Randomly Amplified Polymorphic DNA Loci from a Walnut Backcross [(*Juglans hindsii* × *J. regia*) × *J. regia*]

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Abstract. Twenty-five random decamer primers were used to evaluate the level of polymorphism between Persian walnut and the Northern California black walnut. Sixty-six randomly amplified polymorphic DNA (RAPD) markers were identified using an interspecific walnut backcross population [(*Juglans hindsii* × *J. regia*) × *J. regia*]. Segregation data from these polymorphisms were joined to a previously published set of restriction fragment-length polymorphism (RFLP) marker data to expand the genetic map of walnut to 107 markers in 15 linkage groups.

Randomly amplified polymorphic DNA (RAPD) genetic markers are generated using the polymerase chain reaction (PCR) and polymorphisms are identified by amplifying specific regions of template DNA flanked by primer sites homologous to random oligonucleotide primers (Williams et al., 1990). While RAPD is not codominantly expressed, as are restriction fragment-length polymorphisms (RFLPs), and procedures for the use of RAPD may need optimization for various plant and primer combinations (Yu and Pauls, 1992), its potential for identifying genetic markers is clear (Carlson et al., 1991). Because RAPD markers are similar to RFLPs in their ability to identify and map polymorphisms (Kesseli et al., 1992), RAPD has been used to identify genetic markers (Devos and Gale, 1992; Klein-Lankhorst et al., 1991), to identify and classify germplasm (Yang and Quiros, 1993; Yu and Pauls, 1993), to determine genetic distance (Williams and St. Clair, 1993), and for genetic mapping (Kazan et al., 1993).

The Persian or English walnut (*Juglans regia* L.) is one of the most important nut crops in the United States. *J. hindsii* (Jeps.) Jeps., the northern California black walnut, is a species native to a small area in northern California, but it has become more widely spread by human activity since the mid-1800s. *J. regia* and *J. hindsii* freely intercross to produce a male sterile 'Paradox' hybrid commonly used as a rootstock for cultivated English walnut (McGranahan et al., 1988).

Walnut has 16 linkage groups (2n = 32) (Hans, 1970), disomic segregation in meiosis (Woodworth, 1930), and a genetic map length of from 1300 to 1600 cM (Fjellstrom, 1993). Relatively few genetic loci have been identified for walnut aside from the RFLPs described by Fjellstrom (1993). Arulsekar et al. (1985, 1986) described the inheritance of five isozyme loci with three enzyme systems. Three of the isozyme loci were variable within *J. regia* and two more were variable among *Juglans* species. In addition,

the inheritance of hypersensitivity to Cherry leafroll virus (CLRV) (McGranahan and Woeste, 1996) and heterodichogamy in walnut (Gleeson, 1982) have been described, but the inheritance of other traits is unknown.

A preliminary genetic linkage map for walnut has been published, including 42 RFLPs assigned to 12 linkage groups, with six RFLPs unassigned (Fjellstrom, 1993). This paper describes procedures for identifying RAPD polymorphisms between *J. regia* and *J. hindsii* and characterizes 66 such loci. The existing genetic linkage map for walnut, which is based on RFLPs, is revised and expanded with RAPD marker loci identified in this study.

Materials and Methods

Populations for DNA analysis. The *J. regia* DNA was isolated from 11 cultivars: XXX Mayette, Manregian, Marchetti, Scharsh Franquette, Hartley, Sharkey, Payne, Sunland, Chandler, Eureka, and Conway Mayette. These trees are maintained by the Univ. of California, Davis, Dept. of Pomology. The *J. hindsii* DNA was from 10 trees: 42-01, 93-01, 94-04, 34-07, 35-06, 90-03, 41-01, 43-03, 91-01, and a *J. hindsii* located in front of Wickson Hall on the Univ. of California, Davis, campus. Aside from this latter tree, the *J. hindsii* DNA sources are the property of and are maintained by the National Clonal Germplasm Repository, Davis, Calif.

The inheritance of RAPD fragments was evaluated using a population of 49 interspecific first-backcross hybrids [(*J. hindsii* × *J. regia*) × *J. regia*]. The backcrosses are the open-pollinated seedlings of a single 'Paradox' female. A complete description of this segregating backcross population and its hybrid parent is found in Fjellstrom (1993).

