycle was performed at 95°C for 4 min, prior to 30 cycles of denaturation at 94°C for 1 min, annealing at 48°C, 50°C, or 55°C for 45 s, extension at 68°C, 70°C, or 72°C for 1 min, and a final extension at 68°C to 72°C for 10 min. PCR-amplified products were visualised following electrophoresis on 4% agarose gels (ultraPURE agarose GIBCO-BRL) in 0.5×TBE at 5 V cm^{-1} and staining with EtBr (Seguin et al. 1997), or they were labelled directly by primer terminal binding of γ-[^{33}P]dATP using T_4 polynucleotide kinase before the PCR reaction and run on a 5% denaturating polyacrylamide gels at 55 W.

Amplified fragment length polymorphism (AFLP) procedures

AFLP markers (Vos et al. 1995) were produced as recommended in the Life Technologies AFLP analysis system I (Gibco BRL, Gathersburg, Md.). Restriction enzymes used were *Eco*RI and *Mse*I. *Eco*RI primers were end-labelled with γ-[^{32}P]dATP using T_4 polynucleotide kinase. AFLP products were separated on 5% denaturating polyacrylamide gels for 2 h at 55 W.

Screening procedures

In a progeny from outbred parents, markers that segregate do not necessarily reveal polymorphism between the parents. For this reason, polymorphism was assessed on the basis of sorting blots containing parents and grandparents of the cross plus a sample of 10 progeny individuals. Informative patterns for mapping observed for the parents and the progeny individuals are presented in Fig. 2. Some RFLP probes revealed complex patterns for which loci and alleles were difficult to depict. In this case, segregating bands were individually scored according to configuration types a or b. Loci in the configuration a, c, d, and e were referred to as codominant loci and loci in configuration b as dominant loci. RFLP probes were screened for their ability to reveal codominant loci, which are more informative for mapping than dominant loci. However, type-b markers were scored when they segregated on the selected probes. The same approach was retained for screening informative microsatellites and isozymes. For AFLPs, only pattern types a and b are observable (Fig. 2). AFLP *Mse*I-*Eco*RI primer combinations were screened for their ability to reveal high number of loci in the type-a configuration. However, type-b markers were scored when they segregated on the selected primer combinations.

Linkage analysis

The cross was analysed as a double pseudo-testcross (Grattapaglia and Sederoff 1994). In a first round of analysis, segregating data were used to construct a map for each parent, PB260 and RO38. Each segregating marker was scored individually for all configuration types. The segregation ratio of each marker was tested with a χ^2 test for goodness of fit to the expected 1:1 ratio when the marker is present in one of the two parents or a 3:1 ratio when the marker is present in both parents. Two matrices designed to construct the two parental maps were then constructed, one including markers inherited from PB260 and the other including markers from RO38. Markers derived from configuration type b were not considered in this first round of analysis. For markers derived from configuration type c, the data were considered to be missing for heterozygous individuals for which the parental origin could not be deduced. Parental maps were then established with MAPMAKER software version 3.0 (Lander and Green 1987) using the back-cross option. Marker phase was deduced from the grandparent pattern for most markers used to construct the PB260 map. In a few cases, when the marker was present in both grandparents, the linkage phase was deduced statistically from two-point linkage data. The marker was scored twice, once in a given phase and once in the reverse phase, and the correct phase was chosen according to the highest two-point linkage LOD value. The same process was used to determine the phase of markers in the RO38 map, since grandparents of RO38 were not available in this study. Linkage LOD thresholds were chosen as giving congruent results in the two parental linkage analyses. Markers were initially associated using the "*group*" command (two-point comparison). For each linkage group, marker orders were then defined using the "*order*" command. Three different orders were compared for three different *information criteria* values in MAPMAKER (1 cM, 2 cM, 5 cM) at LOD 2.0. Other markers were then added with the "*try*" command. Final order was confirmed with the "*ripple*" command.

In a second round of analysis, a consensus map between the two parental maps was constructed with JOINMAP software version 1.4 (Stam 1993). With this software, all the patterns presented in

Fig. 2 can be used. The consensus map construction is possible thanks to bridge loci allowing for the merging of homologous linkage groups. Loci in configuration b, c, and d which mapped in homologous groups in parental maps were used as primary bridge loci. Loci in configuration e were used as supplementary bridge loci when they mapped to the same synthetic linkage group using primary bridge loci at an arbitrary distance lower than 10 cM.

Results

Marker screening

A total of 557 RFLP probes were screened for polymorphism on the sorting blots: 71 (13%) gave no clear banding pattern, 113 (20%) were monomorphic, and 373 (67%) were polymorphic with at least one of the three restriction enzymes. Among the polymorphic probes, 169 (45%), 126 (34%), and 78 (21%) detected band pattern differences with one, two, and three restriction enzymes, respectively. This did not allow us to clearly conclude which kind of DNA polymorphism, i.e., sequence insertion/deletion or point mutation, was more common. Of the probes 20% revealed segregating bands in gametes of PB260 only, 41% in gametes of RO38 only, and 38% in gametes of both parents. Parent RO38 thus appears to be more heterozygous than parent PB260, which was expected based on clone ancestry.

Among the 64 AFLP primer combinations tested (8 *Eco*RI and 8 *Mse*I primers), 31 were retained because of the large number of polymorphic bands amplified among the two parents. On average, 13 segregating bands of either type a or b were revealed per primer combination.

Among the 39 microsatellites tested, 18 segregated in the progeny. Eleven segregated in both parent gametes, and 7 segregated in RO38 gametes only. Each of the 18 microsatellites revealed only 1 locus. These 18 loci were all of configuration type a or e.

Among the 13 isozymes tested, 3 of them, AAP, DIA and PGM, did not segregate in the progeny. Four segregated in both parent gametes, 1 segregated in PB260 gametes only, and 5 segregated in RO38 gametes only. These isozymes revealed 10 segregating loci in configuration types a, d, and e.

In summary, 266 RFLP probes, 31 AFLP *Mse*I-*Eco*RI primer combinations, 18 microsatellites, and 10 isozymes were used for segregation analyses in the progeny.

