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RAPD linkage mapping in a longleaf pine × slash pine F_1 family

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Abstract Random amplified polymorphic DNAs (RAPDs) were used to construct linkage maps of the parents of a longleaf pine (*Pinus palustris* Mill.) slash pine (*Pinus elliottii* Englm.) F_1 family. A total of 247 segregating loci [233 (1:1), 14 (3:1)] and 87 polymorphic (between-parents), but non-segregating, loci were identified. The 233 loci segregating 1:1 (testcross configuration) were used to construct parent-specific linkage maps, 132 for the longleaf-pine parent and 101 for the slash-pine parent. The resulting linkage maps consisted of 122 marker loci in 18 groups (three or more loci) and three pairs (1367.5 cM) for longleaf pine, and 91 marker loci in 13 groups and six pairs for slash pine (952.9 cM). Genome size estimates based on two-point linkage data ranged from 2348 to 2392 cM for longleaf pine, and from 2292 to 2372 cM for slash pine. Linkage of 3:1 loci to testcross loci in each of the parental maps was used to infer further linkages within maps, as well as potentially homologous counterparts between maps. Three of the longleaf-pine linkage groups appear to be potentially homologous counterparts to four different slash-pine linkage groups. The number of heterozygous loci (previously testcross in parents) per F_1 individual, ranged from 96 to 130. With the 87 polymorphic, but non-segregating, loci that should also be heterozygous in the F_1 progeny, a maximum of 183–217 heterozygous loci could be available for mapping early height growth (EHG) loci and for applying genomic selection in backcross populations.

Key words Genetic linkage map · *Pinus palustris* · *Pinus elliottii* · Random amplified polymorphic DNA (RAPD)

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Introduction

Longleaf pine possesses many desirable qualities such as excellent timber form, high-naval stores content, moderate to high wood specific gravity, and fusiform rust resistance (USDA 1965). Despite these qualities, complications associated with an extended phase (2–20+years) of juvenile development, referred to as the grass stage, have limited its use in artificial-regeneration programs (Schmidtling and White 1989). During the grass stage, seedlings develop extensive root systems, and apical meristems increase in diameter but not in length (Harlow et al. 1978). The grass stage greatly increases the opportunity for brown-spot needle blight infection [caused by the fungus *Scirrhia acicola* (Dearn.)] (Siggers 1944). This disease can significantly prolong the grass stage and, if severe enough, can kill seedlings. Applications of fungicides to the roots of longleaf pine prior to planting can significantly reduce brown-spot disease and promote early height growth (EHG) (Kais 1975; Kais et al. 1981). Regardless of these efforts, increased seedling mortality (compared to the other southern pines) and the unpredictability of the duration of the grass stage still make planting longleaf pine a risky investment under intensive-management systems.

Inter-specific hybrids of longleaf pine have shown promise for addressing the problem of delayed height growth. Intermediate height growth has been observed in various families of longleaf pine crossed to either loblolly pine or slash pine (Brown 1964; Derr 1966, 1969). Analysis of F_2 and BC_1 hybrids of longleaf pine and loblolly pine yielded an estimate of at least ten loci controlling EHG

[Brown 1964 using methods of Wright (in Castle 1921)]. More recent work with hybrids indicates that there may be fewer than five loci affecting EHG (C. D. Nelson unpublished data, using methods of Lande 1981 and Cockerham 1986). In light of recent theoretical work (Zeng et al. 1990; Zeng 1992) and the fact that current estimates of the number of loci influencing EHG are based on only a few hybrid families, estimates calculated to-date may be too low, but they do suggest that the grass-stage character is a quantitative trait controlled by a finite number of genes (oligogenic vs polygenic).

Recent advances in DNA-based marker technology have made it possible to conduct efficient genetic mapping and quantitative trait loci (QTL)-searching experiments. Both restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNAs (RAPDs) have been used to construct genetic linkage maps for a number of annual crop species (e.g., Bernatzky and Tanksley 1986; Helentjaris et al. 1986; Landry et al. 1987; McCouch et al. 1988; Gebhardt et al. 1989; Keim et al. 1990; Paran et al. 1991), as well as for several perennial tree species (Tulsieram et al. 1992; Faure et al. 1993; Liu and Furnier 1993; Nelson et al. 1993; Hemmat et al. 1994; Nelson et al. 1994). Due to the large number of marker assays required for genetic mapping and QTL-searching experiments, automated approaches afforded by the RAPD technique offer an enormous benefit in terms of time and labor (Grattapaglia et al. 1992; Nelson et al. 1994).