DNA sample preparation and identification of polymorphic fragments. DNA was isolated using the methods of Fjellstrom (1993). After isolation, the concentration of DNA in each sample was estimated using a spectrophotometer (Sambrook et al., 1989), and DNA stocks (150 ng·μl⁻¹) were created by dilution with sterile, distilled water. These stocks were further diluted for use in RAPD reactions using a two step process. Initially, samples were diluted 150-fold and their concentration estimated by visual comparison

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with lambda phage DNA standards after horizontal gel electrophoresis in 0.9 % agarose and 1×TBE (Sambrook et al., 1989). The DNA was visualized using ethidium bromide stain and transillumination of gels with ultraviolet light. The 150-fold diluted stocks were then readjusted to 1.0 to 10 ng·μl⁻¹ using sterile, distilled water. DNA bulks were pools of equal amounts of DNA from each individual accession, and this stock bulk was further diluted for use in RAPD analysis.

Polymorphisms between *J. regia* and *J. hindsii* were identified using DNA bulks of these two species (Grattapaglia et al., 1992; Yu and Pauls, 1993). Only RAPD fragments present in the female parent of the backcross population and the DNA bulk of *J. hindsii* but absent from the *J. regia* bulk were analyzed for their segregation in the BC₁ (Grattapaglia et al., 1992). A chi-square goodness of fit test was used to test the null hypothesis of 1:1 segregation of polymorphic bands.

PCR reaction conditions and electrophoresis. Reaction conditions, buffers and sample preparation for PCR were as suggested by the manufacturer of Stoffel fragment polymerase (Perkin Elmer, Norwalk, Conn.) except concentrations for random decamer primers (0.57 μM) (Operon Technologies, Alameda, Calif.) (Table 1), MgCl₂ (3.6 mM), and each nucleotide triphosphate (0.156 mM). The final reaction volume was 12.5 μl, including 3 μl sample DNA (1.0 to 10 ng·μl⁻¹). The thermal cycling protocol was 1 cycle of 2 min at 94C; 3 cycles of 30 sec at 98C, 30 sec at 35C, and 1 min at 72C; 40 cycles of 5 sec at 94C, 30 sec at 35C, and 2 min at 72C. The final 40 72C steps were extended an additional 2 sec per cycle.

The aqueous phase of the PCR products was mixed with 2 to 4 μl gel loading buffer [0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol (Sambrook et al., 1989)] and electrophoresed in 2% agarose (1% Fisher molecular biology grade; 1% Nusieve, FMC Corp.) gels made with 1×TBE buffer (Sambrook et al., 1989) for 4 h at 3.0 V/cm. Gels were stained with ethidium bromide, and the RAPD fragments were scored visually as the presence or absence of a band. RAPD fragment size was estimated by comparison with a 123 bp ladder DNA size standard (Gibco BRL, Gaithersburg, Md.) or a 1-kb ladder (Promega, Madison, Wis.). Gels were photographed using an ultraviolet transilluminator with plus-X pan, 35 mm, 125 ASA film (Kodak) and an ultraviolet filter with 45 sec exposure.

Evaluation of scoring error. Scoring RAPD fragments can be difficult, and some scoring errors occur because a small percentage of RAPD bands are not clearly +/- (Huen and Helentjaris, 1993). To evaluate the reliability of the amplification of RAPD fragments and their scoring in this experiment, a subset of the primer/DNA combinations was reamplified and scored. An error was recorded when bands were unclear or scored differently in subsequent trials. The error rate was evaluated as the number of errors divided by the number of independent tests. For the purposes of analysis and mapping, untried primer/DNA combinations, as well as those that gave unclear or conflicting results in retrials, were scored as missing data.

Adding RFLP data and mapping. Forty-nine individuals from an interspecific first backcross population were evaluated for 66 RAPD fragments (Table 1). Data from this analysis were added to the linkage data published by Fjellstrom (1993) to develop an integrated genetic linkage map consisting of RAPD markers and RFLPs. The loci described by Fjellstrom (1993) are preceded by the letter "L" in this paper and in the linkage map (Fig. 1). Linkage groups and map distances were estimated using MAPMAKER (Lander et al., 1987) rewritten for the Apple Macintosh (V2.0) provided by S. Tingey (DuPont, Wilmington, Del.). We used the default settings (LOD = 3.00, recombination fraction = 0.4) of the Multipoint/first-order command of MAPMAKER and the Haldane mapping function (Haldane, 1919).