Ploidy level and chromosome pairing

Although the number of bands detected by an RFLP probe gives only a rough estimate of the number of alleles (because one allele may be represented by several cosegregating bands), banding patterns can tentatively be used to characterise the level of ploidy. Among the 266 RFLP probes tested, 184 revealed at the most two bands for each parent and 82 revealed more than two bands for at least one of the two parents. Therefore, the majority of RFLP probes (69%) correspond to diploid-like patterns: this may signify that rubber tree behaves as a diploid and not as a tetraploid for a large part of its genome.

The chromosome pairing behaviour, i.e., disomy versus tetrasomy, was investigated using simple markers in order to validate the mapping strategy. For 141 markers (markers in the configuration c, d, and e), the allelic constitution could be inferred from parent and progeny banding patterns because no more than two alleles were observed for each parent and these two alleles were never absent at the same time in a given descendant. This is expected in the case of disomy. Moreover, mainly two segregation ratios were found: a 1:1 ratio when the band was present in only one parent (7 markers did not fit this ratio); a 3:1 ratio when the band was common to both parents (2 markers did not fit this ratio). These segregation ratios were in accordance with a disomic inheritance of the markers. In the case of tetrasomic segregation, other ratios would have been found: 1:1 or 3:1 when the band is present in only one parent; 7:9, 3:5, 1:3, 3:13, 1:7, or 1:15 when the band is common to both parents. The data is clearly in favour of a disomic pairing behaviour of the chromosomes. This allowed us to use mapping algorithms for diploids without knowing about the diploid or amphidiploid state of the *Hevea* genome.

Construction of the PB260 map

One hundred and sixty-four RFLP, 79 AFLP, 11 microsatellite, and 5 isozyme markers segregated in the gametes of the PB260 parent. A scaffold map was obtained at a LOD of 3.5. The distance linkage criterion, theta, was 0.3. Two hundred and fifty nine loci defined 20 linkage groups, spanning 2000 cM, and 10 markers remained unlinked (Tables 1 and 2). On average, the PB260 map presented 1 marker every 8 cM. Map length was estimated to be 3516 cM at a LOD score of 3.5 according to the method described by Hulbert et al. (1988).

Construction of the RO38 map

Two hundred and forty-seven RFLP, 280 AFLP, 18 microsatellite and 9 isozyme markers segregated in the gametes of the RO38 parent. A scaffold map of 17 linkage groups was obtained at a LOD score of 4 for the male parent. The distance linkage criteria, theta, was 0.3. After comparison with the PB260 map, 1 linkage group in the RO38 map was seen to correspond to 2 distinct linkage groups in the PB260 map. It required a higher LOD score of 5 to be split into 2 groups. Five hundred and fifty-four loci defined 18 linkage groups spanning 2145 cM, and 6 markers remained unlinked (Tables 1 and 2). On average, the RO38 map has 1 marker every 4 cM. The map length calculated according to the method of Hulbert et al. (1988) was 2660 cM at a LOD score of 4.

Table 1 Number of molecular markers implied from parental maps (established with MAPMAKER software) and synthetic map (established with JOINMAP software), and number of bridges used to merge both parental maps

Type[a]	PB260 map: number of markers	RO38 map: number of markers	Synthetic map:[b]	
			number of markers	number of bridges
a	113	412	525	0
b	35	35	36	34
b/a[c]	1	1	1	1
a/b[c]	5	5	5	5
c	9	9	9	9
d	61	61	63	59
e	71	71	78	64
Parental maps cases a c d e	259	554		
Synthetic map cases a b c d e			717	172

[a] See Fig. 2
[b] One, 2, and 7 loci were not used as bridges in configuration types b, d, and e, respectively; the maternal and the paternal markers were thus mapped in the synthetic map
[c] x/y, type x for parent PB260; type y for parent RO38

Table 2 Description of the map data

	PB260 map	RO38 map	Synthetic map
Number of markers	259	554	717
Number of groups	20	18	18
Number of unlinked markers	10	6	9
Number of distorted markers (5%)	2	5	10
Map length[a]	2000	2145	2144
Estimated map length[b]	3516	2660	
Marker density[c]	8	4	3

[a] Corresponds to the sum of linkage group sizes, established with MAPMAKER and a 3% *error detection*
[b] Estimated map length was calculated by the Hulbert et al. (1988) method
[c] Marker density represents the average number of centiMorgans between 2 adjacent markers

Construction of the consensus map

Two hundred and eighty-five RFLP, 359 AFLP, 18 microsatellite, and 10 isozyme markers segregated in the gametes of the PB260 and/or RO38 parent, and these were used for the synthetic map construction. Moreover, 41 dominant loci not used in the parental maps were added, including 12 RFLP and 29 AFLP (Table 1). They corresponded to 35 loci coded as dominant loci for both parents (type b), 1 RFLP locus coded as a codominant locus for RO38 (type a) and dominant for PB260 (type b), and 5 RFLP loci coded as codominant for PB260 (type a) and dominant for RO38 (type a).

We considered as primary bridges those loci which presented a common allele between the two parents segregating in the progeny, that is loci of type b, c, and d revealed by RFLPs and isozymes and type-b loci revealed by AFLPs. According to these criteria, 111 loci could be used as bridges. Nevertheless, 3 loci among the 111 were not retained because: for 2 type-d loci, the allele common to both parents mapped to non-homologous linkage groups in the PB260 and the RO38 maps, possibly due to duplication; for 1 type-b locus, which was unlinked in the PB260 map, altered order and distances were observed in the synthetic map compared to the RO38 map. The synthetic map was first constructed with 108 bridge loci. Then, 71 secondary bridges were added, corresponding to type-e loci. Among these, 7 were discarded because alleles inherited from the two parents mapped at more than 10 cM in the synthetic map realised with the primary bridges. Although this was probably due to statistical mapping imprecisions, we could not ignore possible paralogous clustered loci.

