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Inheritance of RFLP loci in a loblolly pine three-generation pedigree

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Summary. A high-density restriction fragment length polymorphism (RFLP) linkage map is being constructed for loblolly pine (*Pinus taeda* L.). Loblolly pine cDNA and genomic DNA clones were used as probes in hybridizations to genomic DNAs prepared from grandparents, parents, and progeny of a three-generation outbred pedigree. Approximately 200 probes were evaluated for their ability to detect polymorphic loci between DNAs prepared from the two parent trees, 20-1010 and 11-1060, and cut with four different restriction enzymes: *Bam*HI, *Dra*I, *Eco*RI, and *Hin*dIII. More than half of the probes detecting single- or low-copy number sequences (56%) revealed polymorphisms between the two parents with at least one restriction enzyme. If necessary, an additional hybridization to DNAs prepared from the four grandparent trees was conducted to determine the zygosity of parent trees. Ten of these probes were hybridized to progeny DNAs from this cross and, as expected, the markers were inherited as simple codominant Mendelian alleles. Four of the ten probes detected segregation of three alleles at one locus, and four probes detected more than one independently segregating locus. RFLPs can be used immediately to assess genetic diversity in conifer populations and to "fingerprint" genotypes in tree improvement programs.

Key words: *Pinus taeda* L. – Restriction fragment length polymorphisms – genome mapping

Introduction

We are constructing a saturated genetic linkage map for loblolly pine (*Pinus taeda* L.) based on restriction fragment length polymorphisms (RFLPs). Polymorphisms in DNA fragment patterns of restriction digests occur as a result of changes in restriction enzyme recognition sites, due to point mutations or genetic rearrangements such as duplications or deletions. Hybridization with radiolabeled DNA probes containing sequences homologous to regions of the chromosomal fragments permits identification of specific loci.

Detailed linkage maps based on RFLPs have been reported for a number of plants (Helentjaris 1987; McCouch et al. 1988; Chang et al. 1988; Gebhardt et al. 1989). The development of similar maps for conifers, however, has been somewhat hindered because of their large genome size (20–30 pg/nucleus) (Dhillon 1987), long generation times, and outbred mating system.

To date, isozymes have been the best and most widely used genetic markers in loblolly pine (Adams and Joly 1980a, b); however, the number of markers is limited. Adams and Joly (1980a) determined the inheritance of 17 isozyme loci and established linkage relationships among 12 of the loci (Adams and Joly 1980b). These markers have been applied in a variety of genetic studies (e.g., Friedman and Adams 1985; Adams et al. 1988). The efficiency of such studies could be improved if a larger number of polymorphic genetic markers was available. With the application of RFLP markers, this problem could be overcome, because such markers are potentially unlimited in number.

This paper reports the identification and inheritance of 15 RFLP loci in loblolly pine. Mendelian inheritance is inferred from segregation data obtained from a three-generation outbred pedigree. These results are the first report of RFLP inheritance in a forest tree and are a start toward mapping the loblolly pine genome.

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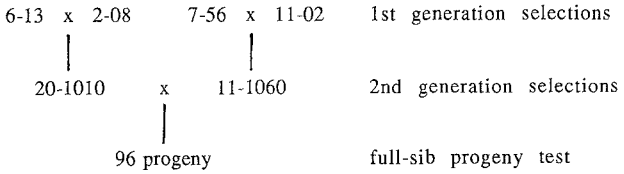

Fig. 1. Loblolly pine three-generation outbred pedigree

Materials and methods

Genetic materials

A single, three-generation outbred pedigree (Fig. 1) was chosen to determine linkage relationships among RFLP loci based on cosegregations in outbred F_2 progeny. This pedigree was developed by the North Carolina State University-Industry Cooperative Tree Improvement Program. Selection was based on evaluations for heterozygosity at isozyme and RFLP loci as discussed below. Clones 6–13, 2–08, 7–56, and 11-02 were first-generation selections (grandparents), whereas 20-1010 and 11-1060 were second-generation selections (parents). All cones were grown in one or more seed orchard sites in the southeastern United States. The F_2 family of 96 progeny grows in Weyerhaeuser Co. evaluation plantations: 48 near Hot Springs, AR, and 48 near Ft. Towsen, OK.