Low- to medium-density RAPD maps have recently been published for several tree species, such as white spruce (Tulsieram et al. 1992), slash pine (Nelson et al. 1993), longleaf pine (Nelson et al. 1994), and apple (Hemmat et al. 1994). In the case of conifers, their unique reproductive biology provides a source of segregating haploid tissue, the megagametophyte (USDA 1974). The megagametophyte is derived from repeated mitotic divisions of a single meiotic product, and has the same maternal genetic complement as the embryo contained in the same seed. Since the megagametophyte is haploid (of maternal origin), segregation and recombination can be evaluated in a sample of seeds from a single tree without the need for controlled pollinations (Guries et al. 1978; Rudin and Ekberg 1978; Conkle 1981). Sufficient DNA for several hundred to several thousand RAPD reactions can be extracted from one megagametophyte, making this an excellent system for evaluating the efficacy of the RAPD technique for constructing genetic linkage maps.

Despite the advantage afforded by the megagametophyte system (uni-parental segregation), this system could potentially be inefficient in terms of QTLs analyses and marker-aided selection (MAS) applications. The genotypic data collected on megagametophytes can be used to search for QTL (of maternal origin) in plants derived from these same seeds (Grattapaglia et al. 1992; Liu et al. 1993). However, the paternal genetic contribution to these plants might potentially confound QTL analyses. The genetic component of an individuals' phenotype results from specific alleles received from both its maternal and paternal parents. If precautions are not taken to control (randomize) the paternal genetic complement (for example, by using polymix pollen sources) this approach to QTL mapping might prove to be mis-leading.

In terms of genetic mapping (and subsequent QTL searching), a more efficient strategy would be to simultaneously map each parent of a specific cross by using their progeny as the mapping population (Carlson et al. 1991; Hemmat et al. 1994). For dominant RAPD markers, the practicality of such an approach is limited by the number of loci found to be in a testcross configuration between parents. Using eight different RAPD primers, Carlson et al. (1991) identified ten loci segregating in at least one of three Douglas-fir F_1 families. Most of these loci (70%) were found to be in a testcross configuration between the parents. Using a total of 64 primers or primer combinations, Hemmat et al. (1994) identified 367 RAPD loci segregating in the F_1 progeny of a single cross between the apple cultivars "White Angel" and "Rome Beauty". Greater than 90% of these loci were in a testcross configuration between the parents. Loci at which both parents were heterozygous (segregating 3:1) were useful in defining homologous counterparts between the two parental maps. These results suggest that the RAPD technique is well-suited to genetic mapping in highly heterozygous outcrossed species.

In the present study, we have used RAPD markers to simultaneously construct linkage maps for the parents of a longleaf pine × slash pine F_1 family. The long-term goal of this research is to employ these markers in a backcross breeding program to accelerate the introgression of positive-effect EHG alleles from slash pine into longleaf pine. In this paper, we present RAPD linkage maps for the parents of an F_1 family and discuss how this information might be used in backcross populations to map EHG loci.

Materials and methods

Plant material

A longleaf pine slash pine inter-specific F_1 family was used as the mapping population. This family was produced by crossing slash pine H-28 (♂) to longleaf pine 3–356 (♀). The longleaf-pine parent had previously been mapped using RAPD markers and the megagametophyte system (Nelson et al. 1994). No mapping information was available for slash pine H-28. Both parents were selected for disease resistance and growth rate at the Southern Institute of Forest Genetics (SIFG) near Gulfport, Mississippi. The cross was completed in the spring of 1990. Seeds were extracted from mature cones in the fall of 1991 and sown into containers in February of 1992. The seedlings were grown in a greenhouse for 4 months and then transplanted to a nursery bed. A total of 98 progeny were available for use in this study.

DNA isolation and purification

Total nucleic acids were isolated from needles as described in Wagner et al. (1987) except that spermine and spermidine were omitted from both the extraction and wash buffers. The RNA component of these extracts was removed by incubation in the presence of RNase A as described in Ausubel et al. (1987). Approximately 2.0 g of DNA was further purified using the Prep-A-Gene DNA Purification Kit (BioRad, Hercules, Calif.) as described by the manufacturer.

Primer Selection and DNA amplification

Primer DNAs were obtained from either Operon Technologies (Alameda, Calif.) or J.E. Carlson (Univ. of British Columbia, Vancouver, B.C., Canada). A total of 288 primers were selected for this study. Of these 288 primers, 102 (35.4%) were chosen because they had been used previously to amplify mappable loci in our longleaf-pine parent (clone 3–356) using the haploid megagametophyte system (Nelson et al. 1994); another 148 primers (51.4%) were chosen because they had previously amplified mappable loci in one of several other pine species (Nelson and Nance, unpublished data). The remaining 38 primers (13.2%) were randomly selected from a set of over 250 additional candidate primers. DNA amplification followed the protocol outlined in Nelson et al. (1994). The only modification consisted of a doubling of the template DNA to 6.25 ng per reaction, to compensate (in theory) for the use of diploid versus haploid material.

