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RFLP inheritance and linkage in walnut

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Abstract Thirty-two low-copy-number genomic DNA clones from a walnut (*Juglans* sp.) *Pst*I genomic library were used to establish a molecular-marker linkage map for walnut. The clones were hybridized to restriction-endonuclease-digested DNA from parent walnut trees involved in an interspecific backcross of (*J. hindsii*×*J. regia*)×*J. regia* in order to identify parental polymorphism. Sixty-three backcross progeny were analyzed to determine the inheritance and linkage of 48 RFLP loci. Sixty-six percent of the walnut cloned sequences detected duplicated, but unlinked, loci. Twelve linkage groups were identified by 42 of the RFLP loci. A Poisson probability method for estimating genome size was utilized to calculate the approximate walnut genome length as 1660 cM and to estimate that 138 markers would be needed to cover 95% of the walnut genome within 20 cM of each marker.

Key words *Juglans* · DNA
Restriction fragment length polymorphisms
Genetic mapping

Introduction

Walnut (*Juglans*) species are cultivated for their nuts (particularly *J. regia* L.) and wood (e.g., *J. nigra* L.) and serve as prominent ecological niche species in hardwood forests. They are monoecious trees that are facultatively outcrossed due to heterodichogamy. *Juglans* species have 2n=32 chromosomes and show disomic segregation behavior in meiosis (Woodworth 1930). These trees have few genetic markers, so genetic studies are limited. While loci controlling heterodichogamy (Gleeson 1982) and virus resistance (McGranahan and Leslie 1990) have been described, the majority of phenotypically-scored traits have an unknown genetic basis. Arulsekar et al. (1985, 1986) identified five isozyme loci using four enzyme systems; glucophosphate isomerase, aspartate aminotransferase, phosphoglucomutase, and esterase. No additional molecular markers have been developed.

The present study was undertaken to develop a more complete set of genetic markers for the study of genetic diversity and phylogeny in *Juglans*. RFLP marker loci were chosen for their stable inheritance, codominant nature, numerous polymorphisms, and ability to be compared directly across a broad range of plant taxa. The nature of gene duplications and differentiation was also studied in the genomes of *J. regia* and *J. hindsii*.

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Materials and methods

Plant material

Sixty-three progeny from an interspecific backcross of (*J. hindsii*×*J. regia*)×*J. regia* were grown from nuts collected from an open-pollinated, male-sterile interspecific *J. hindsii*×*J. regia* tree ('Paradox Mom') found within a *J. regia* 'Hartley' orchard. The specific parental genotypes that created 'Paradox Mom' are not known. This is generally true for 'Paradox' hybrids. An initial assumption was made that since the maternal parent was surrounded by 'Hartley' trees, the primary paternal parent was 'Hartley'. Leaves were collected from these parents and progeny and from another cultivar, *J. regia* 'Franquette', grown near the progeny-source orchard.

Probe construction

Walnut nuclear DNA from *J. regia* 'Early Erhardt' was obtained by the method of Vallejos et al. (1986), except that only one round of CsCl purification was performed. One-hundred-thirty micrograms of nuclear DNA was digested with 400 U of *Pst*I for 5 h, loaded on a 10/40% sucrose gradient, and centrifuged for 18 h at 190000 g (45000 rpm Beckman SW 50.1 rotor) to size separate the DNA. Fractions of the gradient containing 1–3 kb of DNA were pooled, diluted threefold with water, ethanol precipitated, and washed in 75% ethanol before drying and rehydration in TE buffer. The 1–3 kb frag-