Source of probes

We used complementary DNA (cDNA) and genomic DNA probes to identify RFLPs. The cDNA library was prepared from total RNA isolated from 12-day-old loblolly pine seedlings by the method of Alosi et al. (1990). The seedlings were grown under natural day length and harvested at mid-morning. The poly A+ fraction was isolated by three successive cycles of chromatography over oligo-dT cellulose. Complementary DNA was prepared by the method of Gubler and Hoffman (1983) using a Librarian II cDNA cloning kit from INVITROGEN. *Bst*XI nonpalindromic linkers were added, and the cDNA was ligated into prepared pTZ18R-B vector and transformed into bacterial strain DH5α. Recombinant clones were screened for the presence of inserts greater than 500 bp, using a rapid plasmid procedure and subsequent analysis by gel electrophoresis.

The genomic DNA library was prepared from loblolly pine genomic DNA, which was isolated using the CTAB procedure described below. DNA was digested with both *Bam*HI and *Pst*I, sized to between 500 and 2,000 bp, and ligated into *Bam*HI/*Pst*I-digested pUC19. Plasmids were transformed into bacterial strain JM101 and recombinants were selected using IPTG/X-gal.

All cDNA and genomic DNA probes were given an accession number, e.g., pPtIFGc001, where p denotes plasmid, Pt denotes genus and species from which the library was made, IFG refers to the Institute of Forest Genetics, c (or g) indicates whether the clone is of cDNA or genomic DNA origin, and 001 is the accession number. The same numbers, with a c or g prefix only, were used to designate genomic loci as detected by genetic mapping.

DNA procedures

Loblolly pine genomic DNA was prepared from each of the grandparent, parent, and progeny trees by a modification of the method of Wagner et al. (1987). Needle tissue was ground in liquid nitrogen, and 10 g fresh weight was homogenized with a Brinkmann polytron at 4°C in 150 ml of extraction buffer [50 m*M* TRIS (pH 8.0), 5 m*M* EDTA, 0.35 *M* sorbitol, 0.1% BSA, 0.1% mercaptoethanol, 10% (wt/vol) polyethylene glycol MW 8000]. The homogenate was filtered through two layers of cheesecloth and one layer of Miracloth (Calbiochem); a pellet was then collected from the homogenate by centrifugation ($3{,}000 \times g$, 4°C, 10 min). The pellet was resuspended in 15 ml wash buffer [50 m*M* TRIS (pH 8.0), 25 m*M* EDTA, 0.35 *M* sorbitol, 0.1% mercaptoethanol] and brought to room temperature. N-laurylsarcosine was added to a concentration of 1% (wt/vol) and, after 15 min at room temperature, the suspension was brought to a final concentration of 0.7 *M* NaCl, 1% (wt/vol) hexadecyltrimethylammonium bromide (CTAB) and incubated at 60°C for 30–60 min. Following the incubation, an organic extraction with chloroform:octanol (24:1) was performed, and the aqueous layer was precipitated with 2/3 vol isopropanol. The DNA was hooked out of the solution, rinsed in 70% ethanol, and dissolved in an appropriate amount of TE buffer [10 m*M* TRIS (pH 8.0), 1 m*M* EDTA].

Genomic DNA was digested with one of four restriction enzymes (*Bam*HI, *Dra*I, *Eco*RI, or *Hin*dIII), using 10 units per microgram of enzyme and at least a 4-h incubation. Electrophoresis of plant DNA (10 μg/lane) was run overnight in 0.8% agarose, and gels were transferred to Zetaprobe (BioRad) membrane using the alkaline blotting method of Reed and Mann (1985).

For use as probes in hybridizations to loblolly pine DNA, cDNA or genomic DNA inserts were excised with appropriate restriction enzymes and, following electrophoresis in low-melting-point agarose (NuSieve, FMC), were cut out and random-hexamer-labeled to high specific activity (Feinberg and Vogelstein 1984).

Blots were prehybridized for several hours at 65°C in 4 × SSPE [0.72 *M* NaCl, 40 m*M* sodium phosphate (pH 7.4), 4 m*M* EDTA], 5 × Denhardt's (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% BSA), 0.5% SDS, 0.5% BLOTTO ('Carnation' nonfat powdered milk), and 250 μg/ml sonicated and denatured herring sperm DNA. The prehybridization solution was removed and replaced with hybridization solution, which is the same as prehybidization solution, but with the additions of dextran sulfate to 10% and denatured labeled probe to 2×10^6 cpm/ml. Hybridizations were also conducted at 65°C overnight. After hybridization, membranes were rinsed in 2 × SSPE and washed at 65°C for 20 min in 2 × SSPE, 0.5% SDS, and twice for 30 min in 0.1 × SSPE, 0.1%SDS. Blots were then exposed to Kodak XOMAT-AR film with two intensifying screens at −80°C for an appropriate time, usually 1–4 days. For reuse, blots were washed at 65°C in 0.1% SSPE, 1% SDS for 2 h and then prehybridized and hybridized as above.

