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Segregation of random amplified DNA markers in F_1 progeny of conifers

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Summary. The recently developed approach to deriving genetic markers via amplification of random DNA segments with single primers of arbitrary nucleotide sequence was tested for its utility in genetic linkage mapping studies with conifers. Reaction conditions were optimized to reproducibly yield clean and specific amplification products. Template DNA from several genotypes of Douglas-fir (*Pseudotsuga menziesii*) and white spruce (*Picea glauca*) were tested against eight ten-base oligonucleotide primers. Most of the tested primer/parent tree combinations yielded polymorphic PCR products ("RAPD" markers). Selected primers were then used in PCR reactions with template DNA isolated from offspring in Douglas-fir and black spruce diallel crosses among the same parental lines. The diallel study confirmed the appropriate inheritance of RAPD markers in the F_1 generation. The value of these dominant RAPD markers for genetic linkage mapping in trees was established from both theoretical and applied perspectives.

Key words: Conifers – genetic markers – Linkage mapping – Random amplified DNA markers

Introduction

The use of restriction fragment length polymorphisms (RFLPs) to generate genetic linkage maps represents an important contribution of molecular genetics to plant improvement programs. RFLP-based genetic linkage maps have been constructed, or are underway, for maize (Helentjaris et al. 1986; Helentjaris 1987; Burr and Burr 1991; Beavis and Grant 1991), barley (Blake 1990; Nilan 1990), tomato (Bernatzky and Tanksley 1986), wheat (Kam-Morgan and Gill 1989), lettuce (Landry et al. 1987), rice (McCouch et al. 1988), lentil (Harvey and Muehlbauer 1989), *Brassica oleraceae* (Slocum et al. 1990), and *Arabidopsis thaliana* (Chang et al. 1988; Nam et al. 1989).

The most common use for RFLP-based genetic linkage maps in plant improvement is to provide markers for marker-assisted selection. The advantages of marker-assisted selection have been well documented (Beckman and Soller 1986, 1988; Patterson et al. 1988; Soller and Beckman 1990; Lande and Thompson 1990). Studies in tomato (Osborn et al. 1987; Nienhuis et al. 1987; Weller et al. 1988) and corn (Stuber 1987; Grant et al. 1988) have demonstrated that marker-assisted selection is not limited to qualitative traits, that is traits controlled by one or a few genes, but is also applicable to quantitative and semi-quantitative traits. Early selection of progeny in the development of new cultivars for annual crops may save several months to several years of growing time as well as labor and space requirements. For genetic improvement of long-lived species such as coniferous trees, however, the savings could be much more dramatic. Field trial results for *Pseudotsuga menziesii* (Namkoong et al. 1972) and *Pinus ponderosa* (Namkoong and Conkle 1976) suggested that selection for gain in height, volume, and wood quality could not be conducted with confidence until half the rotation age of the respective species (rotation can be 80 years in *P. menziesii*). Imperfect juvenile-mature correlation could result in the inordinate risk of seriously reducing net gain if selection were done too early.

One potential problem in RFLP-based genetic linkage mapping with conifers is the relatively large size of the nuclear genomes of trees relative to herbaceous crops. The genome size of Douglas-fir and white spruce are

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approximately 34 and 17 pg per haploid nucleus, respectively, while that of corn is approximately 6 pg. Thus, one might expect that reproducibly detecting single-copy genes by Southern hybridization and saturating RFLP-based linkage maps may prove more difficult for conifers than for angiosperms and would benefit even more from time-saving and non-hybridization based techniques. The recently developed "Random Amplified Polymorphic DNA" ("RAPD") marker system of Williams et al. (1990) uses the polymerase chain reaction (PCR) technique to generate random amplified polymorphic DNA markers. This approach holds great promise for quickly placing markers on linkage groups, even with large genomes. In the RAPD system, single random ten-base oligonucleotides are used as primers for PCR. The resulting amplification products frequently vary between genotypes and can be used as genetic markers.

