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Construction of a molecular linkage map in coffee

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Abstract A linkage map for coffee (*Coffea canephora P.*) totalling 1402 cM has been developed on the basis of a population of doubled haploids. Both RFLP markers and PCR-based markers (RAPD) were used to construct 15 linkage groups. Coffee genomic and cDNA clones provided the source of the probes. In total, 47 RFLP and 100 RAPD loci have been placed on the linkage map. A rather low DNA polymorphism rate (18% for RFLP markers and 29% for RAPD primers) was detected. Only 81% of RAPD markers and 85% of RFLP markers fit an expected 1:1 ratio ($P < 0.01$). The availability of a molecular linkage map has many implications for the future development of the genetics and breeding of this commercially important crop species.

Key words Coffee · RAPD · RFLP · Linkage map · Doubled haploids

Introduction

Detailed linkage maps based on restriction fragment length polymorphism markers (Heun et al. 1991) and polymerase chain reaction-based markers, called RAPDs (Rafalski et al. 1991), have been developed for a number of plant species.

However, in spite of its commercial importance, little genetic research has been devoted to coffee, particularly to the development and use of molecular markers. Coffee has several features which make it especially difficult for genetic studies. RFLP mapping in *Coffea arabica* ($2n = 4x = 44$) has been hampered by a very low level of polymorphism (Paillard et al. 1993) as well as by complications arising from polyploidy. Also, segregating populations derived from controlled crosses are difficult to develop in a strictly allogamous species with a long juvenile period such as *Coffea canephora.*

It was decided to work on the diploid *C. canephora* species ($2n = 2x = 22$) (Lashermes et al. 1993; Paillard et al. 1994) since study was facilitated by the use of a doubled haploid population obtained by Couturon (1982) and described by Lashermes et al. (1994b). Since only homozygote genotypes are present, dominant markers such as RAPDs are equally informative as co-dominant markers.

The goal in this project was to develop a genetic map for coffee for use in genetics and for future quantitative trait locus (QTL) analysis.

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Materials and methods

Plant material for genetic mapping

Doubled haploids (DHs) were produced from *C. canephora P.* (Couturon 1982). The method of production is based on haploid plants occurring spontaneously in association with polyembryony. The DH population used for the present study was derived from the clone IF200 which was able to produce a large number of DH plants. The high level of heterozygosity of the parental clone and details concerning the characteristics of the DHs are reported in Lashermes et al. (1994b). Eighty five DHs were used for the map construction.

Libraries and RFLP probes

A Lambda ZAP II cDNA library was constructed from RNA extracted either from leaf tissue or from a cell suspension of the genotype 'Arabusta'. This hybrid issued from a cross between *C. arabica* and *C. canephora* produced in the Ivory Coast (Capot 1972). RNA was oligo dT-primed and cloned; the insert size ranged from 0.8 to 4.5 kb. Out of 200 clones checked for repeated sequences, 41% were positive on dot-blot experiments; 75 low-copy cDNA clones were screened for polymorphism and 20% were selected.

Some genomic clones (designated gA) were obtained from a *C. arabica PstI* library; however, most of the genomic clones (designated g) used for map construction were derived from an 'Arabusta' pUC18-*Bam*HI library; in addition, one clone came came from a pUC18-*Pst*I library (designated g55E) and one clone was from a pUC18-*Eco*RI library (designated g94E).

'Arabusta' DNA was partially digested by the enzyme *Mbo*I (Pharmacia) and loaded on a 10–40% sucrose density gradient (Sambrook et al. 1989). As an alternative to a sucrose gradient, *Mbo*I partially digested plant DNA was eluted from a gel. The *Mbo*I inserts were ligated in de-phosphorylated pUC18 cut by *Bam*HI.

DNA inserts isolated from plasmid by means of a PCR reaction were used as probes. They were labelled with [^{32}P]dCTP using the random priming labelling method (Feinberg and Vogelstein 1983). A total of 250 RFLP probes were sreened for the map construction. RFLP probes were selected for their ability to produce both single- or low-copy signals.

