RAPD MAPPING OF GENOMIC REGIONS INFLUENCING EARLY HEIGHT GROWTH IN LONGLEAF PINE x SLASH PINE F_1 HYBRIDS

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Abstract: Despite many desirable qualities, longleaf pine has found limited use in artificial regeneration programs because of its often poor survival rate and extended phase (2-20+ yrs) of reduced height growth referred to as the grass stage. Interspecific hybrids of longleaf pine have shown promise for addressing the problem of delayed height growth. Considerable variation in height growth has been observed in various longleaf pine x slash pine hybrid families suggesting that an interspecific backcross breeding approach might be successfully employed to eliminate the grass stage in longleaf pine. Automation of the random amplified polymorphic DNA (RAPD) technique has now made it possible to quickly identify large numbers of markers that can be used to construct genetic linkage maps, tag genes of interest, and accelerate the introgression of genes in backcross breeding programs. The objective of this study was to use RAPD marker maps for longleaf pine and slash pine to obtain preliminary information on the genomic location of regions influencing early height growth (EHG). Seventy-two F_1 trees generated from an interspecific cross between longleaf pine and slash pine were used. Genetic maps were constructed for each parent with markers segregating in a I:1 Bendelian ratio. Both linked and unlinked markers were used to search for genomic regions influencing EHG. Total height was measured six times over four years (3, 5, 9, 2I, 33, and 45 months). Several regions controlling part of the variation for EHG were identified by single marker regression analyses. Based on age by age analyses, some temporal stability of quantitative trait loci (QTL) expression was observed. One genomic region in longleaf pine was significantly associated with EHG at all six measurement dates. These preliminary results are very encouraging for the application of markers to identify genomic regions influencing EHG. They should also prove to be extremely powerful in the backcross populations where we will be using larger progeny sizes and expect more genes to be segregating.

Keywords: *Pinus palustris* *Pinus elliottii* Engelm., interspecific hybrids, grass stage, RAPD markers.

INTRODUCTION

Despite many desirable qualities, longleaf pine *(Pinus palustris* Mill.) has found limited use in artificial regeneration programs due to complications associated with an extended phase of juvenile development referred to as the grass stage (Schmidtling and White 1989). 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 greatly prolong the grass stage

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and, if severe enough, can kill seedlings. Increased mortality (compared to the other southern pines) and the unpredictability of the duration of the grass stage 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 early height growth has been observed in various families of longleaf pine crossed to either loblolly pine or slash pine (Brown 1964; Den I966; I969). Analysis of various F_2 and BC_1 families suggest that as few as 5-10 loci may control early height growth (EHG) [Brown 1964; C. D. Nelson unpublished data]. Although current estimates of the number of loci influencing EHG are based on only a few hybrid families, 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 QTL searching experiments. Automated approaches afforded by the RAPD technique are offering enormous benefits 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 conifer species such as white spruce (Tulsieram et al. 1992), slash pine (Nelson et al. 1993; Kubisiak et al. 1995), longleaf pine (Nelson et al. I994; Kubisiak et al. I995), Norway spruce (Binelli and Bucci I994), and maritime pine (Plomion et al. 1995). The RAPD technique is well-suited to genetic mapping in highly heterozygous outcrossed species.

In the present study, we used RAPD marker linkage maps constructed for the parents of a longleaf pine x slash pine F_1 family (Kubisiak et al. I995) to perform preliminary searches for genomic regions influencing EHG. We present the results of these searches and relate the findings to our backcross breeding program designed to introgress genomic regions promoting EHG from slash pine into longleaf pine.

MATERIALS AND METHODS

Mapping Population. Seventy-two progeny from an interspecific cross between longleaf pine 3-356 (♀) × slash pine H-28 (♂) were employed for genomic mapping and QTL searching. Seeds were germinated and grown in containers in a greenhouse at the Southern Institute of Forest Genetics previously located in Gulfport, MS. At four months of age, the seedlings were transplanted to a nursery bed at the Harrison Experimental Forest in Saucier, MS and grown for another five months. During this period all seedlings were protected from damping-off and brown spot needle blight and given uniform growing conditions. At nine months, the seedlings were out-planted to a field site on the forest and allowed to grow under natural conditions.

Growth Variables. The growth variables measured included; height in containers at three months, height in the nursery bed at five months and nine months, and height in the field at 21 months, 33 months, and 45 months.

DNA Extraction and RAPD Amplification. DNA was isolated and genetic polymorphisms were amplified using the polymerase chain reaction as described in Kubisiak et al. (1995). Segregating

RAPD markers were identified by the manufacturer primer code corresponding to the 10-mer primer responsible for their amplification, followed by a subscript four digit number indicating the approximate band size in base pairs. Those cases in which the presence or absence of bands was unclear, were recorded as missing data.