Primer screening and marker scoring

To identify primers which amplified polymorphisms, primers were screened against both parents and six F_1 progeny. Three different parent-progeny RAPD banding patterns were scored as putatively polymorphic. When a RAPD band was present in only one parent, and in at least one of the six progeny, the parent was classified as potentially heterozygous for that locus (referred to as testcross loci). RAPD bands which were present in both parents and absent in at least one of the progeny, classified both parents as potentially heterozygous (referred to as 3:1 loci). Bands which were present in only one parent and all of the progeny, tentatively classified each parent as homozygous for alternate alleles (referred to as non-segregating loci). A subset of primers that maximized the number of polymorphisms in a testcross configuration was selected and segregation scored in an additional 80 progeny. The 80 progeny were divided into four template sets (three consisting of 22 progeny each, and a fourth consisting of an additional 14 progeny). Each template set was amplified, along with both parents, on different temperature cyclers. Those polymorphisms which could confidently be scored across all four template sets were included in our analyses. In the case of testcross loci, presence of a band was scored as 'H' (heterozygous) while absence of a band was scored as 'A' (homozygous band absent). Those cases in which the presence or absence of bands was unclear were recorded as missing data.

Marker naming

Each polymorphism was assigned a two-part name according to Nelson et al. (1994). The first part corresponding to the primer with which the polymorphism was amplified (a letter followed by a two-digit number corresponds to an Operon Technologies Inc. primer, and a three-digit number corresponds to a University of British Columbia primer). When only a single polymorphism was amplified by a primer, it was given the letter A. When a primer amplified multiple polymorphisms, the polymorphisms were assigned consecutive letter designations from the smallest-molecular-weight band to the largest (e.g., X19_A, X19_B, X19_C).

Segregation analysis

Each RAPD band was tested for goodness of fit to a 1:1 or 3:1 Mendelian segregation ratio by chi-square (χ^2) analysis (α=0.05). Those loci which appeared to be experiencing segregation distortion were excluded from initial mapping analyses. The testcross data were entered into the computer package MAPMAKER/EXP (version 3.0) and analyzed using a modified backcross format (Nelson et al. 1993). The mapping strategy employed was similar to that suggested in Lincoln et al. (1992). Significant associations between 3:1 loci and testcross loci were determined by χ^2 analysis. By considering only the homozygous band-absent genotypes (–/–) at a 3:1 locus, linkage with a testcross locus was implied if the testcross-locus genotypes deviated significantly from 1:1 in this subset ($P< 0.001$).

Linkage group designations

Linkage groups were assigned three-letter names. The first two letters designate species (Pp=*Pinus palustris*, Pe=*Pinus elliottii*), and the third designates linkage groups in descending size (A=largest). The longleaf-pine linkage groups were assigned names according to Nelson et al. (1994). Linkage groups with letter designations from PpA to PpP are homologous to those identified in Nelson et al. (1994). Longleaf-pine linkage groups which currently show no homology between maps were assigned additional letter designations (PpQ–PpT).