ments were ligated into the *Pst*I cloning site of CIP-treated (Cobianchi and Wilson 1987) pUC18 bacterial vector. Competent cells of *E. coli* DH5α (BRL) were transformed with plasmids and X-gal⁻ colonies were grown to obtain mini-preps of recombinant plasmids (Miller 1987) containing walnut inserts. Identification of genomic clones corresponding to low-copy-member walnut sequences was performed by the method of Landry and Michelmore (1985), hybridizing radiolabelled total genomic *J. regia* 'Early Erhardt' DNA, restricted with *Pst*I and *Hin*dIII, to dot blots of walnut genomic clones. Walnut genomic clones were digested with *Pst*I, electrophoresed at 1.5 V/cm in 1% agarose, 1× TAE for 4 h, and the genomic inserts were isolated using Gene-Clean (Bio 101) silica matrix according to the manufacturer's specifications. Walnut genomic clones are designated as pFP#: p denotes a plasmid-containing clone number # and FP denotes the library from which the clone was isolated. A wheat ribosomal DNA clone, pTA71 (Gerlach and Bedbrook 1979), was also utilized. The plasmid was digested with *Eco*RI and the wheat rDNA insert isolated with silica matrix.

Walnut DNA isolation

Walnut leaves were ground in liquid nitrogen and stored frozen at −70°C before isolation of DNA by a modification of the method of Doyle and Doyle (1987). Five grams of frozen leaves were added to 20 ml of 60°C preheated 2× CTAB buffer (2% CTAB, 1% PVP, 1% β-mercaptoethanol, 0.1% sodium bisulfite, 1.4 M NaCl, 50 mM Tris, 20 mM Na-EDTA, pH 8.0) , and incubated at 60°C for 30 min. The aqueous solution was extracted with 20 ml of chloroform:isoamyl alcohol (24:1), centrifuged for 10 min at room temperature (1800 rpm ICN bench-top centrifuge) and the aqueous layer retained. Fifteen milliliters of isopropanol were added to precipitate the nucleic acids. The precipitate was spooled and washed in 76% ethanol with 10 mM ammonium acetate. The nucleic acid precipitate was air-dried overnight, rehydrated in 1 ml of TE pH 8.0, digested with 10 µg of RNase (Sigma Chemical Co.) at 37°C for 1 h, and ethanol precipitated. The DNA precipitate was washed in 75% ethanol and dried overnight before rehydration in 200 µl of TE pH 8.0. The DNA was quantified spectrophotometrically at 260 nm and visually in an aqueous gel stained with ethidium bromide containing lambda-phage standards, with approximately 25 µg/ml DNA=1.0 Å at 260 nm.

RFLP detection

Six micrograms of walnut DNA were digested with 30 U of restriction enzyme for 6 h and electrophoresed in 0.8% agarose with 1×TAE buffer for 18 h at 0.7 V/cm. Electrophoresed gels were stained with ethidium bromide to visualize the DNA, and the DNA transferred (Southern 1975) to 150×150-mm nylon membranes (Nytran, Schleicher and Schuell). Membranes were rinsed in 2×SSC, dried for 2 h in a 65°C oven, and stored dry at room temperature. Membranes were trimmed to 140×150 mm, loaded into 30×300-mm hybridization bottles, prehybridized for 1 h, and hybridized for 16–20 h at 65°C according to the method of Church and Gilbert (1984). Thirty nanograms of insert DNA were ^{32}P radiolabelled by the random priming method (Feinberg and Vogelstein 1983), denatured by boiling and rapid cooling, and added directly to each hybridization tube. Following hybridization, membranes were washed for 2×15 min in 2×SSC, 0.1% SDS at room temperature, 2×15 min in 1×SSC, 0.1% SDS at 45°C, and 1×30 min in 0.5×SSC, 0.1% SDS at 65°C, rinsed in 2×SSC, blotted on paper towels, and autoradiographed on X-OMATAR film (Kodak) at −70°C using Cronex (DuPont) intensifying screens for 1–5 days. Blots were stripped by soaking in 5 mM of Tris pH 8.0, 0.5 mM EDTA pH 8.0, 0.01× Denhardts, 0.05% Na-pyrophosphate at 65°C for 30–60 min, rinsed in 2×SSC, and stored dry at room temperature. Blots were reprobed from 5 to 10 times.