The RAPD system for genotyping can save much time relative to the standard RFLP approach of cDNA library construction and Southern hybridizations. However, RFLPs (like isozymes) are codominant markers while RAPD markers are dominant, i.e., one cannot immediately deduce homozygosity or heterozygosity at a locus from the presence of a RAPD marker DNA band. Thus, a requisite to the use of the dominant RAPD markers in genome mapping is that for any locus the zygosity of the parent(s) must be determined prior to conducting full-scale segregation analyses with the progeny. When pedigrees are not available, as is often the case in tree genetics, the zygosity of parents at a locus can only be deduced from the progeny. We present evidence in this report that RAPD markers detected in white spruce (*Picea glauca*) and Douglas-fir (*Pseudotsuga menziesii*) often segregate appropriately as independent genetic alleles in F_1 progeny and thus can serve as markers for genetic linkage mapping. From this evidence and theoretical considerations, we argue that dominance should not restrict the use of RAPD markers in genome mapping with conifers.

Materials and methods

Genetic material

A partial diallel between six selected Douglas-fir genotypes was established in 1987 by Dr. Y.A. El-Kassaby of Canadian Pacific Forest Products, Ltd., and Dr. J. Maze of the University of British Columbia. Thirty progeny from six of the crosses in this Douglas-fir partial diallel were sampled. At the Petawawa National Forestry Institute, a diallel between 19 white spruce genotypes was established. Initially, DNA was sampled from five parents in the white spruce diallel and tested for RAPD markers. Following preliminary observations, experiments focussed on two parents (Sb10 and Sb13) and 14 F_1 progeny, 7 each from the reciprocal crosses between these black spruce parents.

DNA isolation

DNA was isolated from needles by the procedure of Wagner et al. (1987) or from buds with a modification of the method of Bousquet et al. (1990). From buds, the preferred tissue for DNA preparations from white spruce, the scales were removed, and the internal tissues transferred aseptically into 1.5-ml sterile microcentrifuge tubes, with 150 µl CTAB isolation buffer [2% (w/v) CTAB (cetyltriammonium bromide, Sigma Chemical Co), 1.4 *M* NaCl, 20 m*M* EDTA, 1% (w/v) PEG 8000 (polyethylene glycol, Sigma) and 100 m*M* TRIS-HCl (pH 9.5)] per two or three buds. The tubes were then frozen in liquid nitrogen for 30 s, followed by grinding of the tissue to a paste with fitted plastic drill bits. The drill bits were rinsed with 700 µl CTAB buffer, the rinse then mixed with homogenate by inversion, and then incubated at 65 °C for 15 min. The homogenate was then extracted with 500 µl chloroform:isoamyl alcohol (24:1) and centrifuged in a microcentrifuge at full speed for 5 min at 4 °C. DNA was precipitated from the aqueous phase by mixing with 500 µl isopropanol and pelleting in a microcentrifuge at 4 °C for 5 min. The DNA pellet was rinsed with 70% ethanol, dried, and resuspended in 200 µl of sterile distilled water and the DNA concentration determined.

For Douglas-fir, DNA was isolated from needles or vegetative buds. Tissues were washed in alcohol, ground to a powder in liquid nitrogen, and if necessary, stored at −70 °C. Powdered tissue was resuspended by grinding in mortar and pestle in CTAB isolation buffer (above), followed by a brief treatment with a Brinkman polytron homogenizer. The homogenate was extracted with chloroform, and the aqueous layer re-extracted twice. DNA was precipitated with isopropanol, rinsed in 70% ethanol, dried and resuspended in TRIS-EDTA buffer. For parental genotypes, the needle DNA samples were further purified by banding in CsCl equilibrium density gradients.