The final synthetic map was thus produced with 172 bridges, that is 128 RFLP, 29 AFLP, 11 microsatellite, and 4 isozyme markers (Fig. 3). Sixteen linkage groups were obtained using a linkage LOD score of 4 and a map LOD score of 0.5. Compared with the parental maps, 1 linkage group required a LOD score as high as 5 to be split into 3 groups. The 717 markers segregating in the F_1 population permitted us to establish a map with 18 linkage groups spanning 2144 cM; 9 markers remained unlinked (Table 2). On average, the synthetic map presents 1 marker every 3 cM.

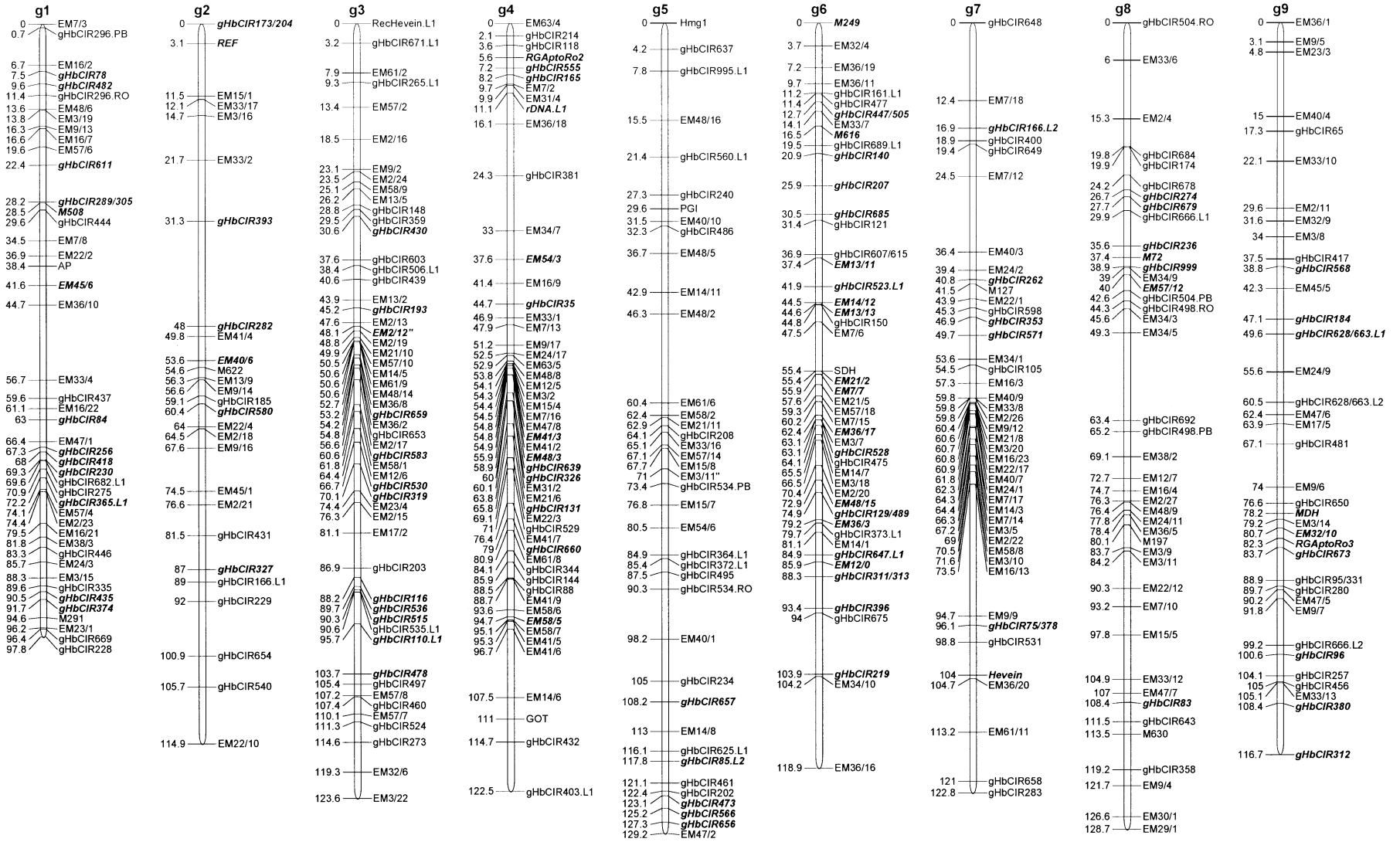


Fig. 3 F_1 synthetic map of 717 markers distributed in 18 linkage groups. The map encompasses 301 RFLP, 388 AFLP, 18 microsatellite and 10 isozyme markers. *gHbCIR* RFLP probe, *RGA* R gene RFLP probe, *EM* AFLP, *M* microsatellite. *Lx* suffix duplicate loci, *PB* and *RO* suffix parental provenance (PB260 and RO38, respectively) for markers present in both parents but not considered as bridges. Bridge markers are indicated (in ***bold*** and *italic*)