Results and discussion

Selection of the loblolly pine pedigree

Efficiency of linkage analysis is improved by selecting a highly heterozygous pair of parent trees. The cross we selected was chosen from among 155 parent pairs. Briefly, the parents were screened for variation at 39 isozyme loci. Individual parent trees had from 0 to 14 heterozygous loci, and the five most heterozygous pairs were selected for subsequent RFLP screening. DNA was prepared from the five selected parent pairs and digested with four restriction enzymes. A total of 40 random cDNA clones was used as probes in hybridizations to parental DNAs, where it was observed that 15 probe/en-

zyme combinations were polymorphic in at least one cross. The most variable parent pair, 20-1010 × 11-1060, possessed 10 of the 15 observed RFLPs and was selected for use in the mapping project. The cross also had the most heterozygous loci based on isozyme data.

Library construction and initial screening of probes

As a source of unique sequence clones, a cDNA library was constructed under the assumption that most sequences transcribed from poly A+ RNA are present in low-copy number. Probes derived from cDNA have an advantage over genomic DNA probes in that they tend to be evolutionarily conserved among related taxa, and are therefore of greater value in comparative RFLP mapping over different conifer genera. One disadvantage of cDNA probes is that coding sequences are not transcribed equally, thus creating redundancy in the library. In fact, from preliminary probe evaluations, we have observed that one sequence has been duplicated several times already in this cDNA library.

An alternative approach would be to use random genomic DNA sequences as a source of probes. Genomic DNA probes can potentially detect more variation, because they are primarily from noncoding DNA sequences which tend to accumulate genetic alterations. Also, a more random distribution of loci may be detected with genomic DNA probes. A disadvantage of genomic DNA probes is that a significant number of repetitive sequences may be included in libraries. To limit the number of cloned repetitive sequences, we used the methylation-sensitive restriction enzymes *Bam*HI and *Pst*I (Helentjaris et al. 1988); these enzymes do not cut C-methylated DNA, which is prevalent in repetitive sequences. Our results indicate that these enzymes preferentially select low-copy sequences in loblolly pine ~78% of the *Bam*HI/*Pst*I clones analyzed to date are unique- or low-copy sequences.

Evaluations for RFLP variation begin with hybridizations of cDNA or genomic DNA probes to Southern blots containing DNA from the two parents that has been cut with four restriction enzymes: *Bam*HI, *Dra*I, *Eco*RI, and *Hin*dIII. Restriction enzymes with 6-bp recognition sequences were chosen over those that recognize 4-bp sequences, because they would have a greater probability of detecting genetic variation caused by insertion/deletion events (McCouch et al. 1988). A typical hybridization pattern observed for cDNA probe pPtIFGc653 is shown in Fig. 2. Approximately 178 probes were evaluated in this manner. About a third of these were discarded because of criteria such as poor signal intensity or high-copy number. Among the remaining single- or low-copy probes, more than half (56%) detected polymorphic loci between parent trees with at least one restriction enzyme. The number of probes evaluated and the percentages of polymorphisms observed with cDNA and genomic DNA probes are shown in Table 1, which considers only variation between the two parents.

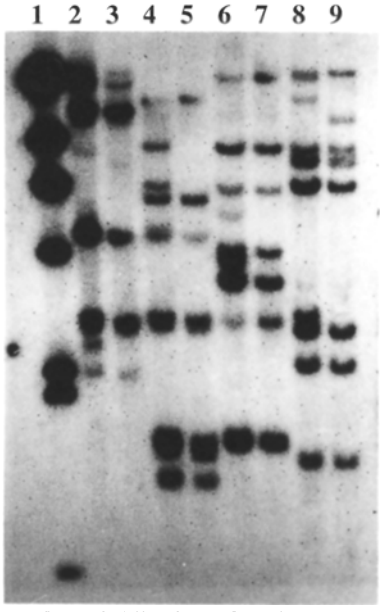

Fig. 2. Hybridization of probe pPtIFGc653 to genomic DNA from loblolly pine parent trees 20-1010 and 11-1060 restricted with *Bam*HI (lanes *2–3*), *Dra*I (lanes *4–5*), *Eco*RI (lanes *6–7*), and *Hin*dIII (lanes *8–9*). Lane *1* = lambda/*Hin*dIII marker

