SINGLE TREE GENETIC LINKAGE MAPPING IN CONIFERS USING HAPLOID DNA FROM MEGAGAMETOPHYTES

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We report a unique application of the Random Amplified Polymorphic DNA (RAPD) technique for genetic linkage mapping of a single spruce tree using haploid DNA from megagametophyte tissue of individual seeds. Sixty-one segregating loci were analysed for reproducibility, inheritance and linkage. Forty-seven of the 61 markers were distributed into 12 linkage groups and covered 873.8 cM. The 14 markers not associated with any linkage group should be assigned to linkage groups as more markers are added to the map. This new approach quickly provides molecular genetic markers that are simple to evaluate for constructing genetic linkage maps and for other related genetic studies in forest tree species.

Genetic linkage maps based on molecular markers now provide the fundamental tool for the localization of genes controlling a number of traits in both plants and animals. The mapped traits can be selected for or followed in a breeding program through the linked markers. This procedure is termed marker assisted selection (MAS) and facilitates the selection of heritable traits that may not be expressed among individuals at any particular time. Forest trees, with their long generation interval, may have much more to gain from MAS in terms of genetic improvement by targeting juvenile plants. The problem of imperfect juvenile-mature correlation encountered in phenotypic selection is non-existent since selection is at the genotypic level. In addition, environmental effects that confound phenotypic selection are also of no consequence with MAS. Despite the significant advantage for MAS in forest tree improvement programs, especially conifers, to date no molecular marker based linkage map has been reported. Linkage studies in forest trees have been limited to isozyme markers[1–8]. These linkage analyses are restricted by the small number of isozyme loci in tree species and the level of polymorphism. The most comprehensive study[5] so far identified five linkage groups in six conifer species, covering a maximum of 226.4 cM.

While genetic linkage maps based on Restriction Fragment Length Polymorphisms (RFLPs) have been constructed for a number of agronomic crops and annual plant species, for example: maize[9–12], tomato[9,13], soybean[14], rice[15], lentil[16], lettuce[17], wheat[18], barley[19], *Brassica*[20–22], and *Arabidopsis*[23,24], in conifers the process is more than a decade behind. Some of the reasons for this may be attributed to complications resulting from: (1) The large size of the genome, for example, it is 3–4 times that of corn, approximately 5 times that of lettuce, 10–15 times that of tomato, and approximately 100 times that of *Arabidopsis*. (2) The lack of pedigree beyond two generations, and (3) long generation times, 15–20 years to sexual maturity.

The recently developed Random Amplified Polymorphic DNA (RAPD) marker system[25] may make possible quick advances in genetic linkage mapping due to its rapidity and simplicity compared to RFLPs. The RAPD marker system is based on the PCR amplification of random DNA segments with single primers of arbitrary nucleotide sequence. Allelic variation between individuals is based on the presence or absence of the amplification product, which is detected as a marker band after electrophoresis and staining on agarose or polyacrylamide gels.

An advantage of the RAPD technique with conifers is the use of haploid DNA, which is available in the megagametophyte of conifer seeds. This is beneficial because the RAPD technique does not readily differentiate between heterozygotes and dominant homozygotes in diploid material. The conifer megagametophyte is 1N, strictly maternal and derived from a single meiotic product and hence permits recombination and segregation to be followed among open-pollinated seeds from a single tree without the need for controlled crosses. This approach, if proved valid, would overcome limitations in the use of MAS and the construction of genetic linkage maps with conifers. It should also be equally applicable in those angiosperms for which haploid cell culture, dihaploid plants or recombinant inbred lines are available.

Our objectives in this study were to assess the utility of RAPD protocols using haploid DNA from white spruce, *Picea glauca* (Moech) Voss., especially in terms of reproducibility, inheritance of marker bands and the level of polymorphisms revealed, and if found satisfactory, to demonstrate the use of the RAPD marker system to construct tree-specific genetic linkage maps.