DNA isolation

Fresh leaves

Leaves were washed and frozen (−80 °C) and 25 g of leaves were ground to a fine powder in liquid nitrogen. The frozen powder was re-suspended in 200 ml of extraction buffer [0.35 M Sorbitol, 0.1 M Tris-HCl (pH 8.0), 0.005 M EDTA, 1 g sodium bisulphite] and filtered. After a 20-min centrifugation (approximately 1000 g) at 4 °C, the pellet was carefully re-suspended in 5 ml of cold extraction buffer, 7 ml of lysis buffer [0.2 M Tris-HCl (pH 8.0), 0.05 M EDTA, 2 M NaCl, CTAB 2%] and 2.5 ml of sodium sarkosyl 5% (w:v). The mixture was then incubated at 65 °C for 0.5 h. Approximately 10 ml of chloroform:isoamyl (24:1) were added and the tube was inverted about ten times to form an emulsion. The emulsion was centrifuged for 15 min at 10000 g at 4 °C.

The upper aqueous phase was transferred to a new tube and DNA, precipitated with 0.45 ml of 3 M sodium acetate and 10 ml of cold ethanol, was hooked out, washed in 70% ethanol and re-suspended in 5 ml of TE. The DNA was then purified by RNAse (200 µg/ml) (for 30 min at 37 °C and by Proteinase K (400 µg/ml) for 1 h at 56 °C. Five milliliters of phenol:chloroform:isoamyl (25:24:1) were added and, after emulsion, the mixture was centrifuged for 15 min at 10000 g and 4 °C. DNA was re-suspended in 0.2–0.5 ml of TE and its concentration was evaluated by measurement of the optical density at 260 nm.

Lyophilized powders

One gram of lyophilized powder from leaves was re-suspended in 15 ml of 65 °C extraction buffer (MATAB 2%, 1.25 M NaCl, 0.1 M Tris-HCl pH 8.0, 0.02 M EDTA, 0.1 M β mercaptoethanol) and incubated at 65 °C for 1.5 h. Extracts were mixed with 7.5 ml of phenol:choloroform:isoamyl alcohol (25:24:1) at room temperature for 10 min.

The emulsion was centrifuged for 10 min and the upper aqueous phase was transferred to a new tube and a phenol:chloroform cleaning was performed. To the final aqueous phase, RNAse (200 µg/ml) was added and incubated for 1/2 h at room temperature. DNA was precipitated with an equal volume of isopropanol; DNA was then hooked out and dissolved overnight in 1 ml of TE. DNA was precipitated with 0.25 M NaCl and 2.5 ml of ethanol. The DNA lifted out again was washed with 4 ml of wash buffer (76% ethanol, 0.2 M sodium acetate). Ethanol was removed and DNA re-suspended in 0.5 ml TE.

The DNA yield was considerably improved when anti-oxidizing substances (β-mercaptoethanol, 0.15 M ascorbic acid or sodium bisulphite) were added to the isolation buffer; 1 g of dried leaves gave an average of 150 µg of DNA whereas 250 µg were obtained from 25 g of fresh non-oxidized leaves.

Plant DNA digestion, electrophoresis, blotting and hybridization

Two different types of filters were prepared: (1) survey filters to test for polymorphism between the IF200 DNA and six DH lines with four different restriction enzymes (*Dra*I, *Eco*RI, *Eco*RV and *Hin*dIII), and (2) progeny filters with IF200 and all genotypes were digested by the same restriction enzyme.

Two restriction enzymes, either *Dra*I or *Eco*RI (Biolabs), were chosen for the RFLP study. Ten micrograms of DNA were used per digestion and electrophoresed on a 0.8% agarose gel in Tris-borate-EDTA (TBE) buffer. DNA was transferred from gels to Hybond-N+ filters (Amersham) or Genescreen plus filters (DuPont NEN) using the DNA alkali blotting procedure recommended by the manufacturer.

Hybridization and washes of membranes were performed according to Amersham's instructions. Filters were exposed to X-ray films (DuPont NEN) and used up to 19 times after removal of probes through a 0.5% boiling bath.