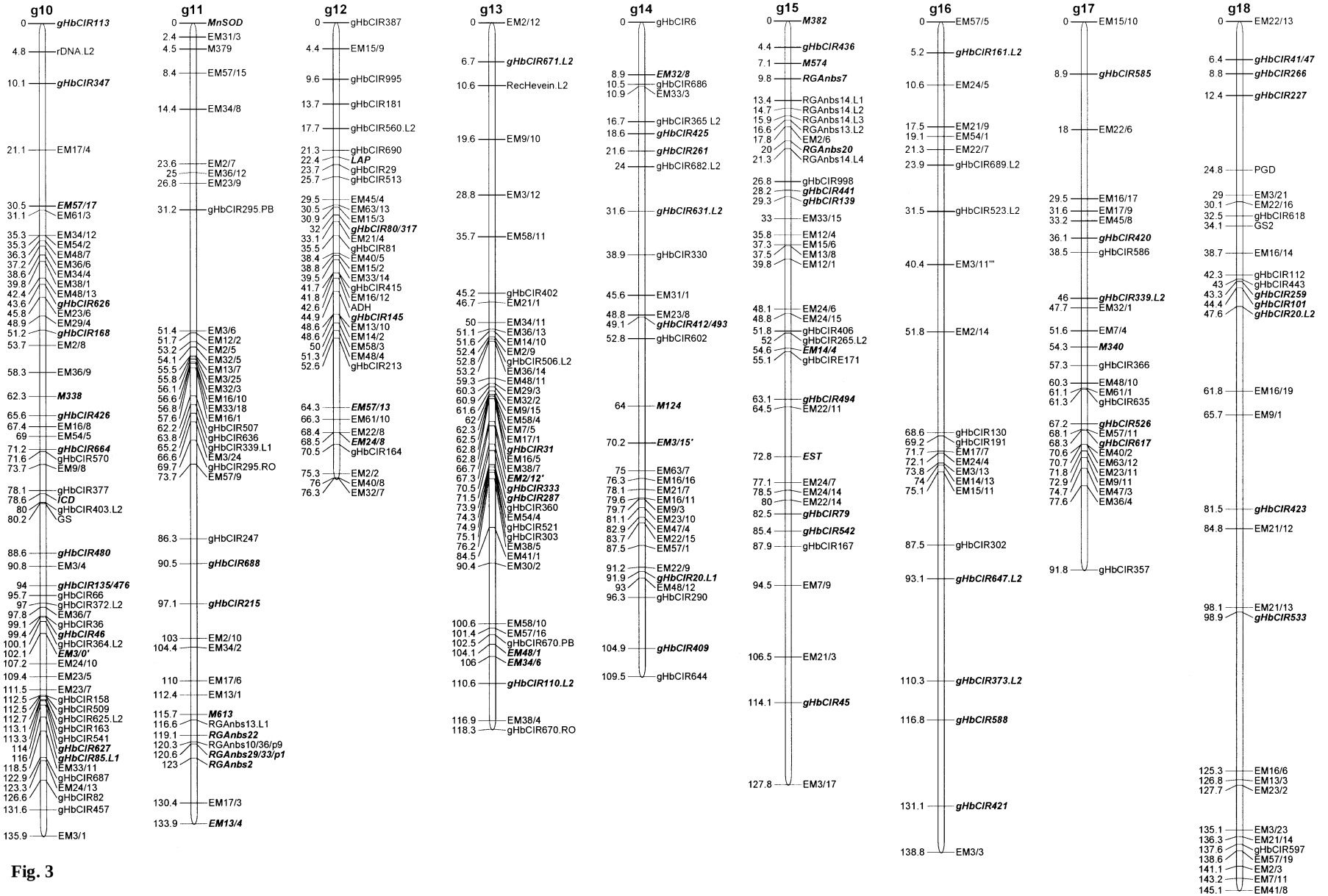

g10
0 gHbCIR113
4.8 rDNA.L2
10.1 gHbCIR347
21.1 EM17/4
30.5 EM57/17
31.1 EM61/3
35.3 EM34/12
35.3 EM54/2
36.3 EM48/7
37.2 EM36/6
38.6 EM34/4
39.8 EM38/1
42.4 EM48/13
43.6 gHbCIR626
45.8 EM23/6
48.9 EM29/4
51.2 gHbCIR168
53.7 EM2/8
58.3 EM36/9
62.3 M338
65.6 gHbCIR426
67.4 EM16/8
69 EM54/5
71.2 gHbCIR664
71.6 gHbCIR570
73.7 EM9/8
78.1 gHbCIR377
78.6 ICD
80 gHbCIR403.L2
80.2 GS
88.6 gHbCIR480
90.8 EM3/4
94 gHbCIR135/476
95.7 gHbCIR66
97 gHbCIR372.L2
97.8 EM36/7
99.1 gHbCIR36
99.4 gHbCIR46
100.1 gHbCIR364.L2
102.1 EM3/0'
107.2 EM24/10
109.4 EM23/5
111.5 EM23/7
112.5 gHbCIR158
112.5 gHbCIR509
112.7 gHbCIR625.L2
113.1 gHbCIR163
113.3 gHbCIR541
114 gHbCIR627
116 gHbCIR85.L1
118.5 EM33/11
122.9 gHbCIR687
123.3 EM24/13
126.6 gHbCIR82
131.6 gHbCIR457
135.9 EM3/1
g11
0 MnSOD
2.4 EM31/3
4.5 M379
8.4 EM57/15
14.4 EM34/8
23.6 EM2/7
25 EM36/12
26.8 EM23/9
31.2 gHbCIR295.PB
51.4 EM3/6
51.7 EM12/2
53.2 EM2/5
54.1 EM32/5
55.5 EM13/7
55.8 EM3/25
56.1 EM32/3
56.6 EM16/10
56.8 EM33/18
57.6 EM16/1
62.2 gHbCIR507
63.8 gHbCIR636
65.2 gHbCIR339.L1
66.6 EM3/24
69.7 gHbCIR295.RO
73.7 EM57/9
86.3 gHbCIR247
90.5 gHbCIR688
97.1 gHbCIR215
103 EM2/10
104.4 EM34/2
110 EM17/6
112.4 EM13/1
115.7 M613
116.6 RGAnbs13.L1
119.1 RGAnbs22
120.3 RGAnbs10/36/p9
120.6 RGAnbs29/33/p1
123 RGAnbs2
130.4 EM17/3
133.9 EM13/4
g12
0 gHbCIR387
4.4 EM15/9
9.6 gHbCIR995
13.7 gHbCIR181
17.7 gHbCIR560.L2
21.3 gHbCIR690
22.4 LAP
23.7 gHbCIR29
25.7 gHbCIR513
29.5 EM45/4
30.5 EM63/13
30.9 EM15/3
32 gHbCIR80/317
33.1 EM21/4
35.5 gHbCIR81
38.4 EM40/5
38.8 EM15/2
39.5 EM33/14
41.7 gHbCIR415
41.8 EM16/12
42.6 ADH
44.9 gHbCIR145
48.6 EM13/10
48.6 EM14/2
50 EM58/3
51.3 EM48/4
52.6 gHbCIR213
64.3 EM57/13
66.3 EM61/10
68.4 EM22/8
68.5 EM24/8
70.5 gHbCIR164
75.3 EM2/2
76 EM40/8
76.3 EM32/7
g13
0 EM2/12
6.7 gHbCIR671.L2
10.6 RecHevein.L2
19.6 EM9/10
28.8 EM3/12
35.7 EM58/11
45.2 gHbCIR402
46.7 EM21/1
50 EM34/11
51.1 EM36/13
51.6 EM14/10
52.4 EM2/9
52.8 gHbCIR506.L2
53.2 EM36/14
59.3 EM48/11
60.3 EM29/3
60.9 EM32/2
61.6 EM9/15
62 EM58/4
62.3 EM7/5
62.5 EM17/1
62.8 gHbCIR31
62.8 EM16/5
66.7 EM38/7
67.3 EM2/12'
70.5 gHbCIR333
71.5 gHbCIR287
73.9 gHbCIR360
74.3 EM54/4
74.9 gHbCIR521