Segregation Analysis. The marker 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 used was similar to that suggested in Lincoln et al. (1992). Linkage groups were assigned three letter names. The first two letters designate species (Pp = *Pinus palustris,* Pe = *Pinus elliottii),* and the third designates individual linkage groups. The longleaf pine linkage groups were assigned names according to designations proposed in Nelson et al. (1994).

Bolecular Evaluation of Growth Data. The degree of association between the marker loci and the EHG data was investigated by employing single-locus ANOVA models in which the individual marker-genotypes were used as class variables (Keim et al. 1990). An association between a marker and the height growth data was considered significant if the probability of observing a F-value as large as or larger than the observed value was 0.05. This threshold was chosen in an attempt to lower the type II error rate (Jansen I994). The association of any marker found to be significantly associated with EHG for at least one age was further examined across all ages. Temporal examination of association as well as additional linkage information provided by the map were used as criteria for assessing whether a genomic region is associated with EHG.

RESULTS

Longleaf Pine and Slash Pine Linkage Baps. A total of I32 and I0I loci were used to construct recombinational linkage maps for longleaf pine and slash pine, respectively. A total of 122 (92.4%) of the longleaf pine loci were found to be linked at LOD> 4.0. These loci mapped to 18 groups and three pairs spanning a total genetic distance of I367.5 Haldane centiMorgans (cM). The genetic distance between markers ranged from 0.0 cM to 35.3 cM, with an average spacing of 11.2 cM. Of the 101 slash pine loci, 9I (90.1%) were found to be linked at LOD >4.0. These loci mapped to I3 groups and six linked pairs spanning a total genetic distance of 952.9 Haldane cM. The genetic distance between markers ranged from 0.0 cM to 38.3 cM, with an average spacing of I0.5 cM.

Genomic Regions Influencing EHG. The distribution of early height growth at each age was found to approximate a normal distribution (data not shown). The mean and standard deviation of height growth at each age is presented for the F_1 family, as well as for open-pollinated families from each parent (Table I). The mean height growth for the F_1 family was roughly intermediate to the open-pollinated families at all ages, but appears to harbor considerable variation suggesting that some genetic loci influencing EHG might be segregating.

Table 1. Mean and standard deviation of height growth at six different ages for three families ' open-pollinated family from longleaf pine 3-356; open-pollinated family from slash pine H-28; and a full-sib family 3-356 xH-28 . Shown are; family (Family), height growth measure (Variable), number of progenies (N), mean height growth in centimeters (Mean), standard deviation about the mean (Std. Dev.).

Family	Variable'	N	Mean	Std. Dev.
3-356 OP	HT3	133	0.303	0.369
3-356 x H-28		72	7.340	1.523
H-28 OP		82	14.183	2.177
3-356 OP		132	1.545	0.695
3-356 x H-28		72	7.625	1.819
H-28 OP		82	25.963	3.766
3-356 OP	HT9	133	3.481	1.535
3-356 x H-28		72	24.153	7.380
H-28 OP		82	59.268	13.075
3-356 OP	HT21	119	7.134	2.724
3-356 x H-28		70	36.614	12.210
H-28 OP		82	80.524	18.363
3-356 OP	HT33	no	37.764	45.788
3-356 x H-28		57	151.386	69.345
H-28 OP		82	221.489	42.155
3-356 OP	HT45	97	74.536	90.224
3-356 x H-28		54	253.315	93.832
H-28 OP		81	339.160	38.622

[1]HT3=height at three months; HT5=height at five months; HT9=height at nine months; HT21= height at 21 months; HT33= height at 33 months; HT45=height at 45 months.

A total of 24 markers located on eight different longleaf pine linkage groups were found to be significantly associated (Prob.>F<0.05) with height growth for at least one age based on single-marker ANOVAs (Table 2). Sixteen of these markers were associated with EHG at only one age and had no apparent pattern of temporal expression. Six markers located on linkage group PpC were significantly associated with EHG at more than one age. Marker A09_I300 was significantly associated with EHG at all six ages. At marker A09_I300, the band-present allele was associated with reduced height growth. By 45 months of age, individuals possessing one copy of this allele were an average of 57.5 cm (0.6I phenotypic standard deviations) shorter than individuals possessing the alternate null allele (Table 3). Two markers (102_0550 and 297_0950) located on linkage group PpI were associated with EHG only at 45 months, however, both clearly exhibit an increasing trend in significance through time suggesting that a region on linkage group PpI may influence EHG. At marker 1020550, the band-present allele from longleaf pine was associated with reduced height growth. By 45 months of age, individuals possessing one copy of this allele were an average of 56.7 cm (0.60 phenotypic standard deviations) shorter than individuals possessing the alternate null allele (Table 3).