Results

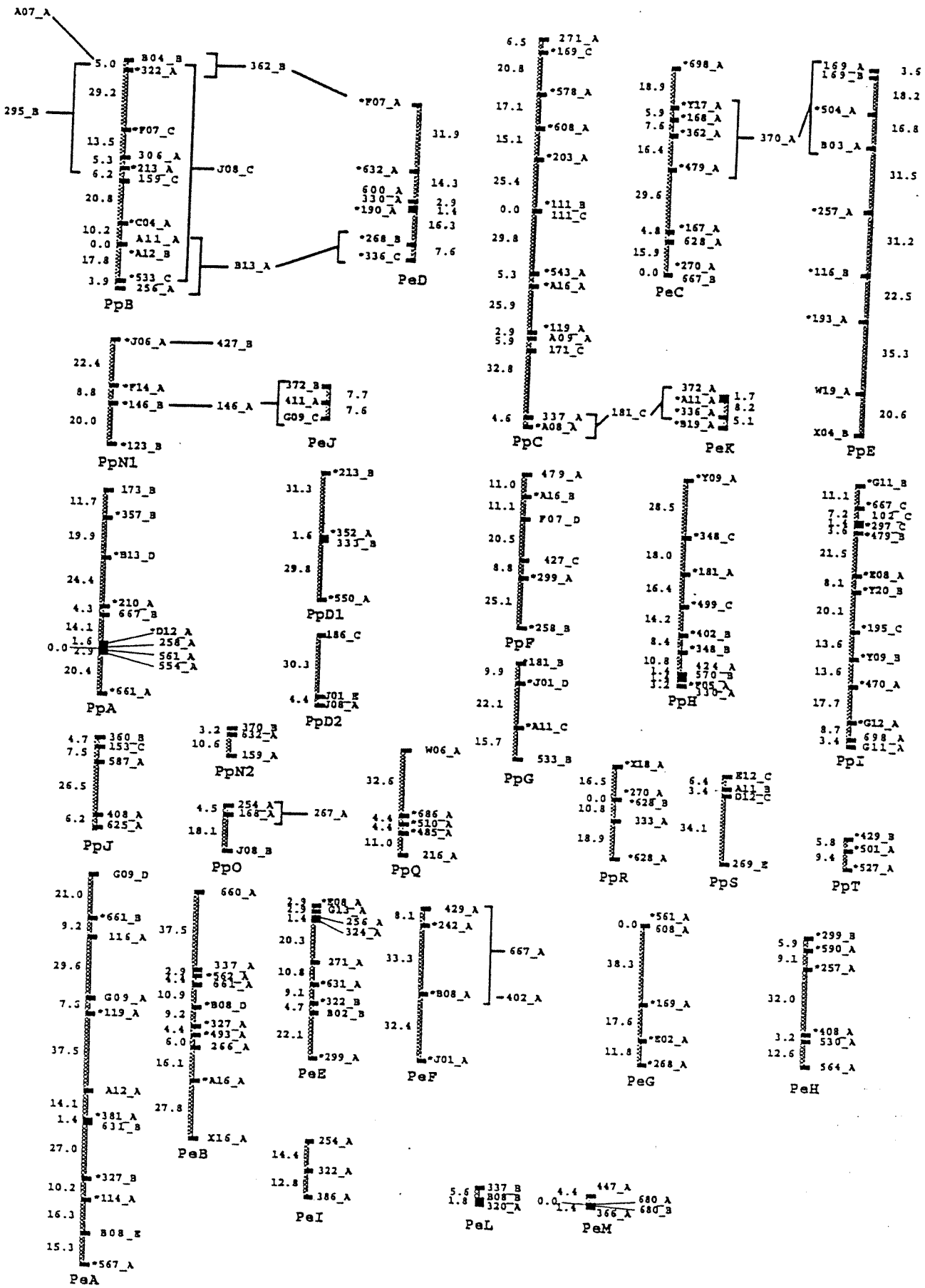
We screened 288 oligonucleotide primers against the parents and six F_1 progeny of a longleaf pine × slash pine cross. Results of primer pre-screening are summarized in Table 1. Of the 288 primers initially screened, 172 primers amplified a total of 318 putatively segregating polymorphisms. Of these 318 polymorphisms, 298 were tentatively classified as testcross loci (possibly segregating 1:1), and the other 20 appeared to be loci heterozygous in both parents (possibly segregating 3:1). Of the 298 putative testcross loci, 167 loci were heterozygous in the longleaf-pine parent and the other 131 were heterozygous in the slash-pine parent. Based on these results, 162 primers were chosen to further characterize these polymorphisms in an additional 66 progeny.

With the 162 pre-screened primers, a total of 281 segregating loci and 87 polymorphic, but non-segregating, loci were scored on an additional four template sets of the mapping population. Of the 281 segregating loci, 267 were in a testcross configuration, and the other 14 were heterozygous in both parents. One hundred fifty-two of the two-hundred sixty-seven testcross loci were heterozygous in the longleaf-pine parent, the other 115 loci were heterozygous in the slash-pine parent. Of the 87 potentially polymorphic, but non-segregating, loci, 43 were unique to the longleaf-pine genome and 44 were unique to the slash-pine genome.

Table 1 Results of pre-screening 288 primers against both parents and six F_1 progeny of a longleaf pine × slash pine cross

Primer group[a]	# Primers		# Putative marker loci
	Screened	Revealing polymorphism	
Longleaf	102	79	162
Other pine	148	80	138
Random	38	13	18
Total	288	172	318

[a] Primer group: longleaf – these primers previously amplified mappable loci in longleaf 3–356; other pine – these primers previously amplified mappable loci in one of several other pine genotypes; random – these primers were randomly selected


PpB
PeD
PeC
PpE
PpN1
PeJ
PpC
PeK
PpD1
PpD2
PpF
PpH
PpI
PpA
PpN2
PpG
PpJ
PpO
PpQ
PpR
PpS
PpT
PeE
PeF
PeG
PeH
PeB
PeA
PeI
PeL
PeM

One-hundred thirteen of the 162 primers chosen to run against the mapping population identified a total of 152 RAPD loci in the longleaf-pine parent (86 primers identified one RAPD locus, 21 identified two loci, and eight identified three loci). Ninety-one primers identified a total of 115 RAPD loci in the slash-pine parent (69 primers identified one RAPD locus, 15 identified two loci, two identified three loci, one identified four loci, and one identified six loci). Thirty-six of the 162 primers identified segregating RAPD loci in both parents.