Table 1. Summary of RFLP variation observed between loblolly pine parents 20-1010 and 11-1060 using cDNA and genomic DNA sequences as probes

	No. of probes evaluated	No. (%) of high copy	No. (%) of single/ low copy[a]	No. (%) polymorphisms
cDNA	146	12 (8)	134 (92)	60 (41)
Genomic DNA	32	7 (22)	25 (78)	11 (34)
Total	178	19 (11)	159 (89)	71 (40)

[a] 28 cDNA and four genomics did not show any discernable hybridization pattern and were assumed to be single- or low-copy sequences

*Hin*dIII detected slightly more variation than did the other three enzymes. Among the probes detecting RFLPs, 72% were polymorphic with this enzyme; *Bam*HI, *Dra*I, and *Eco*RI revealed poymorphisms with 59, 61, and 66% of the probes, respectively. The incidence of polymorphisms detected with more than one restriction enzyme was high; 70% of the probes detecting variation were polymorphic with all four restriction enzymes. The high level of polymorphism using different restriction enzymes allows one to choose a particular combination of probe and enzyme to maximize fragment separation in future analyses and/or reduce the number

of enzymes that must be tested to evaluate the potential usefulness of a clone. This observation is also indicative of insertion/deletion or other rearrangement-type events as a mechanism for RFLP variation, as opposed to point mutation at an enzyme recognition sequence (McCouch et al. 1988). Considering the level of polymorphic loci we have observed in loblolly pine, we have decided that subsequent screening and mapping will be based on *Hin*dIII-digested DNAs.

Zygosity is not always determined from hybridizations to parental DNAs; for example, heterozygotes at one locus cannot be distinguished from homozygotes at two loci. In this situation, an additional hybridization may be made to blots containing DNAs from the grandparent trees. The hybridization pattern observed for probe pPtIFGc1588 on *Hin*dIII-restricted genomic DNA from grandparent and parent trees is shown in Fig. 3. Three alleles were detected at one polymorphic locus.

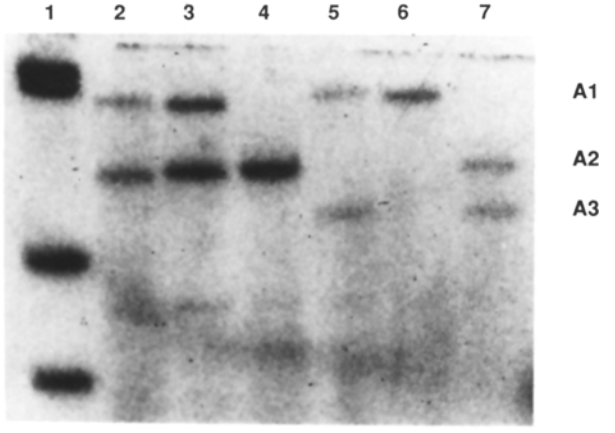

Fig. 3. Hybridization of probe pPtIFGc1588 to *Hin*dIII-restricted genomic DNAs from loblolly pine grandparents 6-13, 2-08, 2-56, and 11-02 (lanes *2*, *3*, *6*, and *7*, respectively), and parents 20-1010 and 11-1060 (lanes *4* and *5*, respectively). Lane *1* = lambda/*Hin*dIII marker

Progeny segregation of RFLP loci

Nine cDNA probes and one genomic DNA probe have been hybridized to blots containing DNAs from 48 progeny of the cross 20-1010 × 11-1060. A total of 15 segregating loci was detected and, as expected, the markers were inherited as simple codominant Mendelian alleles (Table 2). Chi-square values and probabilities for goodness of fit to the proposed segregation ratios are also presented.

Four of the probes (pPtIFGc605, c616, c653, and c669) have detected loci which are segregating for three alleles. In this case the expected segregation ratio is 1:1:1:1. The hybridization pattern for pPtIFGc616 in 19 *Hin*dIII-restricted progeny DNAs is shown in Fig. 4. The parents are each heterozygous for *A1A3* and *A2A3* alleles (data not shown). In a three-allele model the presumptive alleles can only appear two at a time. Two of the three lower fragments are present in each of the progeny except those

Table 2. Segregation of RFLP loci in *Hin*dIII-restricted progeny DNAs of loblolly pine cross 10-1010 × 11-1060 and goodness of fit to expected ratios