75.1 gHbCIR303
76.2 EM38/5
84.5 EM41/1
90.4 EM30/2
100.6 EM58/10
101.4 EM57/16
102.5 gHbCIR670.PB
104.1 EM48/1
106 EM34/6
110.6 gHbCIR110.L2
116.9 EM38/4
118.3 gHbCIR670.RO
g14
0 gHbCIR6
8.9 EM32/8
10.5 gHbCIR686
10.9 EM33/3
16.7 gHbCIR365 L2
18.6 gHbCIR425
21.6 gHbCIR261
24 gHbCIR682.L2
31.6 gHbCIR631.L2
38.9 gHbCIR330
45.6 EM31/1
48.8 EM23/8
49.1 gHbCIR412/493
52.8 gHbCIR602
64 M124
70.2 EM3/15'
75 EM63/7
76.3 EM16/16
78.1 EM21/7
79.6 EM16/11
79.7 EM9/3
81.1 EM23/10
82.9 EM47/4
83.7 EM22/15
87.5 EM57/1
91.2 EM22/9
91.9 gHbCIR20.L1
93 EM48/12
96.3 gHbCIR290
104.9 gHbCIR409
109.5 gHbCIR644
g15
0 M382
4.4 gHbCIR436
7.1 M574
9.8 RGAnbs7
13.4 RGAnbs14.L1
14.7 RGAnbs14.L2
15.9 RGAnbs14.L3
16.6 RGAnbs13.L2
17.8 EM2/6
20 RGAnbs20
21.3 RGAnbs14.L4
26.8 gHbCIR998
28.2 gHbCIR441
29.3 gHbCIR139
33 EM33/15
35.8 EM12/4
37.3 EM15/6
37.5 EM13/8
39.8 EM12/1
48.1 EM24/6
48.8 EM24/15
51.8 gHbCIR406
52 gHbCIR265.L2
54.6 EM14/4
55.1 gHbCIRE171
63.1 gHbCIR494
64.5 EM22/11
72.8 EST
77.1 EM24/7
78.5 EM24/14
80 EM22/14
82.5 gHbCIR79
85.4 gHbCIR542
87.9 gHbCIR167
94.5 EM7/9
106.5 EM21/3
114.1 gHbCIR45
127.8 EM3/17
g16
0 EM57/5
5.2 gHbCIR161.L2
10.6 EM24/5
17.5 EM21/9
19.1 EM54/1
21.3 EM22/7
23.9 gHbCIR689.L2
31.5 gHbCIR523.L2
40.4 EM3/11'''
51.8 EM2/14
68.6 gHbCIR130
69.2 gHbCIR191
71.7 EM17/7
72.1 EM24/4
73.8 EM3/13
74 EM14/13
75.1 EM15/11
87.5 gHbCIR302
93.1 gHbCIR647.L2
110.3 gHbCIR373.L2
116.8 gHbCIR588
131.1 gHbCIR421
138.8 EM3/3
g17
0 EM15/10
8.9 gHbCIR585
18 EM22/6
29.5 EM16/17
31.6 EM17/9
33.2 EM45/8
36.1 gHbCIR420
38.5 gHbCIR586
46 gHbCIR339.L2
47.7 EM32/1
51.6 EM7/4
54.3 M340
57.3 gHbCIR366
60.3 EM48/10
61.1 EM61/1
61.3 gHbCIR635
67.2 gHbCIR526
68.1 EM57/11
68.3 gHbCIR617
70.6 EM40/2
70.7 EM63/12
71.8 EM23/11
72.9 EM9/11
74.7 EM47/3
77.6 EM36/4
91.8 gHbCIR357
g18
0 EM22/13
6.4 gHbCIR41/47
8.8 gHbCIR266
12.4 gHbCIR227
24.8 PGD
29 EM3/21
30.1 EM22/16
32.5 gHbCIR618
34.1 GS2
38.7 EM16/14
42.3 gHbCIR112
43 gHbCIR443
43.3 gHbCIR259
44.4 gHbCIR101
47.6 gHbCIR20.L2
61.8 EM16/19
65.7 EM9/1
81.5 gHbCIR423
84.8 EM21/12
98.1 EM21/13
98.9 gHbCIR533
125.3 EM16/6
126.8 EM13/3
127.7 EM23/2
135.1 EM3/23
136.3 EM21/14
137.6 gHbCIR597
138.6 EM57/19
141.1 EM2/3
143.2 EM7/11
145.1 EM41/8