Polymorphism identification and inheritance analysis

DNA isolated from the interspecific backcross parents, 'Paradox Mom' and 'Hartley', was digested with 3–5 enzymes (*Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I), electrophoresed, Southern transferred, and assayed for RFLP polymorphism by hybridizations made with radiolabelled walnut genomic-cloned inserts corresponding to low-copy-number walnut sequences and *pTA71*. Probe-enzyme combinations showing discernible polymorphism in the interspecific backcross parents were used to analyze RFLP inheritance patterns among their progeny. Forty-eight RFLP loci were tested for single-locus 1:1 Mendelian segregation ratios using chi-square (χ^2) goodness of fit values and are denoted as *fp* plus the plasmid clone number. Linkage analysis was performed with MAPMAKER (Lander et al. 1987) version 1.9 using the Haldane (1919) mapping function.

Genomic size and marker coverage estimation

The total walnut genome size in centimorgans was estimated with the 'method of moments' procedure (Hulbert et al. 1988) and a Poisson probability method described below. The Poisson probability estimate assumes that: (1) randomly-chosen markers will have a Poisson distribution in the genome and makes corrections for the reduced coverage of markers at chromosome ends, in a manner similar to the procedure of Elston and Lange (1975) and Lange and Boehnke (1982), and (2) each chromosome is of equal length. With k markers, the kth marker will fall outside a range of the other k-1 markers with a Poisson probability dependent on the average chromosome length (L), given as

$$p = e^{-[1-(1-d/L)^2]\,(k-1)/n} \quad (1)$$

with p=proportion of k markers unlinked at distance d
n=haploid number of chromosomes

Since the genome length $G=n\,L$, rearrangement of the above equation yields

$$G = \frac{n\,d}{1-\sqrt{1+\dfrac{n\ln(p)}{k-1}}}. \quad (2)$$

For both estimates, Haldane map distance values of 39 cM and 49 cM were used, corresponding to LOD scores of 3.0 and 2.0, respectively. Estimations of marker coverage were made using the same assumptions for the Poisson probability estimate. Formula (3)

$$k = \frac{-n\ln(1-p')}{1-(1-d/L)^2} \quad (3)$$

was used to calculate the number of markers (k) needed to cover a given proportion (p') of the genome within a specified distance (d) from the markers.

Results

After dot-blot hybridization with radiolabeled total genomic walnut DNA, 106 of 128 clones (83%) from the walnut *Pst*I library were scored as low-copy-number sequences based upon the absence of autoradiographic signal, compared to a rDNA standard, after probing with ^{32}P-labeled total genomic DNA. Thirty-two randomly-chosen low-copy-number sequences were used to analyse the degree of genomic duplication and differentiation in the walnut genome. All 32 probes detected interspecific polymorphisms for at least one restriction enzyme/probe combination when hybridized to DNA of the two parents digested with *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I. Forty-eight reproducible polymorphic loci were scored. DNA restricted with *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I had 54, 67, 92, 90, and 85% polymorphic loci, respectively.

The fragment length per locus averaged 4.0, 8.7, 10.3, 8.3, and 9.4 kb, respectively, for these enzymes. There was a general corresponding increase in polymorphism with increasing fragment length, implying that the polymorphism between species was due to changes between the bordering restriction sites (e.g., insertions/deletion of DNA) rather than to sequence changes at recognition sites (McCouch et al. 1988; Miller and Tanksley 1990).