DNA amplification

The conditions reported by Williams et al. (1990) for creating RAPD markers by PCR were optimized for use with conifer template DNA. Tests of reaction components included varying DNA concentration, magnesium concentration, source of enzymes, enzyme concentration, and primer concentration, as detailed in the Results and discussion section. The optimum reaction mix, developed with Douglas-fir template DNA, consisted of 8 µg/ml template DNA, 0.2 µ*M* primer (single, ten base oligonucleotide), 100 U/ml Cetus Amplitaq enzyme, 1.9 m*M* $MgCl_2$ and 200 µ*M* each dATP, dCTP, dGTP and dTTP in a final volume of 25 µl 1 × Cetus reaction buffer overlayed with 25 µl mineral oil. Amplification conditions included a preliminary 7 min denaturation at 94 °C, followed by addition of the enzyme, an additional 2 min soak at 94 °C, and then a total of 45 cycles of: 1 min at 94 °C, 1 min at 35 °C, and 2 min at 72 °C in the Cetus Perkin-Elmer Thermocycler. The amplifications finished with an incubation at 72 °C for 10 min, followed by a 4 °C soak until recovery.

For white spruce samples, the PCR amplifications were conducted with an Ericomp Easy Cycler series twin block thermocycler. With the white spruce samples, resolution of RAPD markers was often improved with a higher stringency secondary PCR amplification in which 2.5 µl of the primary reaction product was mixed with 40 unit/ml Taq polymerase and 0.2 µ*M* of additional primer (same sequence) in a final volume of 25 µl of 1 × reaction buffer. The secondary amplifications went for 20 cycles of: 30 s at 40 °C, 1 min at 72 °C, and 30 s at 94 °C.

Segregation analysis

DNA samples extracted from Douglas-fir F_1 progeny were amplified with a set of eight PCR primers. This set of ten base oligonucleotides (Ap3, Ap4, Ap4c, Ap5a, Ap5h, Ap6, Ap9, and Ap13) was selected from primers used by Williams et al. (1990). Eleven different PCR primers were tested for production of

RAPD markers with the white spruce parental DNAs, from which a set of four oligonucleotide primers were chosen for progeny analysis (FCP1: GCTTACCACC; FCP3: CCATTCACCG; FCPA3: TATCGCACGCT; PRC1: CGCATCCGAA). RAPD markers were detected by electrophoresis of the complete amplification reaction products from progeny and parents of each cross on 1% agarose horizontal gels. Gels were stained with ethidium bromide, photographed, and the distribution of markers among progeny recorded. Goodness-of-fit to the 1:1 pattern of segregation for marker DNA bands, as predicted for Mendelian characters in a test-cross, was determined using the Chi-square test.

Results and discussion

Theoretical considerations for use of dominant molecular markers in genome mapping

Prior to conducting segregation tests with RAPD markers, we asked if genetic theory would support or deny the hypothesis that the lack of codominance in RAPD markers would seriously limit their usefulness in genetic linkage mapping studies with conifers. Where an F_2 generation can be easily obtained, such as with annual orgnisms like *Zea mays,* codominance provided with RFLP markers is clearly an advantage. In a cross between homozygous parents AABB × aabb, the selfing of the F_1 AaBb produces nine F_2 genotypes, all of which are recognizable by RFLPs. However, only four groups of genotypes would be recognizable with RAPD (dominant) markers: AB/--, aB/a-, Ab/-b, and ab/ab. For genome mapping with F_2 populations, fewer recognizable phenotypes translates into a loss of genotype information from RAPD markers relative to RFLPs, unless many more F_2 individuals are sampled.

However, breeding populations in conifers would rarely if ever result from crosses between homozygotes. Conifers show very high levels of heterozygosity (approximately 80% for Douglas-fir – Y.A. El-Kassaby, Canadian Pacific Forest Products, personal communication). Perhaps the lack of codominance with RAPD markers might not represent such a disadvantage for crosses involving heterozygotes. Because of high levels of heterozygosity for many loci in conifers, it could be expected that the segregating progeny often represent a backcross situation. For the backcross situation known as a test-cross, i.e., a cross between a heterozygote and a double recessive (AaBb × aabb), four classes of offspring, AB/ab, Ab/ab, aB/ab, ab/ab, are obtained in a ratio of 1:1:1:1.