Fig. 3

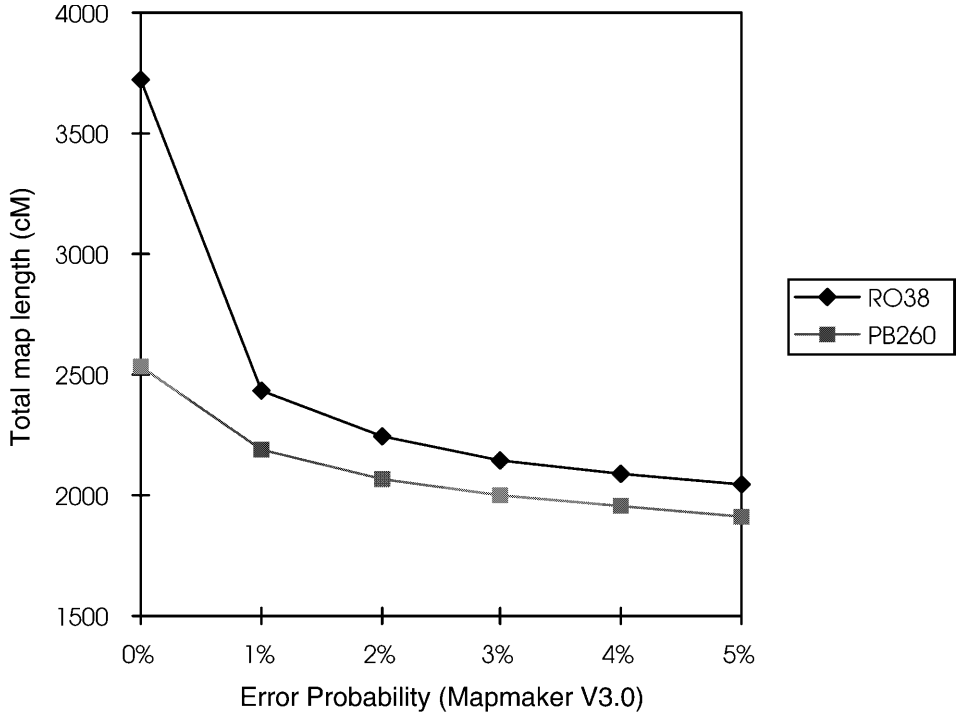


Fig. 4 PB260 and RO38 total map length comparison for different values of *error probability* (see text) used in multipoint analysis using MAPMAKER

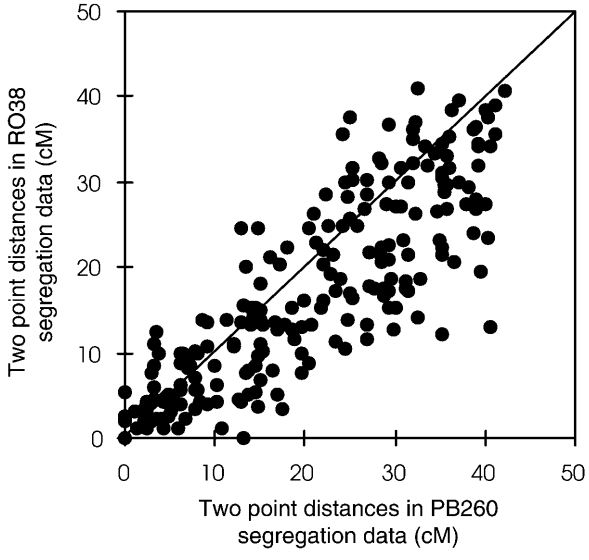


Fig. 5 Comparison of two-point distances (cM) between PB260 and RO38 linkage data. One hundred and thirty-two codominant markers were considered

Comparison of the parental maps

The main difference between the two maps resides in the greater number of loci in the RO38 map due to the higher level of heterozygosity of this interspecific clone. For locus-specific markers, colinearity between the two parental maps was quite good. Other than for 2 markers, no change in the chromosome assignment and only small modifications in the placement of markers were observed. The length of the maps, however, were not the same. Linkage maps for PB260 and RO38 have been established using the *"error detection"* option of MAPMAKER version 3.0 (Lincoln and Lander 1992) with an error probability of 3%. This error rate had been estimated by scoring progeny genotypes obtained from different DNA samples for a same set of markers. The total map length was also compared for different error rates (Fig. 4). PB260 and RO38 map lengths are very different if no error correction is applied (error rate: 0%). If more than 1% of scoring error is admitted, the difference between the two map lengths decreases quickly. The recombination rate observed between pairs of linked loci was compared between the male and the female maps with a paired *t*-test. The codominant loci retained as bridges to construct the synthetic map were used, permitting us to compare 220 intervals located throughout the genome (Fig. 5). An overall significant map length reduction of approximately 17% was observed in the RO38 map ($P<0.001$).

Locus duplication

The duplication scheme of the rubber genome was investigated with the RFLP markers. Among the 266 RFLP probes tested, 26 probes clearly revealed more than 1 locus based on the linkage data. Three types of situations were observed (Table 3). (1) Duplicated loci were detected on 2 different linkage groups of the same parental map. These included 1 probe on the PB260 map, 14 probes on the RO38 map, and 4 probes on both maps. (2) Duplicated loci were observed on non-homologous linkage groups, 1 on each parental map. These included

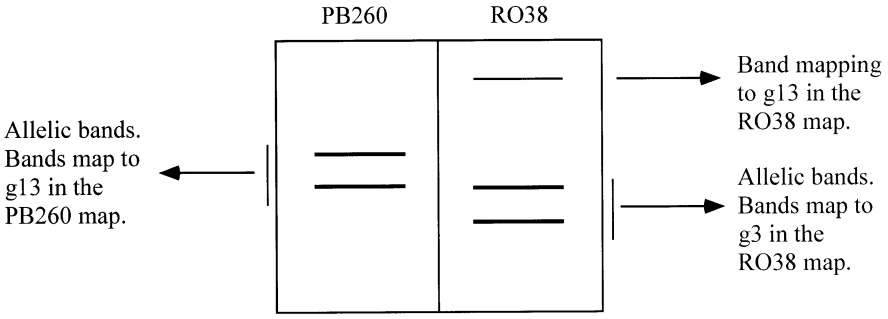


Fig. 6 Parent banding pattern for the marker gHbCIR671

Table 3 Linkage groups and number of duplicated loci

Linkage groups	Number of duplications
g6–g16[a]	5
g3–g13[a]	4
g5–g10[a]	4
g5–g12	2
g1–g14	2
g14–g18	1
g11–g17	1
g8–g9	1
g2–g7	1
g4–g10	1
g11–g15	1
g3–g15	1
g15–g15	1
g9–g9	1