Because the maternal parent tree was open-pollinated, alleles from males other than *J. regia* 'Hartley' could potentially be present in the progeny at some observable level. Although the vast majority of *J. regia* alleles seen in the progeny were those identified in 'Hartley', there were a few progeny with *J. regia* alleles that were not present in either the maternal parent or 'Hartley'. Therefore, more than one *J. regia* pollinator gave rise to the progeny. These alleles were in fact present in *J. regia* 'Franquette', a cultivar found near the progeny-source orchard that had an anther-shedding date closely related to the female-flowering date of the maternal interspecific hybrid. However, the mix of paternal alleles entering the progeny population did not affect the analysis since all but one of the *J. hindsii* alleles were unique. The 1:1 segregation of *J. hindsii* alleles could be followed at all but one locus as all alleles attributable to the males were *J. regia*. The 32 probes detected 36 loci with high autoradiographic intensity (class 1) and 31 loci with low autoradiographic intensity (class 2). The segregation of 35 class-1 loci and 12 class-2 loci was analyzed for 1:1 segregation of heterozygous (*J. hindsii/J. regia*): homozygous (*J. regia*) allelic genotypes (Fig. 1). The 36th class-1 locus at *fp20B* could not be scored because this duplicated locus was heterozygous (AB) in the maternal interspecific hybrid and heterogeneous (AB and AA) in the *J. regia* pollen sources which were present in an unknown ratio. The remaining 19 unscored class-2 loci did not show segregation for *J. hindsii* alleles or else did not hybridize well enough to be reliably scored. *J. hindsii* alleles had less ^{32}P signal than the *J. regia* alleles for up to 25% of mapped loci, perhaps due to reduced homology between the *J. regia* probe and the *J. hindsii* DNA. This was more noticeable for class-2 loci. The segregation data for the forty-eight scored RFLP loci are shown in Table 1. Thirty-six of the forty-eight loci fitted the expected 1:1 Mendelian ratio (P-value>0.05), while 12 of the loci deviated from this expected ratio (P< 0.05). It was more common to see a lower proportion of *J. hindsii* alleles (in 8 of the 12 cases) than *J. regia* alleles for loci with distorted segregation ratios. Further analysis revealed that six of these *J. hindsii* alleles are found together in similar proportions at closely-linked loci.

Linkage analyses showed that 38 loci mapped to ten linkage groups (LOD>3.0, recombination fraction< 0.28, Fig. 2). Two additional linkage groups were identified among the loosely-linked markers in linkage group 3 (LOD=2.69 at rf=0.28) and linkage group 5 (LOD=2.85 at rf=0.27). Six loci were left unassigned to any linkage group.

The total genetic map length of the walnut genome using the 'method of moments' estimation (Hulbert et al. 1988) was 1354 cM for a LOD of 3.0 and 1602 cM for a LOD of 2.0. The Poisson probability method gave larger values, 1963 cM for a LOD of 3.0 and 1706 cM for a LOD of 2.0. The 12 linkage groups span only 302 cM of the walnut genome, with an average of 10.1 cM between each marker. No linkage group was longer than 60 cM, and only five were longer than 20 cM. Recombination was most likely inhibited between loci, due to structural genomic changes between the two species involved in the interspecific cross. This often occurs with interspecific hybridization (Kianian and Quiros 1992). All of the walnut backcross progeny are male sterile as may be expected if *J. regia* and *J. hindsii* have genomic structural differences. Woodworth (1930) also reported meiotic abnormalities among *Juglans* hybrids. The size estimates, ranging from 1354 to 1963 cM, suggest that the walnut genetic map spans approximately 1660 cM, with each chromosome averaging 104 cM in length.