RESULTS AND DISCUSSION

DNA extraction from megagametophytes. The extraction of DNA from megagametophyte tissue was a modification of a protocol used for extraction of DNA from conifer needles[26]. DNA yields per seed varied from 300–500 ng. With use of 2 ng DNA per reaction, 150–250 reactions were possible for DNA extracted from each megagametophyte source. Adequate homogenization of the megagametophyte tissue appears critical for optimal yields. Extraction with ether after phenol/chloroform extraction has also improved yields.

Primer base composition, amplification products and polymorphisms. The nucleotide sequence of each primer was unique and arbitrary. Composition followed the recommendations of Williams et al.[25], that is, 10 base pairs in length, G+C content between 50 and 80% and no palindromic sequences over 6 or more nucleotides.

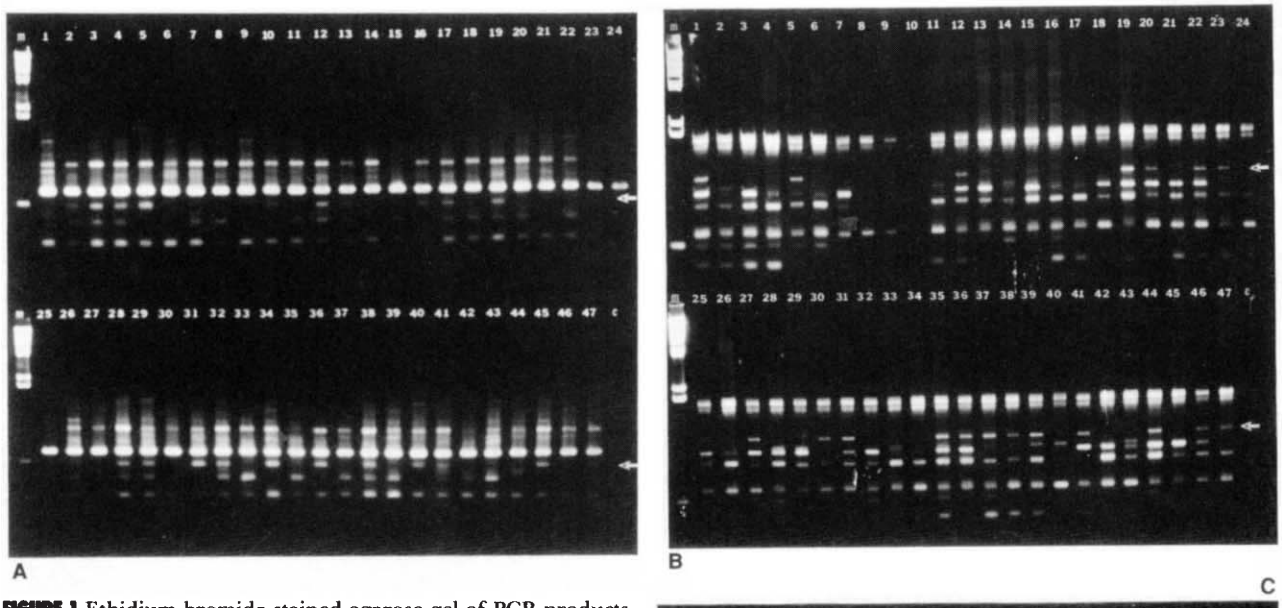

FIGURE 1 Ethidium bromide stained agarose gel of PCR products from 47 megagametophyte DNAs and three individual primers. Five to 12 bands are generally produced ranging from approx. 2.0 kb to approx. 0.2 kb in size as detected on a 1.4% gel. Number of segregating loci varied from 1 to 4. For each photo, lambda Hind III markers are shown at the extreme left lane of each panel (m) and lower extreme right lane of lower panel (c), represents no template control. Arrows indicate segregating loci. A, B and C show the amplification products produced by primers #213, #254, and #230, respectively, with the 47 megagametophyte DNAs. A and B show one segregating band each, while C shows 3 segregating bands.