[a] Duplicated regions

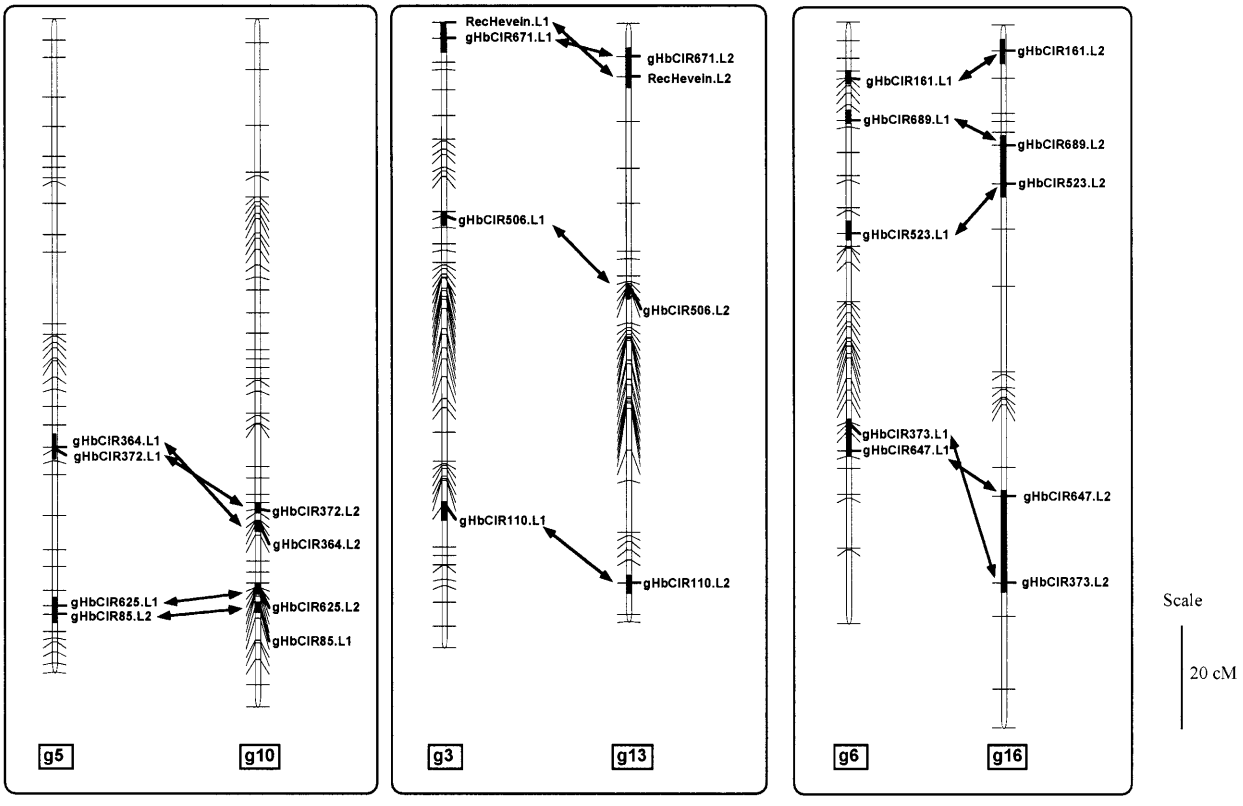
Fig. 7 Duplicated regions in the RO38 map. Four markers were duplicated on the g5 and g10 groups, 4 markers were duplicated on the g3 and g13 groups, and 5 markers were duplicated on the g6 and g16 groups

6 probes. (3) Duplicated loci were observed on the same linkage group of the same parental map. These concerned 2 probes revealing, respectively, 2 loci within group g9 and 4 loci within group g15. The latter probe was a RGA probe (RGAc14) which revealed four loci in a cluster on group g15. One RFLP probe (gHbCIR671) was of both types, (1) and (2) (Fig. 6). Map positions of duplicated loci revealed a clear pattern of regional duplication for 3 pairs of linkage groups, g5 and g10, g3 and g13, g6 and g16 (Table 3 and Fig. 7).

Discussion

Consensus map construction

When mapping is performed based on a cross between heterozygous parents, it is important, for several reasons, to establish parental maps first, before making a synthetic map. First, parental maps allow the examination of the



colinearity between parental genomes and prevent the merging of homologous chromosomes which would have been rearranged. In our case, the parental maps are quite similar except for a few local rearrangments that are probably due to statistical imprecision. Second, bridge markers are required to merge the parental genetic maps and the more bridges used, the better the synthetic map will be. We first defined as bridges those loci that presented a common segregating allele in the meiosis products of both parents (configuration types b, c, and d). We found two cases where apparently homologous alleles corresponding to configuration type d mapped to non-homologous linkage groups in both parental maps. Thus, a band common to the two parents may not always correspond to the same allele at the same locus; it may correspond to alleles of duplicate loci with RFLP, microsatellites, or isozymes. With AFLP, it may also correspond to alleles of completely different loci since, in this case, locus identity is only based on electrophoretic mobility. Thus, it is necessary, before considering a locus as a bridge, to check the consistancy of the map position in both parental maps. Moreover, we considered as supplementary bridges those loci corresponding to the configuration type e and which mapped in homologous linkage groups in both parental maps. The possible error here is to consider as identical those loci which would be duplicated inside the same linkage group. Among the 266 RFLP probes tested, only 2 (less than 1%) revealed duplicate loci inside the same linkage group. This is not uncommon because this kind of clustering has been observed in other plants for resistance gene analogues (Kanazin et al. 1996; Yu et al. 1996). However, it should be pointed out that very closely linked duplicate loci may be overlooked by genetic mapping precision, even though we assume that the error rate is quite low regarding map fusion. With respect to the dominant loci, it is known that recombination rate estimator variance is higher for the codominant-dominant loci associations than for the codominant-codominant loci associations (Ritter and Salamini 1996). However, dominant loci were interesting because 40 of them could be used as bridges to merge the two parental maps. Finally, it is necessary to compare parental recombination rates before merging the maps. If these rates are too different, homologous parental chromosomes should not be merged because this may distort the locus order. In the apple tree map, for example, large differences in the recombination frequencies in both parents for 1 linkage group caused marker order changes in the integrated linkage map (Maliepaard et al. 1998). In our mapping population, the mean recombination rate was 17% lower in the male meiosis than in that of the female, which is a highly significant difference ($P<0.001$). Nevertheless, after merging parental maps, we compared marker orders between the synthetic map and the parental maps and observed only slight differences. The male parent RO38 is issued from an interspecific cross. The general reduction in recombination observed in its map is in agreement with other interspecific mapping data (Bonierbale et al. 1988; Causse et al. 1994). In interspecific crosses, the homology between DNA strands is reduced, and this is generally related to a reduction in the frequency of chiasmata (Causse et al. 1994). Moreover, the female parent of our mapping population, PB260, was issued from crosses performed at the end of the 19th century within a limited genepool (Whickam clones). This certainly contributes to the contrasting of heterozygosity between the two parents. Nevertheless, it would be useful to analyse the recombination rate in the reciprocal cross to test if there is any reduction of recombination in male gametogenesis in rubber tree as has been observed for other species (de Vicente and Tanksley 1991).