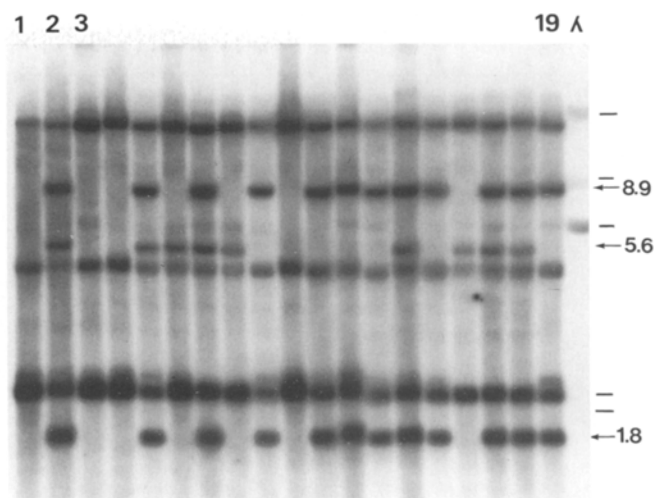


Fig. 1 Southern-blot autoradiograph of DNA from interspecific backcross walnut parents and progeny digested with *Xba*I and hybridized to a ^{32}P-labelled walnut insert from *pFP29*. *Lane 1, J. regia* 'Hartley'; *lane 2, J. hindsii*×*J. regia* interspecific hybrid maternal parent; *lanes 3–19*, backcross progeny demonstrating 1:1 segregation (presence:absence of *J. hindsii* allele) at class-1 locus *fp29A* (8.9- and 1.8-kb fragments co-segregating) and class-2 locus *fp29b* (5.6-kb fragment segregating). *Lane 20*, λ DNA, *Hin*dIII digested (no λ probe used). Lines on right represent λ *Hin*dIII-standard fragment lengths

Discussion

The *Pst*I restriction digestion of total genomic walnut DNA for the construction of low-copy-number genomic clones was very effective, providing 83% low-copy-number clones. Similar results have been noted for *Pst*I genomic libraries in a variety of crop species (Tanksley et al. 1987; Helentjaris et al. 1988; Slocum et al. 1990). The probes usually hybridized to more than one locus and exhibited varying degrees of hybridization signal at the separate loci.

Table 1 Monogenic segregation of walnut RFLP loci in backcross progeny of (*J. hindsii*×*J. regia*)×*J. regia*

Locus [a]	Restriction enzyme [b]	Segregation [c]	χ^2 [d]
fp01	*Eco*RV	37:26	1.92
fp02	*Hin*dIII	22:41	5.73*
fp03	*Hin*dIII	37:25	2.32
fp04	*Hin*dIII	30:32	0.07
fp06A	*Xba*I	30:33	0.14
fp06b	*Xba*I	33:30	0.14
fp07A	*Xba*I	31:31	0.00
fp07b	*Eco*RI	23:40	4.59*
fp07c	*Eco*RI	30:33	0.14
fp07d	*Eco*RI	32:31	0.02
fp07e	*Eco*RI	39:24	3.57
fp09A	*Eco*RV	30:31	0.02
fp09B	*Eco*RV	23:40	4.59*
fp10	*Hin*dIII	37:26	1.92
fp11A	*Hin*dIII	33:30	0.14
fp11b	*Hin*dIII	41:22	5.73*
fp12	*Eco*RV	31:31	0.00
fp14A	*Eco*RV	23:40	4.59*
fp14b	*Eco*RV	31:32	0.02
fp15	*Xba*I	27:36	1.29
fp17	*Eco*RV	26:36	1.61
fp18	*Eco*RI	21:42	7.00*
fp19	*Eco*RV	28:35	0.78
fp20A	*Xba*I	37:26	1.92
fp24A	*Xba*I	24:39	3.57
fp24b	*Xba*I	27:36	1.29
fp25	*Eco*RV	37:24	2.77
fp26	*Xba*I	32:31	0.02
fp29A	*Xba*I	33:30	0.14
fp29b	*Xba*I	23:40	4.59*
fp31	*Xba*I	36:27	1.29
fp32	*Hin*dIII	43:20	8.40*
fp34	*Hin*dIII	37:26	1.92
fp35A	*Eco*RV	31:32	0.02
fp35B	*Eco*RV	28:35	0.78
fp35c	*Eco*RV	38:24	3.16
fp39A	*Xba*I	38:24	3.16
fp39b	*Xba*I	31:32	0.02
fp43	*Hin*dIII	42:21	7.00*
fp45	*Eco*RV	24:39	3.57
fp48	*Hin*dIII	26:37	1.92
fp56A	*Eco*RV	28:35	0.78
fp56b	*Eco*RV	41:22	5.73*
fp59	*Eco*RV	38:25	0.78
fp66A	*Hin*dIII	22:41	5.73*
fp66B	*Hin*dIII	27:33	0.60
fp67	*Eco*RV	23:40	4.59*
TA71	*Hin*dIII	27:36	1.29