Once our PCR reaction conditions were optimized we found that the number of detectable amplification products on horizontal agarose gels generally ranged from 5–12 and only rarely were less than four products generated (Fig. 1, A, B, and C). Each primer produced unique banding patterns. We found no obvious association between primer sequence nor G+C content and number of detectable amplification products nor number of polymorphic bands (Table 1).

Reproducibility of amplification products. One of the first questions that we addressed was reproducibility of the PCR amplification products generated by specific primer/DNA combinations. The concern arose from the possibility that some of the products arose as a result of primer annealing based on partial homology to DNA sequence and the observation that there was a higher proportion of aberrant segregation of bands relative to RFLPs. We needed to demonstrate that under the same reaction conditions we could reproducibly obtain identical amplification products. This was initially tested by setting up five identical reactions (same primer and template) for several primer/template combinations. Also, after scoring segregating bands for all polymorphic primers among the 47 seed genotypes, three primers were chosen at random, reactions repeated under the same conditions and the five segregating bands produced were scored again. Scoring was done independently by two persons and results compared. We found that the PCR amplification products were close to 100% reproducible, which followed Mendelian segregation and therefore were useful as genetic markers.

Screening primers. To identify polymorphic primers 300 primers were screened with an initial subset of DNA from 5 seeds. Since segregation of markers is expected to follow a 1:1 Mendelian ratio of presence:absence of marker band, the use of 5 seeds should give us reasonable confidence (~94% of the time) in identifying primers that detect heterozygous loci in the maternal parent tree. For

TABLE 1 Random sample of primers showing G+C content, total number of amplification products and number of segregating bands per primer.

Primer	Sequence	No. G+C	No. Amplification Products	No. Segregating Loci (1:1)
203	CACGGCGAGT	7	10	0
216	CATAGACTCC	5	7	0
235	CTGAGGCAAA	5	7	0
264	TCCACCGAGC	7	6	0
298	CCGTACGGAC	7	8	0
210	GCACCGAGAG	7	10	1
231	AGGGAGTTCC	6	6	1
271	GCCATCAAGA	5	5	1
289	ATCAAGCTGC	5	6	1
297	GCGCATTAGA	5	11	1
218	CTCAGCCCAG	7	8	2
225	CGACTCACAG	6	8	2
266	CCACTCACCG	7	9	2
300	GGCTAGGGCG	8	8	2
229	CCACCCAGAG	7	5	3
244	CAGCCAACCG	7	12	3
278	GGTTCCAGCT	6	11	3
285	GGGCGCCTAG	8	9	3
295	CGCGTTCCTG	7	7	3
256	TGCAGTCGAA	5	8	4