DNA amplification and RAPD markers

The amplification procedure was similar to that described by Williams et al. (1990). The PCR procedure was performed with 25 ng of DNA, a final 0.15 mM of each dNTP, 45 ng (0.3 µM final) of primer (decamer oligonucleotide purchased from Operon Technologies Inc. or Genset), 2 units of *Taq* polymerase (Stratagene) in buffer, and sterile H_2O up to 50 µl. A first denaturation step was done for 2 min at 94 °C. Then amplification was achieved for 45 cycles of denaturation (1 min, 94 °C), primer annealing (1 min, 37 °C), primer extension (2 min, 72 °C), then one additional cycle the same except for a 7-min extension time; 45 µl of the amplification products were loaded on 1.4% agarose gels.

Data analysis and map construction

Goodness of fit to a 1:1 ratio for DH loci was determined by means of a chi-square test (χ^2). Segregating RFLP and RAPD markers were scored for each of the DH lines as either A or H and analysed on a Vax/Vms version 5 (supplied by Digital) of Mapmaker (Lander et al. 1987; Lincoln et al. 1990). Two-point analysis was used to identify linkage groups with recombination values (θ) of 0.4 and with a LOD score of 4.0 ($\log_{10}$ of the likelihood ratio). Starting with the most tightly linked markers, multipoint analysis was performed and the most likely order was established with an exclusion threshold of −2.0 LOD score units. The Kosambi function (Kosambi 1944) was used for converting recombination fractions into map distances or centi-Morgan (cM) values.

Results and discussion

Restriction fragment length polymorphism

Probe choice

Two-hundred and fifty genomic probes and 75 cDNA probes were screened as RFLP probes to detect polymorphism between IF200 and the DH lines. Approximately 41% of the cDNA clones gave patterns on genomic Southerns indicative of repeated DNA. The use of *Pst*I digests has led to the cloning of single- or low-copy clones in many plants, such as tomato (Zamir and Tanksley 1988) and maize (Burr et al. 1988). In coffee, among 29 *Pst*I probes, 31% gave repeated signals.

DNA polymorphism has been detected as frequently with genomic DNA probes (15–20%) as with cDNA probes. In tomato (Miller and Tanksley 1990) and in lettuce (Landry et al. 1987), the cDNA clones detected more polymorphism than did random genomic probes, whereas the opposite was observed for bread wheat (Gill et al. 1991).

The *Eco*RI library was a source of a higher frequency of repeats (80%) whereas the *Bam*HI library appeared to be the best choice for avoiding the cloning of repeated sequences and obtaining a higher polymorphism rate.

One-hundred and forty seven RFLP clones were grouped on the basis of the number of fragments detected by any endonuclease; most of the clones (47%) were single loci, 18% were duplicated loci, and 35% were multiple loci. Seven cDNA probes out of eleven were multiple loci. A few PCR products from RAPDs were tested as probes, but all contained repetitive elements.

Complex RFLP patterns corresponding to unlinked homologous sequences seemed to be frequent in the coffee genome. A segregation study of some RFLP probes showed that, in certain cases, when the hybrid IF200 had two alleles A and H, some of the doubled haploids were neither A or H, as expected, but AH. Such probes detected duplicate loci of which one mapped to a chromosome and the second remained unlinked. Those probes probably referred to gene families dispersed on different chromosomes.

Choice of plant DNA restriction enzymes

Four enzymes were tested (*Dra*I, *Eco*RI, *Eco*RV and *Hin*dIII) for their ability to give polymorphism. For the 100 probes employed, *DraI* gave a 58% polymorphism rate, *Eco*RI 60%, *Eco*RV 25%, and *Hin*dIII 37%.

Figure 1 shows the segregation of marker g1069D in 17 DHs digested with *Dra*I.

Random amplified DNA polymorphism

A total of 250 10-base random primers were screened against IF200 and a sample of eight DHs. Based on the quality and intensity of the amplification (Fig. 2) a total of 73 primers detecting polymorphism were selected. The polymorphism rate obtained with RAPD markers was higher than with RFLP markers; 29% of the primers tested gave polymorphic patterns. Minor fragments, which seemed to possess the greatest propensity for irreproducibility, were not considered in this analysis. RAPD reactions amplified an average of 4.5 visible bands on an ethidium bromide-stained agarose gel. Among the amplification products, 1–3 products per primer were polymorphic with a mean size of 1 kb.