Will RAPD markers provide more or less information than RFLP markers from test-crosses? In Table 1 we compare the formulas and values for standard errors (SE) and mean amount of information (i_p) that can be obtained from each of three classes of progeny: test-cross, codominance and dominant/recessive. In this analysis, the recombination value, p, was set at the level of 0.2 to represent a good linkage situation; the number of individuals in the progeny set, n, was set at 50, and the loci are in the repulsion phase. The derived standard error of 0.057 for a test-cross is almost equal to the SE for codominance and over twice as good as that for the dominant/recessive class of progeny. In fact, about 280 individuals would be required to give a comparable SE for the dominant/recessive case as for 50 progeny in a test-cross. Alternatively, for the dominant/recessive case when both parents carry the dominant RAPD marker and segregation has been observed in the progeny, it should be possible to determine the parental genotypes if additional crosses are available.

Table 1. Calculation of standard error ($SE=(1/ni_p)^{1/2}$) and mean amount of information (i_p) for each individual obtainable from genetic markers in three progeny classes in repulsion phase

Progeny	i_p [a]	n	p	(i_p)	SE
Test-cross	$\frac{1}{p(1-p)}$	50	0.20	6.25	0.057
Codominance	$\frac{2(1-3p+3p^2)}{p(1-p)(1-2p+2p^2)}$	50	0.20	9.56	0.046
Dominant/ recessive	$\frac{2(1+2p^2)}{(2+p^2)(1-p^2)}$	50	0.20	1.10	0.135

[a] from RW Allard 1956

From such theoretical considerations it would appear that lack of codominance should not preclude the usefulness of RAPD markers in genome mapping studies with forest trees species. This conclusion will hold true in practice only if enough RAPD marker loci can be easily identified in the proper test-cross configuration in the parent trees being studied (i.e., AaBbCcDdEe. . . in parent 1 × aabbccddee. . . in parent 2). The opportunity for achieving such an arrangement with a sufficient number of loci to complete a linkage map should be very good as the number of RAPD markers that can be screened are virtually unlimited and the level of heterozygosity in conifers is quite high. A method for identifying heterozygotes based on relative intensities of DNA bands might simplify the task, but only if reaction conditions could be precisely reproduced sample to sample so that results would be strictly quantitative. Our preliminary results with RAPD markers in conifers indicate that the quantitative reproducibility of PCR products with random primers was not sufficient for identifying heterozygotes. Quantitation of RAPD marker PCR products via incorporation of isotope-labelled deoxyribonucleotide triphosphates, limited amplification cycles, and densitometry from autoradiograms is an approach that we have not yet fully explored (Hayashi et al. 1989).

Heterozygosity in parent trees should be discernible either by pre-screening a few progeny from each family

or by prescreening the grandparents where available. Most genetic improvement programs for conifers are still at the second generation, however. Grandparents are not available for the diallels we have worked with in Douglas-fir and white spruce. In this study, we report our evaluation of the practicality of meeting the requirement for test-cross cases in mapping conifer genomes with RAPD markers by screening Douglas-fir and white spruce F_1 progeny.

Optimization of amplification conditions for conifers

The DNA isolation procedures with dormant vegetative buds of Douglas-fir and white spruce proved to be the most efficient method for obtaining PCR template. Forty or more DNA preparations could be conducted in 4 h or less with yields of 20–30 μg per bud.

The first attempts that we made with template DNA from Douglas-fir revealed many polymorphisms between several individuals (data not shown) when the reactions conditions recommended by Williams et al. (1990) were utilized. However, to use the RAPD system routinely with conifer template DNA it was apparent that we needed to find reaction conditions that would increase the reproducibility of bands and decrease background staining in gel lanes due to non-specific amplification products.