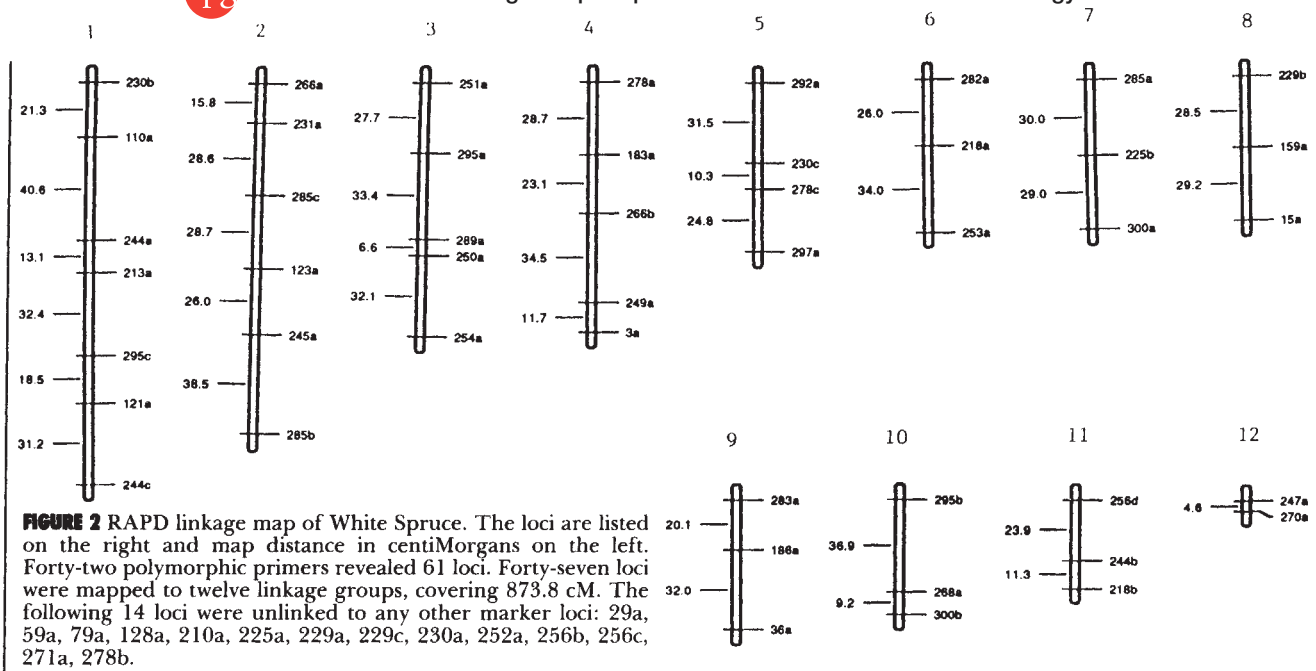

FIGURE 2 RAPD linkage map of White Spruce. The loci are listed on the right and map distance in centiMorgans on the left. Forty-two polymorphic primers revealed 61 loci. Forty-seven loci were mapped to twelve linkage groups, covering 873.8 cM. The following 14 loci were unlinked to any other marker loci: 29a, 59a, 79a, 128a, 210a, 225a, 229a, 229c, 230a, 252a, 256b, 256c, 271a, 278b.

the first 200 primers (#1–#200), 26 primers revealed polymorphism among the 5 haploid genotypes. Often complete reactions had failed or products were not discernible. However, amplification conditions were subsequently further improved; the amount of enzyme per reaction was halved while Mg^{2+} and primer concentration were increased (see Experimental Protocol). Under these conditions, 43 of the third set of a hundred primers (#201–#300), detected one or more bands segregating among the 5 genotypes. Spruce is a recently domesticated, open-pollinated species with high levels of genetic variation based on both phenotypic[28,29] and isozyme[30,31] analyses. Thus higher levels of polymorphism seen with the third set of primers are more consistent with the high level of heterozygosity expected with spruce. Also, homozygosity due to inbreeding is not expected to be a limiting factor in using this approach with conifers.

Scoring segregating markers and linkage mapping. Of the 69 primers that were initially characterized as polymorphic, 27 revealed segregating bands that lacked goodness of fit to a 1:1 segregation ratio. Ratios computed for some were highly skewed while others showed lower levels of distortion. The remaining 42 primers revealed 61 marker bands that conformed to Mendelian segregation. Thirty-one of the 42 primers detected one segregating locus (Fig. 1 A and B), 4 detected 2 loci each while 6 detected 3 loci each (Fig. 1 C), and one primer revealed 4 segregating loci.

In the computation of linkages among segregating loci we did not include those that did not fit the 1:1 Chi square expected ratio. In both agronomic and forest tree species the occurrence of distorted ratios in segregating RFLP and isozyme alleles is not uncommon[5,7,12,15,19,20,22,27,32,33]. The actual mechanism underlying this phenomenon is not well understood. There appears to be a lack of consensus among researchers as to whether markers expressing segregation distortion should be used in linkage mapping studies.