Chi-square analysis indicated that 132 of the 152 longleaf-pine testcross loci were segregating at the expected 1:1 Mendelian ratio (n=86, α=0.05), as were 101 of the 115 slash-pine testcross loci. Those loci which appeared to be experiencing segregation distortion were excluded from initial mapping analyses. Two-point analysis of the longleaf-pine testcross loci classified 129 of the 132 loci into 18 groups (three or more loci) and three pairs with a linkage criteria of LOD 4.0 and distance of 35 cM. Ninety-one of the 101 slash-pine testcross loci were classified into 13 groups and seven pairs. Orders of loci that were consistent for all three-point tests (LOD=3.0 distance 39 cM) were taken as framework orders. This analysis resulted in the ordering of 64 framework loci within 13 longleaf-pine linkage groups, and 41 framework loci within nine slash-pine linkage groups (see markers with prefix *, Fig. 1). Those loci which were clearly linked to a particular group, but could not be confidently ordered using framework thresholds, were placed in their most likely positions. An additional 52 loci were placed in longleaf pine and 38 in slash pine. Linkage of those loci initially ungrouped (based on two-point analyses), as well as linkage between all possible pairs of groups, was tested. Only one further linkage was suggested. The LOD for linkage between the grouped slash-pine markers (268_A, E02_A, 169_A) and (608_A, 561_A) was 3.1, corresponding to a genetic distance of 38.3 cM (see linkage group PeG, Fig. 1).

Linkage analysis of the 132 testcross loci heterozygous in the longleaf-pine parent suggested a genetic map consisting of 18 groups and three linked pairs (122 markers) spanning a total of 1367.5 cM. The weighted-average distance between markers within the 21-longleaf-pine linkage groups is 13.0 cM (13.6 cM in the groups and 9.3 cM in the pairs). Using methods described in Hulbert et al. (1988), genome-size estimates for longleaf pine were 2373, 2348 and 2392 cM for LOD scores of 2.0, 3.0 and 4.0, respectively. Assuming that each unlinked marker accounts for 30 cM, and that 24 of the 42 ends of our 21 linkage groups and pairs cover true telomeric regions (15 cM/un-

Table 2 Results of chi-square (χ^2) analyses between 3:1 loci and testcross loci. By considering only the homozygous band-absent genotypes (–/–) at a 3:1 locus, linkage with a testcross locus was implied if the testcross-locus genotypes significantly deviated from 1:1 in this subset (P< 0.001)

3:1 loci	Testcross loci	Chi-square	p-value	Linkage group
146_A	146_B	10.7143	~ 0.001	PpN1
146_A	411_A	13.7619	< 0.001	PeJ
146_A	372_B	12.8000	< 0.001	PeJ
146_A	G09_C	12.8000	< 0.001	PeJ
181_C	337_A	11.0000	0.0	PpC
181_C	A08_A	9.8000	< 0.01	PpC
181_C	336_A	18.0000	0.0	PeK
181_C	A11_A	16.2000	< 0.001	PeK
181_C	372_A	15.2105	< 0.001	PeK
267_A	254_A	12.0000	0.0	PpO
267_A	168_A	8.3333	< 0.01	PpO
295_B	322_A	15.0000	0.0	PpB
295_B	A07_B	11.2667	< 0.001	unordered(PpB)
295_B	F07_C	10.2857	< 0.01	PpB
295_B	213_A	9.0000	< 0.01	PpB
362_B	B04_B	22.0000	0.0	PpB
362_B	322_A	15.6957	< 0.001	PpB
362_B	C04_A	15.6957	< 0.001	Pair
362_B	F07_A	12.5652	< 0.001	PeD
370_A	169_B	19.1739	< 0.001	PpE
370_A	B03_A	18.1818	< 0.001	PpE
370_A	169_A	15.6957	< 0.001	PpE
370_A	504_A	14.7273	< 0.001	PpE
370_A	173_B	12.5652	< 0.001	unordered (PpE)
370_A	362_A	21.0000	0.0	PeC
370_A	168_A	15.6957	< 0.001	PeC
370_A	479_A	15.6957	< 0.001	PeC
370_A	Y17_A	12.5652	< 0.001	PeC
402_A	B08_A	11.8421	< 0.001	PeF
427_B	J06_A	11.2667	< 0.001	PpN1
427_B	299_C	16.0000	0.0	Pair
427_B	116_B	11.2667	< 0.001	Pair
667_A	242_A	13.000	0.0	PeF
667_A	429_A	13.0000	0.0	PeF
667_A	B08_A	12.0000	0.0	PeF
A07_A	322_A	11.2667	< 0.001	PpB
B13_B	256_A	16.0000	0.0	PpB
B13_B	533_C	12.2500	< 0.001	PpB
B13_B	590_A	12.2500	< 0.001	unordered(PpB)
B13_B	A11_A	12.2500	< 0.001	PpB
B13_B	336_C	16.0000	0.0	PeD
B13_B	268_B	12.2500	< 0.001	PeD
J08_C	213_A	17.0000	0.0	PpB
J08_C	306_A	17.0000	0.0	PpB
J08_C	F07_C	16.0000	0.0	PpB
J08_C	A11_A	13.2353	< 0.001	PpB
J08_C	322_A	13.2353	< 0.001	PpB
J08_C	533_C	11.2667	< 0.001	PpB
J08_C	B13_B	13.2353	< 0.001	PeP