To determine the effect of magnesium, we tested four magnesium concentrations (1.5 m*M*, 1.9 m*M*, 2.3 m*M*, 2.7 m*M*) in the RAPD protocol, using one PCR primer (Ap3) versus three Douglas-fir genotypes (Fd25, Fd439, Fd48). To determine the optimum DNA concentration, we tested five concentrations of DNA from one Douglas-fir parent (12.5, 25, 50, 100, 200 μg) in separate reactions with two primers (Ap3 and Ap4). We also looked at the effect of enzyme source by comparing the production of RAPD markers from DNA using the Cetus Perkin Elmer enzyme (AmpliTaq) with two Douglas-fir genotypes (Fd37 and Fd196) and one PCR primer (Ap9) with amplification products from thermostable DNA polymerases of several other commercial suppliers. Finally, we tested different approaches to purifying the synthetic oligonucleotide primers (Sep packs, G-25 Sephadex drip and spun columns, Pharmacia NAP-10 columns, ethanol precipitation, and no purification).

The optimal reaction conditions that we arrived at for generating RAPD markers from conifer template DNA are given in the Materials and methods. Some of our observations that are pertinent to obtaining reproducible and readily scorable amplification products with conifers include:(1) enzyme concentration and enzyme source are critical; (2) 1.9 m*M* Mg^{2+} is optimal; higher concentrations result in background smearing, while lower concentrations produced less intense PCR bands; (3) reproducibility and resolution are greatly enhanced with higher DNA template concentration; (4) lower molecular weight DNA bands are usually more reproducible as RAPD markers than higher molecular weight bands; (5) variability may be caused by the thermocycler itself; and (6) secondary amplification of the PCR product at a higher stringency, using the same primer, can reduce background and improve resolution of RAPD markers.

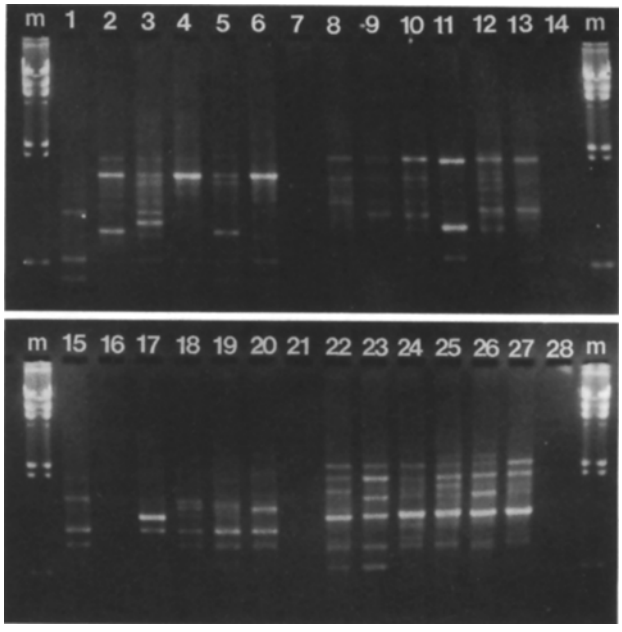

Fig. 1. DNA polymorphisms between six parental Douglas-fir genotypes from four PCR primers. Shown is an ethidium bromide-stained agarose gel of PCR products from primer Ap4c, *lanes 1–7*; primer Ap5a, *lanes 8–14*; primer Ap5h, *lanes 15–21*; primer Ap13, *lanes 22–28*; parental genotype Fd-25, *lanes 1, 8, 15, and 22*; parental genotype Fd-37, *lanes 2, 9, 16, and 23* (lack of amplification left lane 16 blank); parental genotype Fd-48, *lanes 3, 10, 17, and 24*; parental genotype Fd-120, *lanes 4, 11, 18, and 25*; parental genotype Fd-196, *lanes 5, 12, 19, and 26*; parental genotype Fd-439, *lanes 6, 13, 20, and 27*; and minus template DNA controls, *lanes 7, 14, 21, 28*

Segregation analyses

As a first step, we determined which of the eight PCR primers could produce scorable RAPD markers with template DNA from six Douglas-fir parental genotypes (Fd-25, -37, -48, -120, -196, -439). Figure 1 shows the electrophoretic results of reactions with four of the eight primers. We found that six of the eight primers revealed scorable polymorphisms. Although not all parents gave unique products with every primer, certain primers, e.g., Ap4C, revealed more polymorphisms than others. Other primers did not produce scorable markers from any of the parents, e.g., primer Ap4, which differed from the very productive 4c primer at only the third nucleotide (data not shown). Primers Ap3, Ap4c, Ap5a, and Ap9 were chosen to conduct a test of marker segregation in F_1