Segregation distortion

Among all probes screened, 12% of the RFLP markers and 20% of the RAPD markers showed a segregation which deviated from the expected 1:1 ratio ($P < 0.01$) (Table 1).

An analysis of the segregation data for most of the RAPD and RFLP markers is shown in Fig. 3. The frequency of distribution of loci was evaluated for 87 RAPD markers and 46 RFLP markers. Since it was not possible to trace parental alleles, the most frequent allele was denoted as A and the percentage of this allele in the DH population was calculated. The segregation ratio of 17 RAPD loci versus three RFLP loci was highly distorted. Skewed segregation ratios have been frequently reported in the literature (Helentjaris et al. 1986; McCouch et al. 1988; Kam-Morgan et al. 1989; Keim et al. 1990; Prince et al. 1992). Heun et al. (1991) noted that 10% of the loci mapped in a doubled haploid population of barley (*Hordeum vulgare*) deviated from the expected 1:1 ratio at $P < 0.01$.

Since the coffee DH population appeared spontaneously through polyembryony (Couturon 1982), gametic selection during this process might explain the disturbances. However, the clone IF200 was chosen for producing high numbers of DHs and for its good combining ability. It has been suggested by Lashermes et al. (1994a) that the DHs produced were an unbiased sample of the parental gametes with regard to agronomic traits.

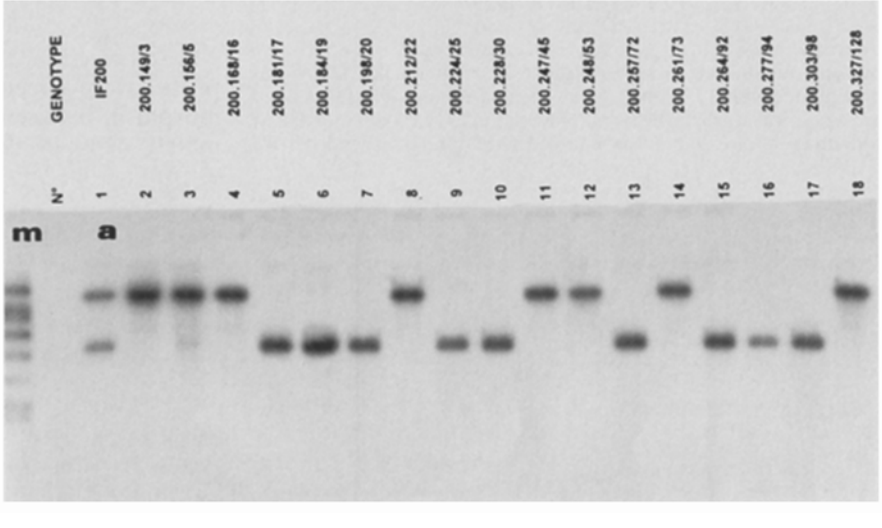


Fig. 1 Autoradiogram derived from probing *DraI*-digested DNA from IF200 (*lane a*) and 17 doubled haploid progenies showing a simple banding pattern against genomic probe g1069D. *Lane m* contains a standard molecular DNA marker