Table 1. RAPD fragments polymorphic between *Juglans regia* and *J. hindsii*, the random decamer primer used to identify the polymorphism, the size of the polymorphic fragment, segregation of the fragment in the first backcross, and χ^2 test of 1:1 segregation for each locus.

Locus	Primer	Fragment size (bp)	Segregation*	χ^2
K151160	OPK-15	1160	21/20	0.00
O101110	OPO-10	1110	27/20	0.38
O101180	OPO-10	1180	10/31	9.76**
R121060	OPR-12	1060	20/15	0.46
R12425	OPR-12	425	22/10	3.78
R12340	OPR-12	340	17/13	0.30
S01520	OPS-01	520	16/13	0.14
S01395	OPS-01	395	15/24	1.64
S021120	OPS-02	1120	23/24	0.00
S02700	OPS-02	700	14/10	0.38
S03285	OPS-03	285	24/19	0.37
S041635	OPS-04	1635	25/16	1.56
S05940	OPS-05	940	19/24	0.37
S061500	OPS-06	1500	19/21	0.03
S06940	OPS-06	940	21/22	0.00
S06480	OPS-06	480	30/14	5.10*
S06440	OPS-06	440	18/24	0.60
S07760	OPS-07	760	24/22	0.02
S07590	OPS-07	590	27/20	0.77
S07530	OPS-07	530	25/20	0.36
S07345	OPS-07	345	19/26	0.80
S07310	OPS-07	310	29/15	3.84*
S131250	OPS-13	1250	18/29	2.13
S13800	OPS-13	800	28/18	1.76
S13750	OPS-13	750	26/21	0.34
S13490	OPS-13	490	27/21	0.52
S15940	OPS-15	940	24/15	2.70
S15440	OPS-15	440	23/15	1.29
S15285	OPS-15	285	21/19	0.03
S181180	OPS-18	1180	10/10	0.00
S181110	OPS-18	1110	24/22	0.04
S18785	OPS-18	785	24/23	0.00
S18590	OPS-18	590	18/27	1.42
S18385	OPS-18	385	19/27	1.07
U19575	OPU-19	575	21/25	0.20
W13940	OPW-13	940	31/16	4.17*
W13610	OPW-13	610	25/20	0.35
W13560	OPW-13	560	34/10	12.02***
X041850	OPX-04	1850	11/9	0.05
Y011060	OPY-01	1060	15/21	0.70
Y01590	OPY-01	590	28/21	0.73
Y03590	OPY-03	590	22/24	0.02
Y03285	OPY-03	285	29/16	3.20
Y05560	OPY-05	560	27/22	0.33
Y05520	OPY-05	520	26/21	0.34
Y05335	OPY-05	335	28/20	1.02
Y07475	OPY-07	475	17/25	1.16
Y07385	OPY-07	385	24/18	0.60
Y09335	OPY-09	335	24/20	0.20
Y11545	OPY-11	545	26/19	0.80
Y11415	OPY-11	415	27/17	1.84
Y13675	OPY-13	675	26/16	1.93
Y13560	OPY-13	560	10/32	10.5***
Y13540	OPY-13	540	22/20	0.02
Y13395	OPY-13	395	22/21	0.02
Y14520	OPY-14	520	25/16	1.56
Y14415	OPY-14	415	21/24	0.09
Y20475	OPY-20	475	14/26	3.03
Y201120	OPY-20	1120	25/17	1.17
Y20780	OPY-20	780	19/22	0.10
Y20360	OPY-20	360	26/19	0.80
Z031260	OPZ-03	1260	26/21	0.53
Z03520	OPZ-03	520	31/18	2.93
Z03375	OPZ-03	375	23/26	0.08
Z03340	OPZ-03	340	28/21	0.73
Z03285	OPZ-03	285	15/33	6.02*
Totals			1307/1199 ^y	4.60**

*Ratio of heterozygous *J. hindsii*-*J. regia*/homozygous *J. regia* genotypes.

^yTotals do not include values from loci that deviate significantly from 1:1 expectation.