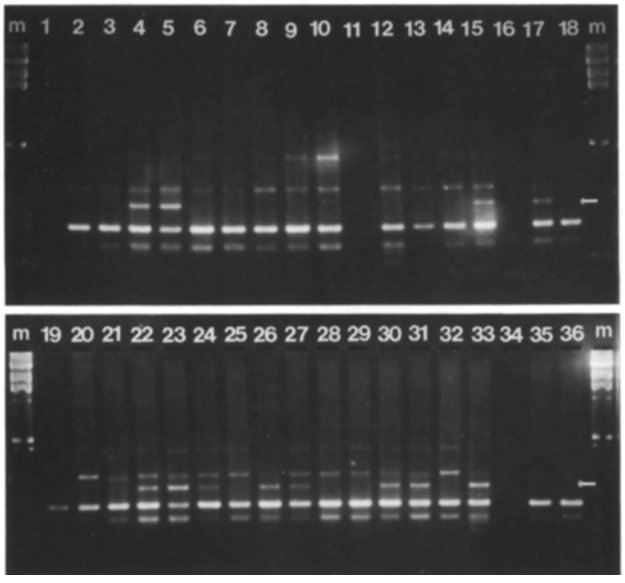

Fig. 2. Segregation of a polymorphic DNA band (RAPD marker) produced by primer Ap9. Shown is an ethidium bromide stained agarose gel of PCR products from Douglas-fir parent Fd-37, *lanes 17 and 35*; parent Fd-196, *lanes 18 and 36*; F_1 progeny, *lanes 1–15 and 19–33*; and no template DNA controls, *lanes 16 and 34*. *Arrows* indicate position of the polymorphic DNA band

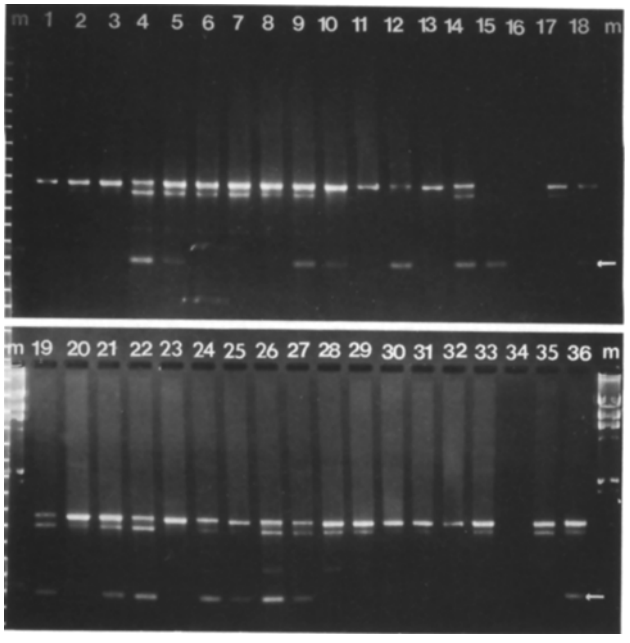

Fig. 3. Segregation of a polymorphic DNA band (RAPD marker) produced with primer Ap3. Progeny, parents, and lane arrangement are identical to those of Fig. 2

progeny from a partial diallel of the six Douglas-fir parental genotypes.

Segregation of RAPD markers in 30 F_1 progeny from individual crosses in a diallel between the Douglas-fir parents were analyzed by horizontal agarose gel electrophoresis. Also included on each gel were the PCR products from the two parental genotypes and contamination controls (PCR reactions without template DNA). Figures 2 and 3 show the electrophoretograms of amplification products from two sets of progeny. This data reveals how easily the RAPD markers can be scored as segregating genetic alleles (by presence or absence of an ethidium bromide-stained parental DNA band among progeny).