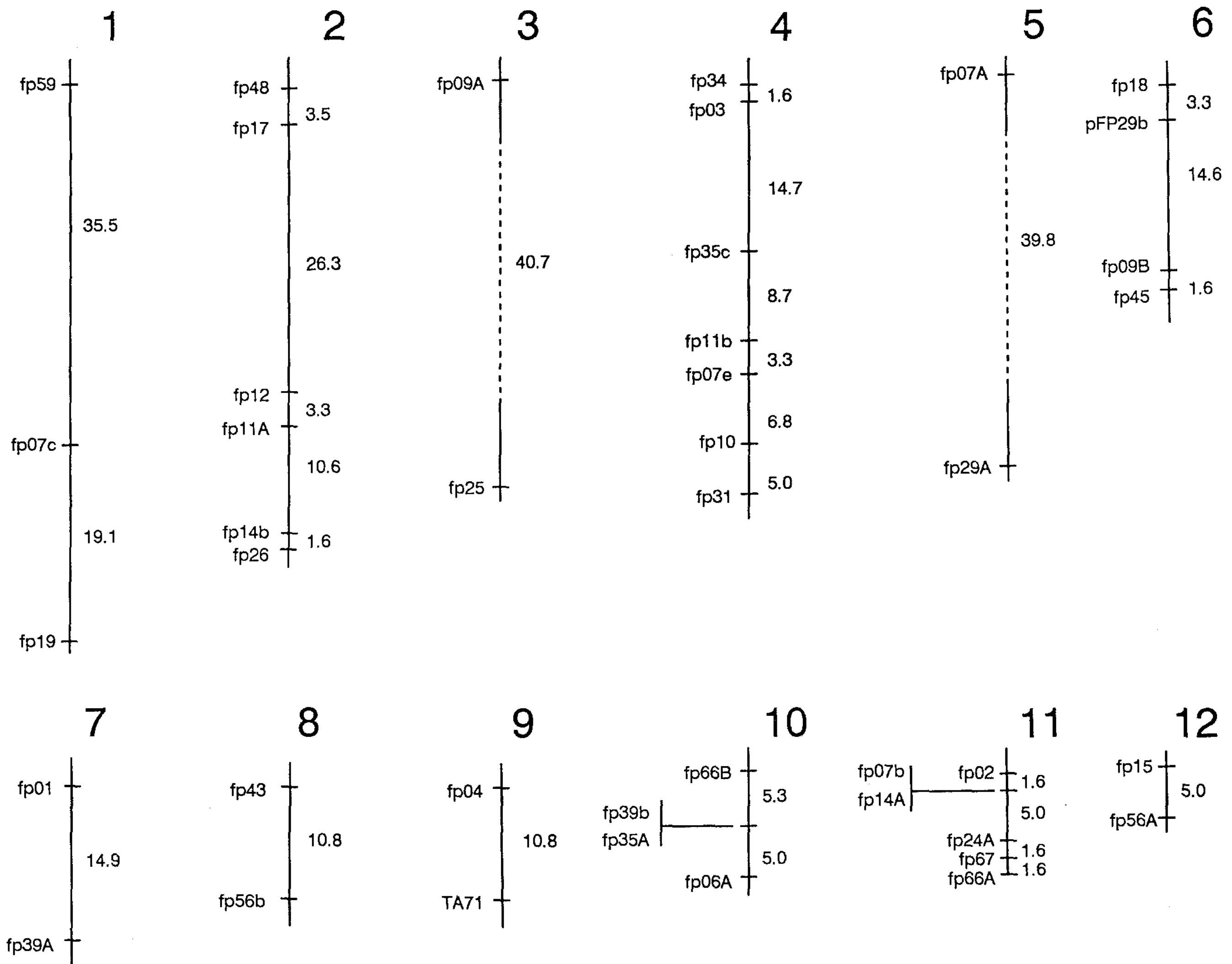
[a] Capital and lowercase letters following plasmid number designate class-1 and class-2 loci, respectively
[b] Restriction enzyme used to cut probed walnut DNA
[c] Ratio of heterozygous *J. hindsii*/*J. regia*: homozygous *J. regia* allelic genotypes
[d] Based upon a 1:1 expected segregation ratio, * indicates deviation from expected ratio at the P=0.05 level of significance