Fig. 1 Genetic linkage maps of longleaf-pine clone 3–356 (linkage groups Pp_) and slash-pine clone H-28 (linkage groups Pe_). Linkage groups are arranged in descending order (A=largest). Longleaf-pine linkage groups assigned letter designations from PpA to PpP are homologous to those identified in Nelson et al. (1994). Longleaf-pine linkage groups which currently show no homology between maps were assigned additional letter designations (PpQ–PpT). Primer names and Haldane centiMorgan (cM) distances are provided. Framework markers (LOD 3.0, distance 35 cM) are indicated by an *asterisk*. Association of 3:1 loci is indicated by *heavy lines*

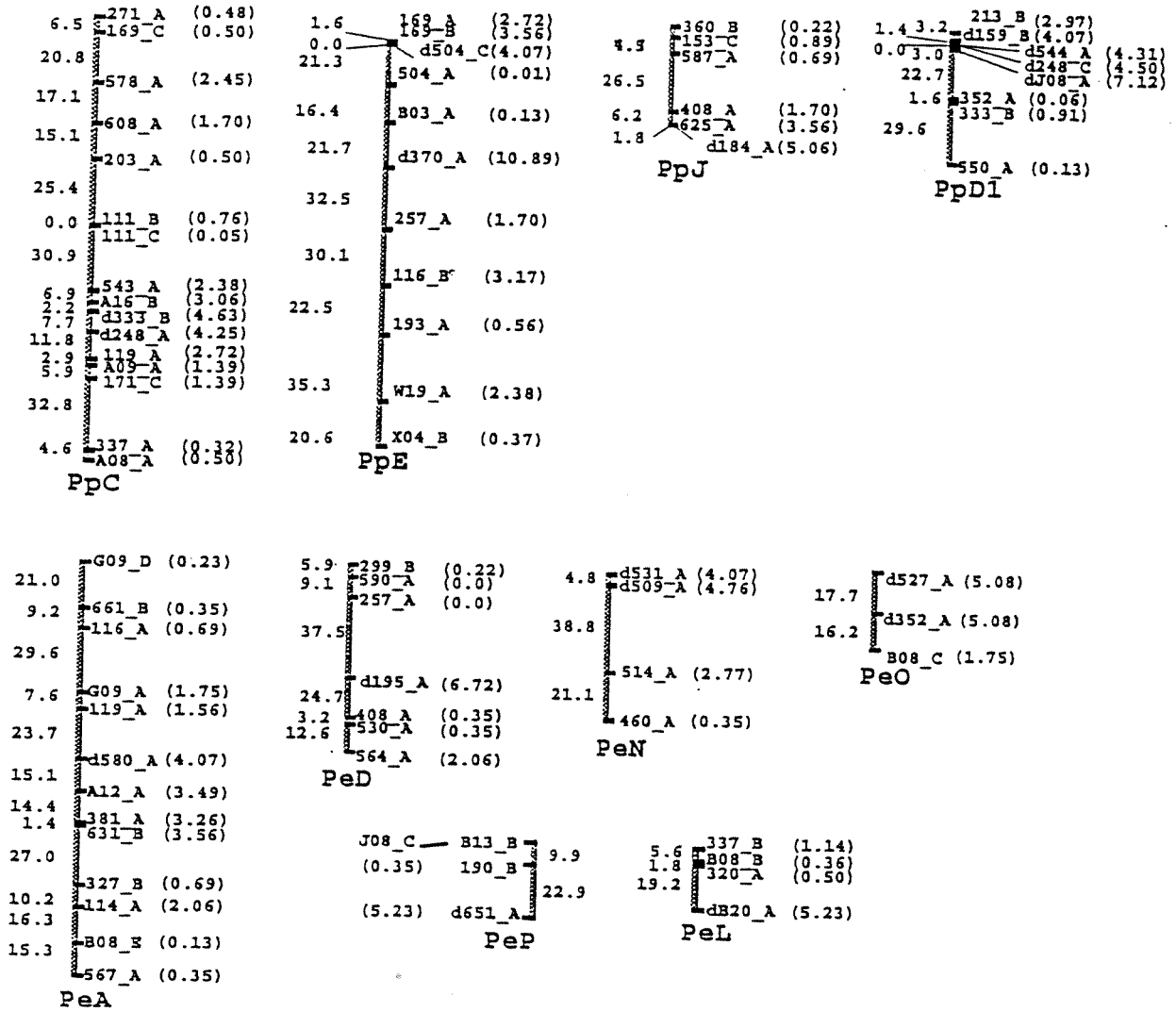


Fig. 2 Revised maps showing linkage of marker loci displaying significant segregation distortion ($\alpha \leq 0.05$). Marker names and Haldane cM distances are provided. Distorted markers are indicated by a prefix *d*. Chi-square (χ^2) values are provided in *parentheses*

accounted end), the total map coverage is estimated at 1937.5 cM or 80.9% of our largest genome-size estimate.