Table 2. Longleaf pine markers significantly associated with early height growth (Prob.>F<0.05) for at least one date based on single marker analysis of variance. Shown are; marker (Marker), linkage group (LG), marker order in the linkage group (MO), height at three months (HT3), height at five months (HT5), height at nine months (HT9), height at 21 months (1-1121), height at 33 months (HT33), height at 45 months (HT45).

			Prob.>F					
Marker	LG[1]	MO[2]	HT3	HT5	HT9	HT21	HT33	HT45
322_0525	PpB	NA	0.161	0.591	0.166	0.277	**0.048**	0.186
169_1500	PpC	1	0.083	**0.019**	0.568	0.645	0.411	0.649
608_0600	PpC	2	0.268	**0.042**	0.976	0.681	0.164	0.108
111_0850	PpC	3	**0.020**	0.391	0.054	0.086	0.714	0.778
111_0900	PpC	4	**0.020**	0.391	0.054	0.086	0.714	0.778
5430725	PpC	5	**0.003**	0.023	**0.018**	0.053	0.436	0.642
A16_1100	PpC	6	**0.007**	**0.010**	**0.024**	0.051	0.145	0.215
3330650	PpC	7	**0.003**	**0.008**	**0.011**	**0.028**	0.259	0.222
119_0525	PpC	8	**0.046**	**0.013**	0.108	**0.044**	0.071	0.057
A09_1300	PpC	9	**0.041**	**0.008**	**0.049**	**0.019**	**0.039**	**0.025**
171_1500	PpC	10	0.124	0.059	0.077	**0.012**	0.062	**0.043**
A080900	PpC	11	0.138	0.140	0.151	**0.022**	0.173	0.211
159_0550	PpD		**0.036**	0.883	0.959	0.943	0.322	0.454
544_1700	PpD	2	**0.019**	0.912	0.431	0.772	0.652	0.916
J08_0700	PpD	3	**0.048**	0.789	0.453	0.834	0.467	0.959
W19_0600	PpE	NA	0.755	0.325	0.293	0.056	0.082	**0.025**
257_1000	PpE	NA	**0.019**	0.149	0.205	0.084	0.498	0.769
A11_1400	PpG	NA	0.278	0.811	**0.021**	0.095	0.779	0.653
499_0800	PpH	NA	**0.025**	0.112	0.780	0.863	0.707	0.941
X04_1000	PpI	1	0.511	0.369	**0.010**	0.134	0.170	0.217
1020550	PpI	2	0.755	0.325	0.293	0.056	0.082	0.025
297_0950	PpI	3	0.981	0.408	0.270	0.054	0.108	**0.040**
1230525	PpN1	NA	0.752	0.161	0.093	**0.035**	0.208	0.340
360_1100	UL	NA	**0.036**	0.206	0.849	0.533	0.114	0.343

[1]LG=linkage group; UL=unlinked
[2]MO--marker order; NA=not applicable

Table 3. Mean height growth at 45 months of age for individuals harboring alternative marker genotypes. Shown are; species, marker, marker genotype (Genotype), number of individuals with particular marker genotype (N), mean height at 45 months of age for individuals with particular marker genotype (HT45), standard deviation (Std. Dev.).

Species	Marker	Genotype'	N	1-1145 (cm)	Std. Dev.
Longleaf pine	A09_1300	a	32	276.75	89.48
		A	22	219.23	91.35
	102_0550	b	29	279.55	79.03
		B	25	222.88	101.78
Slash pine	452_1700	c	25	285.76	80.24
		C	23	232.52	101.72
	X04_0550	D	25	290.08	60.38
		d	29	221.62	106.27

'a = null allele at marker A09_1300 associated with increased height growth
b = null allele at marker 102_0550 associated with increased height growth
c = null allele at marker 452_1700 associated with increased height growth
D = band-present allele at marker X04_0550 associated with increased height growth

A total of I5 markers located on six different slash pine linkage groups were found to be significantly associated (Prob.>F.<0.05) with height growth for at least one age based on single-marker ANOVAs (Table 4). Ten of these markers were associated with EHG at only one age and had no apparent pattern of temporal expression. Two markers (E02_0700 and 268_I200) located on linkage group PeG were significantly associated with EHG at 3 and 5 months, however, there effect diminished once the trees were put into the field. An unlinked marker (638_0330) was significantly associated with EHG at 9 and 2I months, however, no association was noted at 33 or 45 months. Two markers 452_I700 and X04 0550 showed trends of an increasing association through time. Barker 452_I700 is one member of a linked pair. At this marker, the null allele was associated with increased height growth. By 45 months of age, individuals possessing one copy of this allele were an average of 53.24 cm (0.57 phenotypic standard deviations) taller than individuals possessing the alternate band-present allele (Table 3). Marker X04_0550 is an unlinked marker. The band-present allele at this locus was associated with increased height growth. By 45 months of age, individuals possessing one band-present allele were an average of 68.46 cm (0.73 phenotypic standard deviations) taller than individuals possessing one copy of the alternate null allele (Table 3).