Map description

Genetic length of the 18 chromosomes is fairly homogeneous in the F_1 map. The average map length per chromosome is about 120 cM, which is in accordance with what has been found in other crop species (Maliepaard et al. 1998). Some regions of about 20 cM in length are still without any marker and should be completed with BSA of AFLPs (Harushima et al. 1998). Clusters of markers, mainly AFLP markers, can be found in some linkage group regions. This has already been observed for other crops and may correspond to reduced recombination frequency regions such as centromeres (Keim et al. 1997).

Estimations based on flow cytometry suggest that the amount of DNA in a haploid nucleus (1C-value) is about 2 pg for both species, *H. brasiliensis* and *H. benthamiana* (M. Seguin, personal communication). This value is in accordance with the amount of DNA found by Bennett and Leitch (1997) for *H. brasiliensis*. With the RO38 estimated map length of 2660 cM, 1 cM corresponds, on average, to approximately 700 kb, which is quite similar to the estimation found for tomato (Causse et al. 1994).

The 266 RFLP markers are well distributed over all 18 linkage groups and constitute a framework of markers which could be used for any other *Hevea* genetic map. Nevertheless, these RFLP markers were insufficient to saturate the map, and AFLP markers appeared as a very useful tool to quickly saturate the map. However they reveal dominant markers and will be less informative than RFLP loci with three or four alleles for the quantitative trait loci (QTL) analysis. The few microsatellites mapped are widely distributed along the map. Development of additional microsatellites from an enriched library is in progress. They should soon be integrated into the map and should facilitate map construction in the future.

Hevea ploidy

Most species of the Euphorbiaceae family have a basic chromosome number, n, of between 7 and 11 (Ong 1975). Rubber tree and cassava, the two most economically important crops of the Euphorbiaceae, have 36

chromosomes. The cassava genome is assumed to be of tetraploid origin. Despite this, the first cassava genetic map encompasses only 6 duplicated loci for 236 RFLP probes tested (Fregene et al. 1997). Preferential pairing and disomic inheritance were also observed for the majority of the chromosomes.

Cytological studies in different *Hevea* species have shown that bivalents are mainly formed and that tetravalents are formed only rarely as a result from pairing between non-homologous chromosomes during prophase I and metaphase I of meiosis (Bouharmont 1960; Ong 1975). These observations suggest that large DNA segments are shared in common between different chromosomes. Rubber tree was therefore considered as an amphidiploid, resulting from the fusion of two related genomes. The very low number of tetravalents observed would dismiss the hypothesis of autotetraploidy. Recent *in situ* hybridisation studies have revealed that the *Hevea* genome possesses two distinct 18S-25S rDNA loci and one 5S rDNA locus (Leitch et al. 1998). The author suggested a possible allotetraploid origin for *Hevea*, with the loss of one 5S rDNA locus during the evolution process. Nevertheless, no potential ancestor for *Hevea* with 2n=18 is known.

The genetic inheritance of 13 isozymes was analysed in a previous study (Chevallier 1988). All enzymes showed a 1:1 segregation ratio, and only 2 enzymes revealed duplicated loci. This favoured a diploidy and disomy in rubber tree. Our data reinforce these observations. Whatever the evolutionary origin of rubber tree, it behaves as a diploid. On the basis of the number of bands detected by RFLP probes, one-third of the probes reveal complex banding patterns which may correspond to more than 1 locus. A partially non-random arrangement of duplicate loci was observed in our study with certain chromosome pairs (Fig. 7). This suggests that the chromosomal segments containing these sequences possess extensive regions of homology and almost certainly had a common ancestor. Nevertheless, nothing proves that non-homologous chromosomes implicated in tetravalent formations are the same as non-homologous chromosomes which share duplicated regions (Helentjaris et al. 1988). At this stage, we cannot say whether duplicate regions represent vestiges reflecting an ancient polyploid origin or whether they represent duplications that occurred later. Finally, our results suggest that rubber tree, similarly to cassava, is not a typical amphiploid such as wheat or cotton. Our RFLP-based genetic map showed that the *Hevea* genome presents only a segment of the homeologous chromosomes whose origin is still unknown, and it behaves as a diploid genome. A similar mapping study with apple tree (x=17), member of the Rosaceae family (x=7, 8, or 9), showed a disomic inheritance of RFLP markers and duplication of linked markers (Maliepaard et al. 1998). Previous studies suggested an allopolyploid origin for apple tree and provided evidence that at least one progenitor was a Spiraeoid (x=9) (Morgan et al. 1994). Perhaps a similar search for duplications and parallel linkages in Euphorbiaceae-related genera, such as cassava, or even some n=9 species, using the rubber tree probes which identify duplicated sequences, would be most informative.

The parental maps and the synthetic map presented here will be used for QTL analysis of resistance to South American Leaf Blight. These maps will also be useful to complete the first analysis of *Hevea* germplasm diversity. They will enable us to choose new markers mapping in genome regions which were not represented in the first analysis. Finally, cross-hybridisation of rubber tree and cassava genomic RFLP probes has been tested, suggesting the feasibility of a comparison map between the two genomes (M. Seguin and J. Tohme, personal communication). Despite their very different DNA content (0.84 pg for cassava and 2 pg for *Hevea* for 1 C), the similarity of mapping results between the two crops, with few duplications and a diploid-like segregating behaviour of molecular markers, is encouraging for further genome comparative analyses.

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