The walnut genome probably underwent duplication at some time in the past creating homeologous loci, followed by sequence divergence that reduced the homology between the loci, resulting in one locus that hybridizes strongly with the probe and one locus of varying divergence that hybridizes weakly. Duplicated structural genes with sequence divergence have been the most likely source for multilocus hybridizations of cDNA probes in tomato (Bernatzky and Tanksley 1986). Whether duplicate loci arose from the fusion of two diploid progenitor species during the formation of a polyploid progenitor species or from internal stepwise duplications of walnut chromosomes and chromosome segments is not clear. All members of the Juglandaceae are higher-order polyploids, with 2n=32 or 64 chromosomes (Hans 1970). No putative lower-order diploid progenitors exist for comparison.

One quarter of the probes showed segregation ratios that deviated from the expected 1:1 Mendelian backcross ratio for single genes. Such deviations have been seen in other genome mapping studies involving interspecific crosses (Helentjaris et al. 1986; Havey and Muehlbauer 1989; Durham et al. 1992). Distorted segregation ratios in such crosses are attributable to positive or negative selection on genes linked to molecular loci (Jarrell et al. 1992). These loci may be mapped to adjacent loci, although estimated recombination distances between the loci may be altered. The linkage arrangements among the duplicated walnut loci were not similarly distributed. No duplicated locus was found on the same linkage group and no linkage-order conservation was observed between adjacent duplicated loci, suggesting that genomic duplication may have occurred early in the evolution of the genus. This type of independent distribution of duplicated loci is more typical of that seen in the tomato genome (Bernatzky and Tanksley 1986) compared to that in the genomes of maize (Helentjaris et al. 1988) and *Brassica* (Slocum et al. 1990) where adjacent duplicated loci have shown linkage-order conservation on different chromosomes.

Compared to the Poisson probability estimates, the 'method-of-moments' estimates gave lower values for the walnut genome length even though it has been demonstrated that 'method-of-moment' estimations could overinflate calculations of genome length (Chakravarti et al. 1991). Because of recombination suppression in the interspecific progeny, the 'method-of-moments' estimates are probably not an overestimation of genome length. On the other hand, the Poisson probability estimate utilizes information on the number of chromosomes and takes into account the reduced span of linkage due to the effects of chromosome ends and it is likely to give a more accurate estimation of genetic map length from partial linkage data. Further testing of genetic maps with greater marker saturation could demonstrate the usefulness of each estimator. Based on a genome size of 1660 cM, the walnut map would need 138 markers to cover 95% of the genome within 20 cM of each marker.

The expansion of the walnut genetic map with more markers should be readily achieved due to the high percentage of segregating polymorphic molecular loci. The use of RAPD markers is presently being pursued in this interspecific backcross population (G. McGranahan and K. Woeste, personal communication). The genomic probes from this study also hybridize well to other important Juglandaceae members such as *J. nigra* and *Carya illinoensis* (R. Fjellstrom, unpublished) and will be useful for comparing genome organization among these valuable species.



Unlinked markers: fp06b, fp07d, fp20A, fp24b, fp32, fp35B

Fig. 2 Linkage map of 48 RFLP walnut loci. The 12 linkage groups are labelled by order of length on top, with loci on the left, and map distance (cM) on the right of each linkage group. *Solid lines* indicate linkages with LOD scores of 3.0 or greater, *broken lines* indicate linkages with LOD scores between 2.6 and 3.0. Loci for which no linkages were detected are listed on the botttom right

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