Of the 61 segregating loci, 47 were mapped to 12 linkage groups (Fig. 2), while the remaining 14 were unlinked, using a recombination value of 0.4 and log likelihood ratio (LOD) score of 3.0. The 12 linkage groups covered a total of 873.8 cM. The order of marker loci within a linkage group was assigned based on best LOD score of all possible arrangements. In nearly all cases the order chosen was greater than 3 times better than the next possible order. We cannot say with certainty that these 12 groups represent each of the 12 pairs of homologous chromosomes of white spruce. As more marker loci are added to the map some of these linkage groups may merge and the unlinked markers will be assigned to linkage groups. Based on the haploid DNA content, it is estimated that the conifer genome size is approx. 2500 cM[34]. Thus, our preliminary map has covered ~1/3 of the genome and it is therefore not surprising that 14 loci were not included among the 12 linkage groups.

Applications of RAPD marker system in forest trees. This is the first report of a genetic linkage map for a conifer (Ron Sederoff and David O'Malley at North Carolina State University are also using this approach to develop a genetic linkage map for loblolly pine; personal communication) and demonstrates the power of RAPD markers and haploid DNA for genome mapping. The use of RAPD markers in conifers presents a realm of opportunities in genetics and breeding previously not possible with isozymes. The study of simply inherited traits or more complex quantitative traits could be accomplished as demonstrated for a number of agronomic crops[32,35–38]. Since the models used for annual inbred species may not be appropriate for open pollinated forest species, new methods need to be developed either based on the available breeding populations or modified population structures. MAS permits for early selection, an advantage that is of considerable importance in conifers due to their long rotation age. The high number of marker bands produced with each primer using the RAPD technique makes this approach ideal for genotyping/fingerprinting parental trees, clones or any seed orchard material for identification purposes and for analyzing pollen contribution in controlled crosses. RAPD analysis of variation at the nuclear genome level has advantages over RFLPs in genetic diversity studies, since a single primer usually produces multiple loci and hence a larger portion of the genome can be analyzed. In evolutionary and population genetics studies the RAPD analysis of the nuclear genome can very quickly add a new dimension to the available data currently based on organelle genomes and isozymes, and possibly assist in clarifying some of the current anomalies

that appear to be due to inadequate data.

The advantages that haploid DNA offers when using the RAPD marker system for genetic studies will not be limited to conifers but should also find application in angiosperm plants through the use of anther culture. Haploid plants have been produced from anther culture from 85 genera belonging to 38 families[42]. Since we have shown that a linkage map can be constructed from one microgram or less of DNA per haploid genotype, even microcalli from anthers would be a suitable source of template DNA. This could extend the RAPD genome mapping technique to those many plant species that only respond transiently to another culture by producing small haploid calli but not necessarily haploid plants.

EXPERIMENTAL PROTOCOL

Plant material. Seeds from the weevil tolerant parental tree PG29 were collected from open-pollinated cones from the Province of British Columbia Forest Service interior spruce breeding orchard at Vernon, B.C.

DNA isolation. DNA was extracted from megagametophytes of individual seeds by a modified CTAB procedure[26]. Seeds were imbibed in water for 4 hours to overnight at room temperature prior to dissection for removal of the diploid embryo and outer brown scale covering the megagametophyte. The isolated tissue was ground in a (Kontes) microcentrifuge tube containing 30 µl wash buffer (50 mM Tris·HCl, pH 8.0, 25 mM EDTA, 0.35 M sorbitol, 0.1% β-mercaptoethanol) using a motorized pestle grinder (Kontes). An additional 236 µl of wash buffer was added to the homogenate, followed by 53 µl of 5% sarkosyl, mixed by inversion and incubated at room temperature for 3–5 minutes. After addition of 46 µl 5 M NaCl and 37 µl 8.6% CTAB in 0.7 mM NaCl and mixing by inversion, the tubes were incubated at 65°C for 15 minutes. RNA was digested by addition of 2 µl bovine pancreatic RNAse (1 mg/ml, Boehringer Mannheim, analytical grade) and incubation at 37°C for 15 minutes. The homogenate was extracted with one volume phenol:chloroform:isoamyl alcohol (24:24:1) and phases separated in a microcentrifuge run at maximum speed for 5 minutes. A second extraction with 1 ml of ether was then performed. DNA was precipitated by addition of 2.5 volumes ice cold 100% ethanol and incubation at −20°C for 1 hour to overnight. The DNA was pelleted and washed with 1 ml 70% ethanol, briefly dried and resuspended in 1× TE (10 mM Tris·HCl, pH 8.0, 1 mM EDTA). DNA concentration was estimated by electrophoresis of 1/40 th of the sample on 0.8% agarose gel and visual comparison with standards.