Linkage analysis of the 101 testcross loci identified in the slash-pine parent suggested 13 groups and six linked pairs (91 markers) spanning a total of 952.9 cM, with a weighted-average distance between markers of 16.1 cM (12.9 cM in the groups and 20.2 cM in the pairs). Genome-size estimates for slash pine were 2292, 2342 and 2372 cM for LOD scores of 2.0, 3.0 and 4.0, respectively. Again, using a 30-cM map scale we estimate the total map coverage to be 1462.9 cM (or approximately 61.7% of the slash-pine genome).

Chi-square analyses performed between 3:1 loci and testcross loci suggested a number of significant associations (see Table 2). Most 3:1 loci were found to be associated with more than one testcross locus per linkage group ($P < 0.001$), providing evidence against chance associations. Ten 3:1 loci were found to be associated with testcross loci on five different longleaf-pine linkage groups (see Fig. 1). Seven of the 3:1 loci were also found to be significantly associated with testcross loci on five different slash-pine linkage groups. Those 3:1 loci which showed significant association to markers in both parents were used to infer homologous linkage groups between maps. Six of the ten 3:1 loci which were found to be associated with longleaf-pine testcross loci also suggested significant association to slash-pine testcross loci (refer to Table 2, and Figs. 1 and 2). Four of the longleaf-pine linkage groups appear to be potentially homologous counterparts to five different slash-pine linkage groups (PpB-PeD,PeP; PpC-PeK; PpE-PeC; PpN1-PeJ).

Discussion

The high level of DNA polymorphism detected by the RAPD technique in the genus *Pinus* allowed us to simultaneously construct medium-density linkage maps for the parents of a longleaf pine × slash pine cross using their F_1 progeny as the mapping population. High heterozygosity

in conifers has been documented by several investigators (Conkle 1981; Carlson et al. 1991; Devey et al. 1991; Tulsieram et al. 1992; Gerber et al. 1993; Nelson et al. 1993, 1994), and numerous loci might be expected to be segregating in the progeny of any conifer cross. In this interspecific cross, longleaf pine 3–356 had about 22.3% more heterozygous loci than did slash pine H-28 (166 versus 129 loci, respectively). This excess can be partially explained by the criteria used for initially choosing candidate primers. Approximately 35% of the primers used in this study were chosen because they were already known to amplify heterozygous loci in longleaf pine 3–356 using a segregating haploid population of megagametophytes (Nelson et al. 1994). Preliminary comparisons between the megagametophyte- and F_1-based maps indicates that a number of marker loci were lost in the F_1-based map due to either slash pine H-28 being homozygous band-present for these loci (i.e., not segregating in the progeny), or due to poor amplification of DNA derived from diploid tissues.

The dominant nature of RAPD markers does not appear to present a problem when mapping in highly heterozygous outcrossed species, due to the large number of loci found to be in a testcross configuration between the parents (Carlson et al. 1991; Grattapaglia et al. 1992; Hemmat et al. 1994). Since two classes of loci are identified (one set heterozygous in one of the parents, and a second set heterozygous in the other parent) parent-specific linkage maps are produced. By taking advantage of the information provided by loci heterozygous in both parents (segregating 3:1) it is possible to infer homologous linkage groups between parents (Hemmat et al. 1994). Combining previously parent-specific linkage groups would greatly increase the number of markers associated with specific groups and the overall map, in general. In this study, the number of loci found to be heterozygous in both the longleaf-pine and slash-pine parents was limited (14 of 281 loci, or 5.0%). A similar study in apple (Hemmat et al. 1994) found only 8.7% of the segregating markers to be heterozygous in both parents (39 of 448 loci). The limited number of loci found to be heterozygous in both parents may be an artifact of the rather diverse origin of the parents used in these studies, as a preliminary study involving intra-specific crosses of yellow birch (*Betula alleghaniensis*) found 5 of 14 loci (35.7%) to be heterozygous in both parents (Roy et al. 1992).

Currently, our maps are incomplete, as they include 18 groups and three pairs (122 markers) for longleaf pine and 13 groups and six pairs (91 markers) for slash pine. Based on karyotype analyses, pine species are known to contain 12 (similar-sized) pairs of homologous chromosomes (Saylor 1972; Kormutak 1975). The number of linkage groups identified in each of our maps (approximately 20) is comparable with previously published findings. Using the megagametophyte system, Nelson et al. (1993) mapped 73 RAPD markers to 13 groups and nine pairs in slash pine, and in a similar study involving longleaf pine mapped 133 markers to 16 groups and six pairs (Nelson et al. 1994). Obviously, more markers are required to bridge the gaps between current groupings and expand the coverage towards 100%. Estimates of the number of markers required to obtain 90% coverage of the pine genome (average spacing of 20–30 cM) suggest that approximately 200 to 300 markers will be required (Neale and Williams 1991; Nelson et al. 1993). At the current levels of genomic coverage (60–85%), screening more primers for additional polymorphisms will not be efficient. Approaches such as increasing the sample size for terminal and unlinked markers, or applying bulked segregant analysis (Michelmore et al. 1991) to search for polymorphisms near the remaining terminal and unlinked markers, may prove to be a most efficient means to achieve such ends.