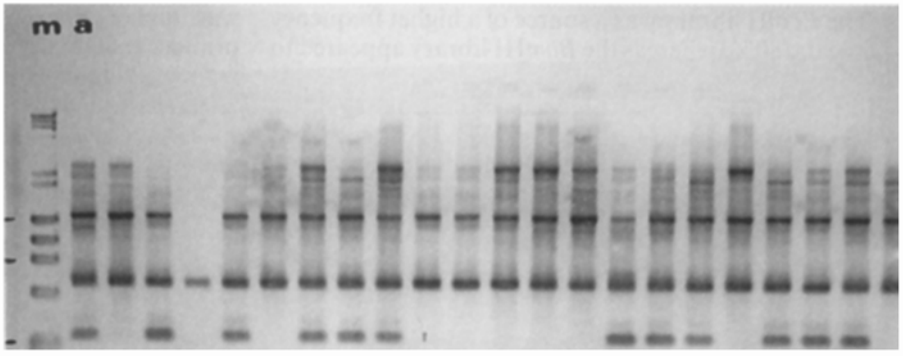


Fig. 2 RAPD gel profile. Assay with primer OPA 07 (Operon Technology) was performed on genomic DNA of the parent IF200 (*lane a*) and 21 doubled haploid progenies. *Lane m* contains a mix of λ DNA digested with *Hin*dIII and ϕX 174 DNA digested with *Hae*III

Table 1 RAPD markers list. Some primers gave several polymorphic amplification products, denoted a, b, c. Lines with bold characters indicate distorted markers

Locus name	Sequence	χ^2 $\alpha = 0.01$ 1:1 ratio	Segregating products kb	Locus name	Sequence	χ^2 $\alpha = 0.01$ 1:1 ratio	Segregating product kb
OPA03	AGTCAGCCAC	0.42	1.2	OPK11	AATGCCCCAG	5.76	3
OPA07	GAAACGGGTG	3.16	0.3	OPK14a	CCCGCTACAC	0.3	2.2
OPB07	GGTGACGCAG	4.25	1	**OPK14b**		**7.53**	**0.8**
OPB10	CTGCTGGGAC	2.33	1.2	**OPK14c**		**7.2**	**0.7**
OPB15	GGAGGGTGTT	0.19	1.5	OPL4	GACTGCACAC	5.31	1
OPB18	**CCACAGCAGT**	**21.26**	**1.5**	**OPL7a**	**AGGCGGGAAC**	**16.9**	**1.5**
OPC01	TTCGAGCCAG	1.99	1.3	OPL7b		0.012	0.8
OPC02	GTGAGGCGTC	0.1	0.9	OPL8	AGCAGGTGGA	4.76	1
OPC5	GATGACCGCC	0.29	1.3	OPL10	TGGGAGATGG	0.012	0.5
OPC11	AAAGCTGCGG	1.22	1.5	OPL12	GGGCGGTACT	0.31	0.85
OPD2a	GGACCCAACC	4.24	0.8	**OPL14**	**GTGACAGGCT**	**15.8**	**1.3**
OPD2b		**16.1**	**0.7**	OPM2	ACAACGCCTC	0.22	0.8
OPD11	**AGCGCCATTG**	**9.89**	**0.6**	**OPM12**	**GGGACGTTGG**	**9**	**0.5**
OPD15	CATCCGTGCT	3.8	3	OPM15	GACCTACCAC	0.19	0.8
OPE3	CCAGATGCAC	2.33	1	**OPM18**	**CACCATCCGT**	**13.5**	**1.3**