Table 2. Chi-square analysis for goodness of fit to a 1:1 ratio of segregating PCR bands in Douglas-fir

Cross	Primer	Band present	Band absent	Total	Chi-square
25 × 439	Ap5a	20.0	7.0	27.0	6.26*
25 × 439	Ap4c	15.0	13.0	28.0	0.14
25 × 439	Ap4c	20.0	8.0	28.0	5.14*
25 × 439	Ap9	15.0	14.0	29.0	0.03
37 × 196	Ap9	11.0	17.0	28.0	1.29
37 × 196	Ap3	16.0	14.0	30.0	0.13
37 × 196	Ap4c	13.0	17.0	30.0	0.53
37 × 196	Ap4c	17.0	13.0	30.0	0.53
37 × 196	Ap4c	21.0	9.0	30.0	4.80*
48 × 196	Ap3	7.0	15.0	22.0	2.83

* Significant at the 0.05 level

The data for assortment of ten segregating RAPD markers in progeny of three Douglas-fir crosses is summarized in Table 2. Results for Chi-square analysis of goodness of fit to a 1:1 segregation ratio of the marker bands are given. For these ten Douglas-fir RAPD markers, seven cases fit a 1:1 segregation while three cases did not, tested at the 0.05 level of significance. The 1:1 pattern follows the expected Mendelian behavior for segregation of alleles in a test-cross when the maternal parent is heterozygous for the allele (presence of the DNA band) and the paternal parent is homozygous recessive for the allele (i.e., lacking the DNA band). The majority of segregating RAPD markers that we detected in this study with Douglas-fir were of the test-cross class (over 70%). Certain markers did not show 1:1 segregation as expected, e.g., primer Ap5a with cross 25 × 439. While the small progeny size may be implicated as a possible explanation for such deviations of segregating loci from the expected ratio, this phenomenon appears to be of a common occurrence in both isozyme and RFLP analysis (Edwards et al. 1987; Slocum et al. 1990; Tulsieram et al. 1991). The generally favored postulate for such aberrations is a mechanism occurring prior to zygote formation. We also observed independent assortment of multiple RAPD markers produced by the same primer within one family, e.g., with primer 4c (Table 2). This primer revealed three markers that could be scored from the Fd-37 × Fd-196 cross, and two scorable markers from the Fd-25 × Fd-439 cross.

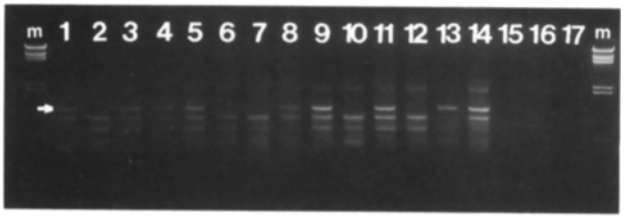

Fig. 4. Segregation of a polymorphic band (RAPD marker) in white spruce produced with primer PRC1. Shown is an ethidium bromide stained agarose gel of PCR products from F_1 progeny, *lanes 1–6 and 9–16*; parent Sb-10, *lane 7*; parent Sb-13, *lane 8*, and minus-template DNA control, *lane 17*. Products in lane 15 and 16 were low in quantity. *Arrow* indicates the position of the polymorphic DNA band

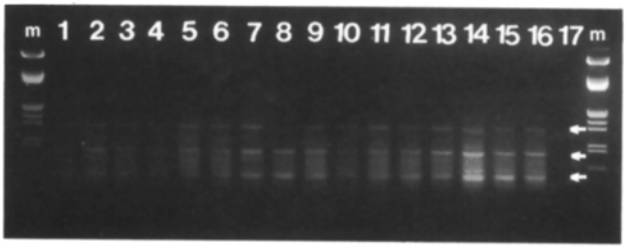

Fig. 5. Example of non-segregating polymorphic bands in white spruce. Shown is an ethidium bromide stained agarose gel of PCR products with primer PCP1. Progeny, parents, and lane arrangement are identical to those of Fig. 4

In addition to the ten segregating RAPD markers in Douglas-fir, we also observed two instances where parents demonstrated polymorphism for a PCR band, but the band was persistent in all F_1 progeny. In these cases, the crosses clearly involved a dominant homozygous allele and its homozygous recessive state.