DNA amplification. The optimized PCR conditions for the use of megagametophyte DNA were a modification of the procedure reported by Williams et al.[25]. This involved a reduction in the amount of genomic DNA and Taq DNA polymerase relative to our previous report with conifer needles or buds[27]. The components of our optimized PCR reactions consisted of 2 ng DNA, 2.3 mM MgCl, 0.3 µM primer, 200 µM dNTPs and 0.625 U Perkin Elmer Cetus Amplitaq enzyme per reaction volume of 25 µl. Template DNA (in 5 µl TE) was overlayed with 25 µl oil and denatured at 94°C. The remaining components made up in a master mix were then aliquoted to individual tubes containing the DNA. The tubes were mixed by tapping and then spun briefly. Amplification involved 45 cycles of 1 minute at 94°C, 1 minute at 36°C and 2 minutes at 72°C in a Perkin Elmer Cetus DNA Thermal Cycler 480™. Amplification finished with an incubation at 72°C for 10 minutes followed by 4°C soak until recovery. Amplified products were separated by gel electrophoresis on 1.4% agarose and detected by ethidium bromide staining.

Primers. The oligonucleotide decamers were synthesized on a Applied Biosystems Inc. PCR-MATE DNA synthesizer and purified using NAP-5 (Pharmacia) disposable columns. The sequence of each primer was arbitrary and generated on a random basis within the constraints of G+C content between 50 and 80% and no palindromic sequences including 6 or more nucleotides.

Screening of primers and scoring of segregating markers. In order to identify primers that detect polymorphism among seeds from a single tree, each primer was used in PCR reactions with DNA from 5 seeds. Those that detected variation among the 5 genotypes were labelled as polymorphic and used to score for segregation of the polymorphic band among 47 seeds. Presence of a band was scored as a plus (+) while absence of the band was scored as minus (−). In cases where presence or absence of bands were unclear, they were recorded as missing data.

Statistical analysis. Goodness of fit to a 1:1 Mendelian ratio of segregating loci was tested by Chi square analysis at the 0.01 significance level. Markers that did not segregate according to the expected ratio were excluded from the linkage analysis. The Macintosh Version 1 of the Mapmaker Program[39] was used to compute linkages. Raw data was presented to the Mapmaker Program designated as "A" for + alleles and "B" for − alleles (i.e. to mimic homozygote A and homozygote B individuals in an F2 intercross; A. Rafalski, personal communication). The two-point analysis was used to sort loci into linkage groups using the linkage criteria of 0.4 recombination value and log likelihood (LOD) of 4.0. The Linkage 1 computer program[40] was also used to confirm the two-point linkages obtained from the Mapmaker Program. Each linkage group obtained from the two point analysis was subjected to three-point (LOD = 3) and multipoint analysis to order loci on each linkage group. The selection from alternate maps was on the basis of highest LOD score. Distances between markers were expressed in cM as derived from the Kosambi function[41]. Because the MapMaker Program conducts multipoint linkage analysis based on the method of maximum likelihood and because we are mapping a single tree from a sufficiently large set of haploid genotypes, it was not necessary to assign phase to the markers prior to determining linkage and map distances using the program. Thus pedigree information regarding markers in the tree's grandparents was also not necessary.

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