OPE14	**TGCGGCTGAG**	**17.28**	**0.6**	OPN5a	ACTGAACGCC	0.78	1
OPE16b	GGTGACTGTG	1.42	0.7	OPN5b		0.78	0.8
OPE16c		0.58	0.6	OPN5c		0.19	0.7
OPE18	GGACTGCAGA	1.47	0.9	OPN6	GAGACGCACA	1.42	1.1
OPF01a	ACGGATCCTG	2.06	3	OPN11	TCGCCGCAAA	5.76	0.3
OPF01b		1.25	1	**OPN14a**	**TCGTGCGGGT**	**25**	**1.1**
OPF01c		1.25	0.8	OPN14b		2.77	0.4
OPF06a	GGGAATTCGG	0.58	0.8	OPN15	CAGCGACTGT	0.76	0.3
OPF06b		4.76	2	**OPN16a**	**AAGCGACCTG**	**8.24**	**1.2**
OPF09	CCAAGCTTCC	0.53	0.9	OPN16b		0.049	0.7
OPG02	GGCACTGAGG	2.71	2	OPO6	CCACGGGAAG	0.31	0.6
OPG03a	GAGCCCTCCA	1.99	0.7	OPO10	TCAGAGCGCC	5.98	2.1
OPG03b		**7.35**	**0.6**	OPO20	ACACACGCTG	6.37	1.5
OPG5	CTGAGACGGA	3.8	0.9	OPP6	GTGGGCTGAC	4.88	0.5
OPG6	**GTGCCTAACC**	**20.25**	**3**	OPP15	GGAAGCCAAC	2.04	0.7
OPG9a	CTGACGTCAC	4.87	1.1	OPP17	TGACCCGCCT	3.86	1.1
OPG9b		2.71	0.5	OPQ1	GGGACGATGG	6.53	0.8
OPH3a	**AGACGTCCAC**	**9**	**0.8**	**OPQ6**	**GAGCGCCTTG**	**22.3**	**1.1**
OPH3b		0.012	0.7	OPQ12	AGTAGGGCAC	2.33	0.4
OPH4a	GGAAGTCGCC	0.11	2	OPQ14	GGACGCTTCA	2.45	1.5
OPH4b		0.6	0.8	OPQ18	AGGCTGGGTG	1.71	0.8
OPH7	**CTGCATCGTG**	**11.3**	**0.9**	**OPR1**	**TGCCCCTCCT**	**7.53**	**1.5**
OPH8a	GAAACACCCC	1.46	2.2	OPR2	CACAGCTGCC	0.97	0.7
OPH8b		0.3	0.8	OPR8	CCCGTTGCCT	0.3	1
OPH11	CTTCCGCAGT	3.02	1.1	OPR12	ACAGGTGCGT	5.9	0.6
OPH19	CTGACCAGCC	2.41	0.8	OPR13	GGACGACAAG	0.65	0.9
OPI12	AGAGGGCACA	3.85	1.2	OPR20	ACGGCAAGGA	0.32	0.8
OPJ10	AAGCCCGAGG	0.6	1.8	**OPS13**	**GTCGTTCCTG**	**8.8**	**0.95**
OPJ19	**GGACACCACT**	**15.42**	**0.6**				