Prior to analyses, we anticipated that selective forces might cause markers to deviate from their expected segregation ratios, most likely as a result of pollen-ovule incompatibility (Richards 1986). There appears to be a lack of consensus among researchers as to whether markers experiencing segregation distortion should be used in linkage studies (Tulsieram et al. 1992; Faure et al. 1993; Nelson et al. 1993). Regardless of the fact that various genetic models (single-locus and multi-locus) have been proposed to explain the existence of segregation distortion (Richards 1986; Lin and Ikehashi 1993; Pham and Bougerol 1993), and that the use of loci whose alleles deviate markedly from their expected ratio has no effect on estimates of recombination (Ott 1991), distorted markers have not, in general, been included in linkage analyses. Our initial mapping efforts focused only on those loci segregating at their expected Mendelian ratios. However, further linkage analyses were performed which included distorted markers. By including distorted markers we hoped to map additional regions of the parental genomes (those experiencing selective drag), possibly allowing us to further converge our maps towards 12 linkage groups. Of the 20 testcross loci in longleaf pine which were not segregating at the expected 1:1 ratio (n=86, α=0.05), nine mapped (see markers with a prefix d in the revised linkage groups, Fig. 2). Likewise, 8 of the 14 slash-pine testcross loci, displaying significant distortion, mapped.

Initial mapping analyses (those excluding distorted markers) identified a total of 21 linkage groups in longleaf pine and 19 linkage groups in slash pine. Including the distorted markers did not allow further convergence of either map. In fact, the number of linkage groups increased from 19 to 22 in slash pine as some of the distorted markers were found to be associated with previously unlinked markers. Inspection of the segregation ratios of markers found to be linked within 10 cM of the distorted markers shows them to be slightly distorted (see χ^2 values, Fig. 2). The apparent gradation in the level of distortion as one moves along a particular linkage group would seem to reinforce the validity of the suggested linkages.

In terms of genetic mapping, QTL searching, and applying genomic selection in BC_1 populations, we plan to focus on primers which amplify more than one heterozygous locus (within a specific parent, or between parents) as these primers would maximize the efficiency of such applications. Results from this study indicate that multiple RAPD loci amplified by the same primer (within a specific

parent) tend to be unlinked. For example, of the 23 primers which amplified more than one heterozygous locus in longleaf pine, five primers amplified loci which mapped to the same linkage group, only two of which were linked at less than 20 cM (loci 111_B-111_C at 0 cM, PpC; loci 169_A-169_B at 3.6 cM, PpE). Of the 19 primers which amplified more than one heterozygous locus in slash pine, only one amplified loci linked at less than 20 cM (680_A-680_B at 0 cM, PeM). These results appear to be similar to those obtained in other studies (Tulsieram et al. 1992; Nelson et al. 1993; Hemmat et al. 1994). Utilization of such "multi-locus" primers would simultaneously maximize the number of informative loci available for linkage applications, while minimizing the total number of RAPD reactions required.

In our mapping population, we found the number of heterozygous loci per F_1 individual to range from a low of 96 to a high of 130. Therefore, we might expect to have segregation information for a maximum of 96–130 mapped loci available for use in BC_1 populations. In addition, assuming that each RAPD band represents a single genetic locus, the 87 polymorphic (between parents), but non-segregating, loci should be heterozygous in the F_1 progeny. Of course, the actual number of loci useful for genetic mapping and QTL searching in BC_1 populations will depend upon the F_1 individual chosen, and the genotype of the recurrent parents. Our plans are to focus on those F_1 individuals (intermediate for EHG) which are heterozygous for a maximum number of marker loci. In order to avoid possible complications which might arise as a result of inbreeding (poor-seed set and reduced seedling-vigor), we plan to use unrelated recurrent parents in our backcross pedigrees (Bernatzky and Mulcahy 1992). Potential recurrent parents will be genotyped at all loci known to be heterozygous in the selected F_1 parent. Those recurrent parents which are homozygous band-absent at the most loci will then be selected.

To identify loci influencing EHG we plan to produce and test two divergent backcross families. In one family, a longleaf pine will be used as the recurrent parent, and in the other family, a slash pine will be used as the recurrent parent. Use of the same F_1 individual as the male parent in each of the backcross families should allow for the identification of EHG loci in the most comparable genetic background (with positive-effect EHG alleles coming from the hybrids' slash-pine parent and negative-effect (grass stage) EHG alleles coming from the hybrids' longleaf-pine parent). By analyzing both backcross families, we expect to find "real" (not false-positive) EHG loci as they should map to the same marker loci in both families.

Successful completion of this research should result in the development of longleaf-pine hybrid genotypes which exhibit vigorous early height growth as a result of their harboring high numbers of positive-effect EHG alleles. These genotypes could be selected for further backcrossing, clonally propagated and used directly in production plantings, or crossed to fix loci influencing EHG. Selected progeny, fixed at EHG loci, could then be used as parents in seed orchards as well as in further backcrosses.

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