*Pooled χ^2 for all loci that do not deviate from 1:1 expectation. Homogeneity $\chi^2 = 39$, df = 58^{ns}.

***Significant at $P = 0.05$, 0.01, 0.001 percent probability respectively.

Fig. 1. Linkage map of 107 RAPD and RFLP walnut loci. The 15 linkage groups are numbered on top. Map distances (cM) between loci are on the left; locus name is on the right. All loci are linked with a LOD score of 3.0 or greater. Loci for which no linkage was detected are L6B, L7D, L29A, L20B, R121060, S01520, S07345, S131250, W13560, Y14520, and Y20780.

Results and Discussion

RAPD markers readily detected polymorphisms between *J. regia* and *J. hindsii* when DNA bulks of the species were compared using a subset of 25 random decamers. Every primer that amplified reproducible fragments generated at least one polymorphism. Sixty-six polymorphisms that produced a strong, consistent signal were characterized and evaluated further. The RAPD fragments varied in size from 285 to 1850 base pairs (bp) with most between 300 and 800 bp. Most of these bands were relatively easy to score and retests were usually consistent with previous results. Thirty-six of the RAPDs were retested in a sample of the population without scoring error. The scoring error rate over all the loci was 42/1607 (2.7%).

Seven of the RAPD loci—O101180, S06480, S07310, W13940, W13560, Y13560, Z03285 (13% of the total)—had segregation ratios that deviated significantly from the expected 1:1 Mendelian backcross ratio for single genes. Fjellstrom (1993) reported that 25% of the RFLP loci showed significant distortion, with an excess of *regia* homozygotes. This type of distortion is not unusual in wide crosses (Helentjaris et al., 1986; Jarrell et al., 1992).

Linkage analysis placed 107 RAPD and RFLP markers into 15 linkage groups (Fig. 1). Linkage groups were numbered following Fjellstrom (1993) whenever possible. Fjellstrom's linkage group 9 was joined to group 5 as reported here, but all other RFLP loci remained in their previously assigned linkage groups. RFLP markers L20A, L24B, L32, and L35B, which could not be assigned by Fjellstrom, were placed into linkage groups 13, 14, 15, and 1, respectively. In this analysis, RAPD markers R121060, S01520, S07345, S131250, W13560, Y14520, Y20780, L6B, L7D, L29A, and L20B were unassigned. Map distance estimates (Fig. 1) are biased by the factors that contribute to linkage disequilibrium: small sample size, interpopulation mating, few generations of recombination (Falconer, 1981) and any genomic differences between the parental species that lead to regions of repressed recombination. For these reasons, estimates of the recombination distances among RAPD loci are approximate, especially if the loci have distorted segregation. Markers O101180, Y13560, and Z03285 were placed in linkage group 11. This group contains 9 loci, and all show significant segregation distortion towards the *regia* allele. Loci with significant segregation distortion in favor of the *hindsii* allele were not linked. When the segregation of the RAPD markers is summed over all the loci for the entire backcross population a significant distortion ($\chi^2 = 4.6$, $P \leq 0.05$) in favor of *hindsii/regia* heterozygotes is revealed (1307 heterozygous : 1199 homozygous loci). Loci with distorted segregation were omitted from the above analysis, and the remaining loci were not significantly nonhomogeneous ($\chi^2 = 39$ with 58 degrees of freedom). It is possible this distortion reflects selection during propagation of the BC1 since Paradox hybrids are more vigorous than either *J. regia* or *J. hindsii*.

The first backcross population and the methods used to identify the polymorphisms reported here have several limitations. While useful for the assessment of introgression, the RAPD markers identified in this study are all derived from *J. hindsii* and cannot be used to evaluate *J. regia*. The first backcross population used to determine their segregation is relatively small and several of the individuals are unthrifty, suggesting genomic abnormalities. Any further mapping of *J. regia* should be based on a larger population not derived from an interspecific cross. This could be done using RFLPs and the existing *J. regia* germplasm (Fjellstrom, 1993). Placing morphological traits and previously characterized isozyme markers into the existing linkage groups will also increase the usefulness of the map. Since initial linkage assignments have been

made, a subset of markers can be used to build a scaffold map with evenly spaced markers covering the entire genome.

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