Map construction

One-hundred and forty seven markers (31 g, 16 c and 100 RAPD) were mapped to 15 linkage groups spanning 1402 cM (Fig. 4). Among all linkage groups, the average distance between two markers was 10 cM. Four linkage groups spanned between 160 and 210 cM, eigth between 40 and 110 cM, and three groups were smaller. The

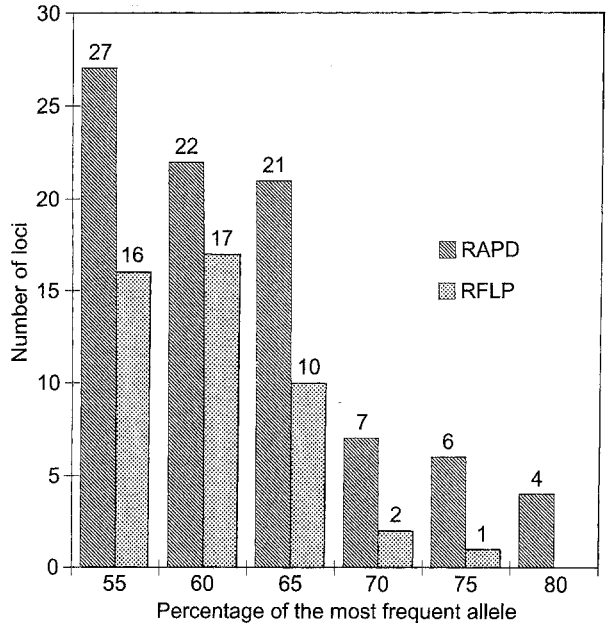


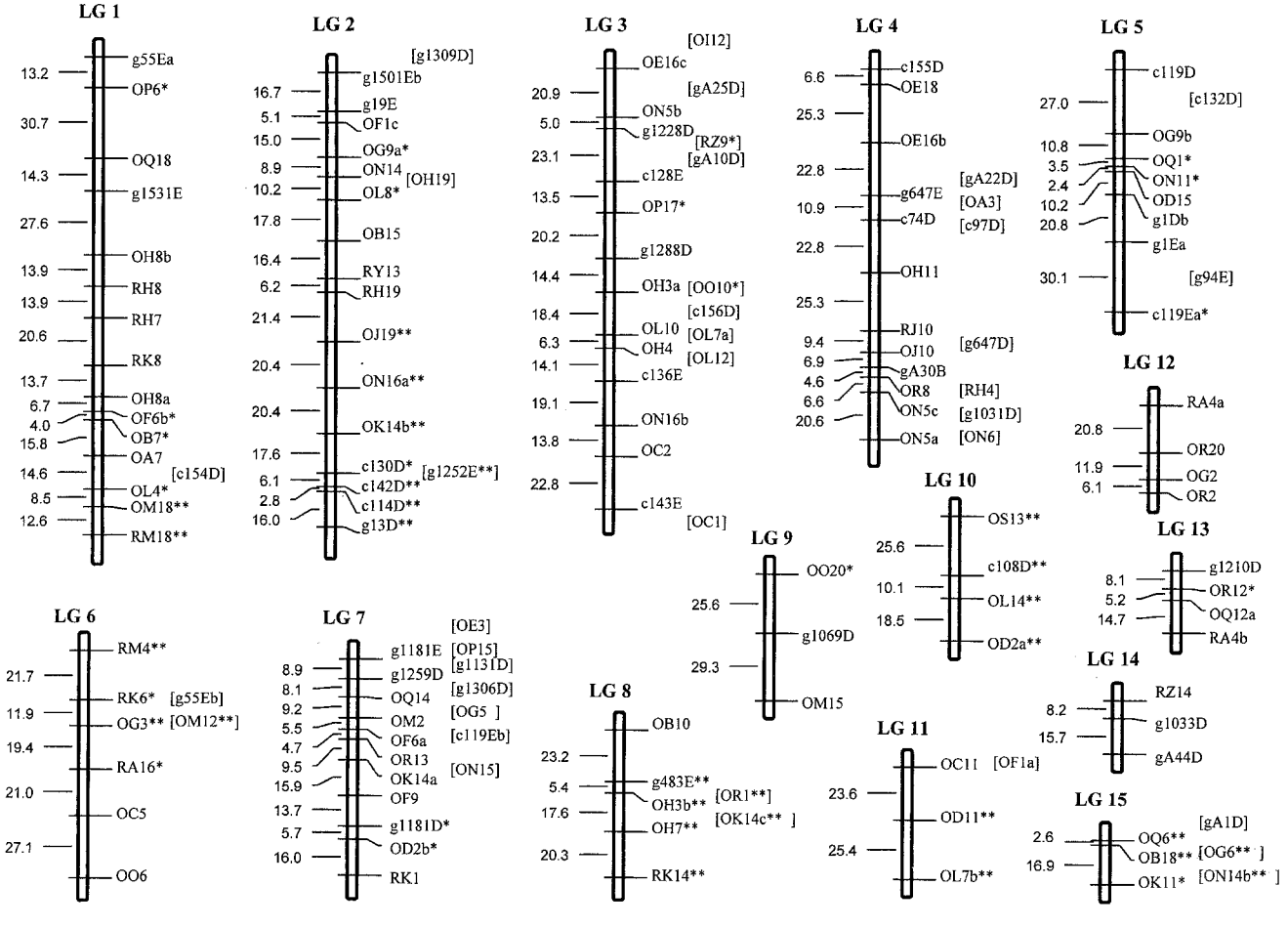
Fig. 3 Frequency distribution of 87 RAPD loci and 46 RFLP loci in the doubled-haploid population according to their deviation of single-gene segregation from the expected 1:1 ratio

relative lengths of the *C. canephora* chromosomes have been shown (Bouharmont 1959) to vary by a factor of two (12.9%–6.7% of the total length of the idiogram).

RFLPs probes from both genomic and cDNA libraries appeared to be evenly distributed throughout the genome (Fig. 5). Every linkage group, except for the two small groups 11 and 12, was covered with RFLP and RAPD markers at the same time.