Our results with white spruce were very similar to that obtained with Douglas-fir. Four primers (PRC1, FCP1, FCP3, and FCP13) were tested with progeny template DNA from the cross Sb10 × Sb13. The four primers clearly revealed three polymorphic markers. As shown by the gel in Fig. 4, the polymorphism detected with primer PRC1 showed Mendelian segregation in an approximately 1:1 ratio. This proved that there was a test-cross condition at this loci, that parent Sb13 was heterozygous for presence of the band, and that parent Sb10 was homozygous recessive for absence of the band. Primer FCP13 also revealed a polymorphism that segregated as Mendelian alleles in this cross (data not shown), although in this case Sb10 was the heterozygous parent and Sb13 homozygous recessive. Because only 14 F_1 progeny were available from the white spruce diallel, it was imprudent to apply a statistical test for significance to the data. The polymorphic RAPD marker produced by primer FCP1 was present in parent Sb10, but was absent in parent Sb13 (Fig. 5). Progeny analysis revealed persistence of the parental band in all 14 offspring (Fig. 5). This, therefore, reveals homozygosity for presence of the band in parent Sb10 and homozygosity (homozygous recessive) for absence of the band in parent Sb13. The polymorphism from this primer also confirms the dominant nature of these RAPD marker alleles.

The data obtained in this preliminary study with Douglas-fir and white spruce suggest to us that the optimism from theoretical considerations for use of RAPD markers in genome mapping have been born out. Loci in the test-cross situation appear to be relatively common in the conifers tested. At least 7 of 12, or over 50%, of the polymorphic loci that we followed in this preliminary study proved to be test-cross cases. Half of these test-cross cases had the heterozygous loci in one parent and half in the other, as expected by random chance. Furthermore, over 80% of the random primers tested in this study revealed polymorphisms between the six Douglas-fir parents (as shown in Fig. 1 and additional data not reported here). The knowledge that random primers generate so many polymorphisms and that test-cross cases for RAPD markers in conifers are so common suggests that a suitable number of loci that are in the proper test-cross arrangement can be obtained for use in constructing a genetic linkage map for Douglas-fir and white spruce. We would suggest that preliminary screening of small samples of progeny be conducted to determine the mode of segregation of RAPD markers in the F_1 prior to full-scale segregation analysis. This data would suggest that from 600 unique random primers, 200 markers could be placed on a genetic linkage map for a typical conifer. If these primers were utilized in combination, even more markers could be mapped (A. Rafalski, E. I. DuPont de Nemours & Co., personal communication). Furthermore, we have observed that the amplified DNA bands can be labelled and used as probes to generate additional RFLP markers (data not shown).

Briefly, the steps involved in using RAPD markers for linkage mapping include: (1) identify primers that reveal present/absent DNA band polymorphisms between the selected parents (i.e., A_ × aa); (2) screen the polymorphisms for test-cross cases, i.e., for "Aa" heterozygotes; and (3) conduct segregation analysis among progeny with all test-cross RAPD markers. In cases where one wishes to use all possible RAPD markers that reveal test-cross cases, one could conceivably construct two linkage maps based on the heterozygous loci in each parent. Linkage groups in each map could then be correlated by choosing PCR products from several loci in each linkage group to generate RFLPs that can be followed for linkage. Similarly, data from RAPD markers could be pooled with data from RFLPs to create saturated linkage maps.

To conclude, our experience with the RAPD marker system suggests several possible applications with conifers: (1) genetic linkage mapping (especially in "test-cross" classes); (2) genetic diversity (fingerprinting) studies; (3) fast generation of hybridization probes from total

DNA for RFLP analyses; (4) generation of single copy RFLP probes or single copy RAPD markers from cDNA (using a RAPD 10 mer together with an oligo-dT primer); and (5) determining the degree of heterozygosity within individual parent trees by amplifying DNA from single gametes. These applications will be reported on in detail in subsequent publications.

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