Locus	Observed segregation	Expected ratio	χ^2	Probability
c602	28:20	1:1	1.333	0.10–0.25
c605-1	12:9:16:10	1:1:1:1	2.447	0.25–0.50
c605-2	23:24	1:1	0.021	0.75–0.90
c616	13:12:7:16	1:1:1:1	3.500	0.25–0.50
c624	21:24	1:1	0.200	0.50–0.75
c653-1	13:7:15:12	1:1:1:1	2.957	0.25–0.50
c653-2	23:25	1:1	0.983	0.75–0.90
c655	24:24	1:1	0.0	1.0
c658-1	22:24	1:1	0.087	0.75–0.90
c658-2	21:18:6	1:2:1	11.800	0.01–0.001
c669-1	22:26	1:1	0.333	0.50–0.75
c669-2	14:25:9	1:2:1	1.125	0.50–0.75
c669-3	8:12:14:14	1:1:1:1	2.000	0.50–0.75
c670	23:22	1:1	0.022	0.75–0.90
g003	41:54	1:1	1.779	0.10–0.25

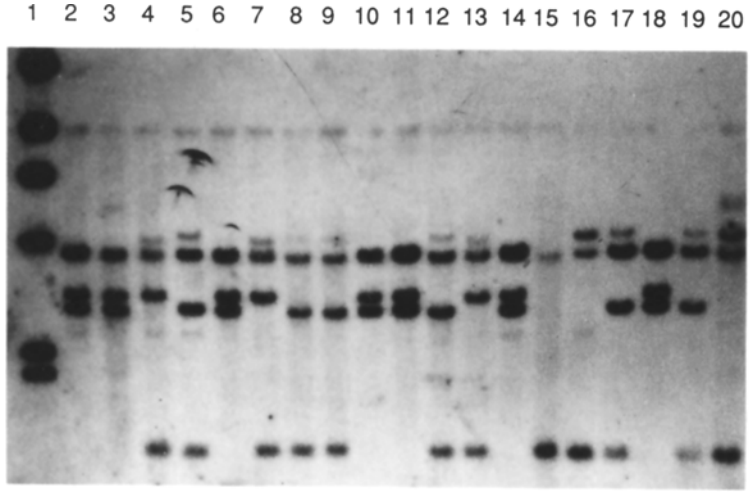

Fig. 4. Hybridization of probe pPtIFGc616 to *Hin*dIII-restricted genomic DNAs from 19 full-sib progeny of loblolly pine cross 20-1010 × 11-1060. Lane *1* = lambda/*Hin*dIII marker

in lanes 15, 16, and 20, which are homozygous for *A3*. Additionally, the upper two bands are also segregating but are completly linked to the A locus.

More than one independently segregating locus can be detected with a given probe by cross hybridization to related sequences at other loci. In cases such as this, the resulting hybridization patterns are quite complex and are not always interpretable. Four of the ten probes listed in Table 2 detect duplicate or triplicate loci, an incidence that seems high for diploid species. Moreover, with many of the probes evaluated, there were often several bands detected. A large number of duplicated RFLP loci have been reported in maize and may have been a result of internal duplication or perhaps an allopolyploidization event (Helentjaris et al. 1988). Our results are preliminary, but suggest that duplication of structural gene loci in *Pinus* may be common. These events may have contributed to the large size of its genome.

Conclusions

The results described here demonstrate that RFLPs can provide the necessary number of markers to construct a detailed genetic map of loblolly pine for applications in forest genetics research and tree improvement. We have estimated the number of markers needed to saturate the loblolly genome, with an average spacing of 20 c*M*, to be between 200 and 300 (Neale and Williams 1991).

The level of variation we are detecting is high, particularly when it is noted that the observed differences are only between two individual trees. Probes hybridized to DNAs from F_2 progeny have detected allelic variation and often more than one independently segregating locus. Hybridization patterns are generally complex, involving several fragments. A high proportion of duplicated sequences and complex hybridization patterns suggests that large multigene families are common for this species.

In most cases a single probe revealed polymorphisms with more than one restriction enzyme. For example, nearly one-third (31%) of the probes detecting RFLPs were polymorphic with all four restriction enzymes. A high degree of polymorphism detected under different genomic digest conditions can be explained by insertion/deletion events, as has been reported in angiosperm species (Helentjaris et al. 1985; McCouch et al. 1988). Similar levels of variation are detected with both cDNA and genomic DNA sequences used as probes; however, the number of genomic DNA probes evaluated to date is still small.

We would expect a general colinearity of genetic maps developed for other *Pinus* species, and perhaps for other conifer genera as well, so that as the probes from this work become available they may be used to rapidly construct maps in related species. Some preliminary work in comparative mapping has been initiated in this laboratory.

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