Seven genomic clones, one c-DNA clone and six RAPD markers remained unassigned to linkage groups. This observation, together with the discrepancy between the haploid chromosome number (n = 11) and the current number of linkage groups (15), suggests that additional markers should be mapped to fill the gaps. Lowering the threshold LOD score to 3.0 resulted in the merging of linkage groups LG11, LG13 and LG3, of groups LG10 and LG6, and of groups LG1 and LG5, leading to a correspondence between number of linkage

Fig. 4 Genetic linkage map of *C. canephora*. Map distances in cM (Kosambi function) are indicated on the left side of linkage groups and the locus names are on the right side. RFLP markers are prefixed with "*c*" for loci detected by 'Arabusta' cDNA clones, "*g*" or "*gA*" for loci detected by random genomic clones of 'Arabusta' and *arabica* respectively. RAPD markers are labelled "*O*" or "*R*". Markers that could not be ordered unambigously are indicated in *brackets*. Loci marked * and ** deviated significantly from a 1:1 ratio at $P < 0.001$ and $P < 0.01$ respectively



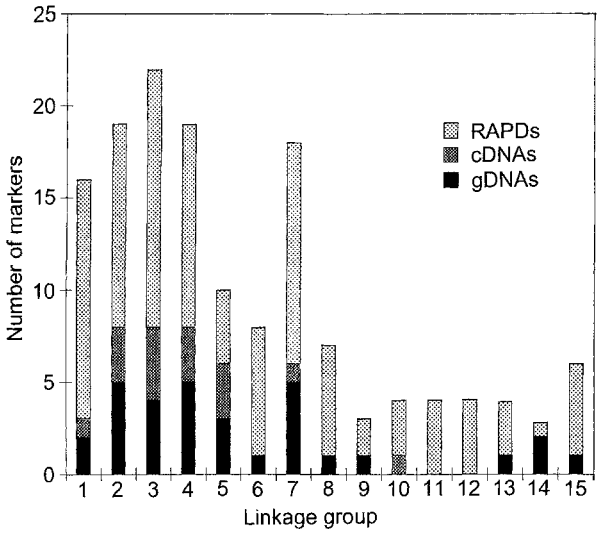


Fig. 5 Histogram depicting the number of loci detected with genomic, cDNA and RAPD clones for each of the linkage groups

groups and number of chromosomes. The longest gap corresponded to a 30.7-cM interval on group LG1 and there were a total of 11 gaps that exceeded 25 cM. While mapping additional markers may eventually fill these gaps, another approach to reduce the linkage group number would be to increase the number of progeny.

The map was first established with markers giving a 1:1 ratio. When distorted markers were subsequently introduced they did not change the order of the mapped linkage groups and appeared more or less clustered in some regions of the chromosomes. Most of the loci showing deviant ratios clustered on parts of LG1, LG2, LG6 and LG8 ($P < 000.1$).

In the present study, 147 markers (1/3 RFLPs, 2/3 RAPDs) were used to construct a molecular map of the coffee (variety robusta) genome. The diploid size of the *C. canephora* genome has been estimated by flow cytometry to be 1.33 ± 0.02 pg or 10^9 bp (E. Earle, personal communication). This places the *C. canephora* genome among the small plant genomes.

The map obtained has a good density since, if one considers that 1 cM in *C. canephora* is about 460 kb, 70 markers would have been needed to find linked markers about 20 cM apart (Botstein et al. 1980). Among linkage groups the average distance between two markers varied from 3.25 to 18.3 cM.

Conclusions

Although rather difficult to obtain, a population of doubled haploids in a strictly allogamous perennial woody species seems to be of high value for mapping and for following recombination frequencies when many loci are segregating (Snape 1988). The present work provides a starting point for further molecular study of coffee genetics. Although additional markers are needed to cover the genome more completely, this map opens the way to a more detailed analysis.

The ease of obtaining crosses between the two species, *C. arabica* and *C. canephora* indicates the affinity of both genomes. Since most of the mapped RFLP probes came from the hybrid 'Arabusta' and from *C. arabica*, it might be possible in the future to use these probes for *C. arabica* mapping. This would lead to a genetic analysis of agronomic and technological characters and the localization of quantitative trait loci.

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