Table 4. Slash pine markers significantly associated with early height growth (Prob.>F<0.05) for at least one date based on single marker analysis of variance. Shown are; linkage group (LG), marker order (MO), height at three months (HT3), height at five months (HT5), height at nine months (HT9), height at 21 months (HT21), height at 33 months (HT33), height at 45 months (HT45).

			Prob .>F					
Marker	LG[1]	MO[2]	HT3	HT5	HT9	HT21	HT33	HT45
6600400	PeB	NA	0.986	0.974	0.279	0.053	**0.016**	0.053
698_2200	PeC	NA	**0.025**	0.119	0.404	0.767	0.547	0.286
631_0800	PeE	NA	0.202	**0.024**	0.543	0.260	0.314	0.962
E02_0700	PeG	NA	**0.048**	**0.003**	0.764	0.156	0.597	0.906
268_1200	PeG	NA	**0.012**	**0.002**	0.855	0.502	0.888	0.590
G09_0750	PeJ	NA	**0.009**	0.421	0.202	0.146	0.646	0.524
460_0600	PeN	NA	0.224	0.366	0.128	0.486	0.230	**0.031**
485_1100	LP	NA	0.222	0.393	0.142	0.366	0.263	**0.032**
452_1700	LP	NA	0.943	0.568	0.297	0.019	**0.014**	**0.049**
299_1300	LP	NA	0.793	0.936	0.673	0.754	0.342	**0.026**
X04_0550	UL	NA	0.365	0.391	**0.014**	0.003	**0.008**	**0.006**
G090500	UL	NA	0.129	**0.040**	0.454	0.277	0.066	0.103
B08_2200	UL	NA	**0.023**	0.145	0.489	0.924	0.617	0.456
638_0330	UL	NA	0.187	0.146	**0.021**	0.004	0.295	0.634
314_0950	UL	NA	0.486	0.095	0.076	0.047	0.150	0.183

[1]LG=linkage group; LP=linked pair; UL=unlinked
[2]MO-marker order; NA not applicable

For the four marker loci A09_I300, 102_0550, 452 _ 1700 and X04_0550 the mean and standard deviation of height growth for individuals possessing zero, one, two, three, or four positive-effect height growth alleles are displayed in Table 5. Individuals possessing four positive effect height growth alleles were an average of 152.3 cm (1.62 phenotypic standard deviations) taller than individuals harboring no positive-effect alleles. At 45 months of age, four of the eight shortest trees and four of the eight tallest trees could be identified by alleles at these four loci (data not shown). The other four individuals in each tail of the distribution could be

identified by alleles at three of these loci, and were most likely recombinants at the fourth locus.

Table 5. Mean height growth at 45 months of age for individuals harboring different numbers of putative alleles for early height growth (EHG) based on molecular marker genotypes: Shown are ; marker genotype (Genotype), number of positive-effect EHG alleles (NPA); number of individuals with particular marker genotype (N), mean height at 45 months of age for individuals with particular marker genotype (HT45), standard deviation (Std. Dev.).

Genotype	NPA	N	1-1T45 (cm)	Std. Dev.
a,b,c,D	4	8	317.63	52.47
a,b,c,d	3	11	297.45	52.81
a,b,C,D				
a,B,c,D				
A,b,c,D				
a,b,C,d	2	16	266.62	95.14
a,B,c,d				
A,b,c,d				
a,b,C,D				
A,b,C,D				
A,B,c,D				
a,B,C,d	1	7	203.00	101.69
A,B,C,D				
A,b,C,d				
A,B,c,d				
A,B,C,d	0	6	165.33	101.91

'a = null allele at marker A09_1300 associated with increased height growth
b = null allele at marker 102_0550 associated with increased height growth
c = null allele at marker 452_1700 associated with increased height growth
D = band-present allele at marker X04 0550 associated with increased height growth

DISCUSSION

In the present study, we utilized recombinational linkage maps constructed for the parents of a longleaf pine x slash pine F_1 cross to search for genomic regions influencing (EHG). Using the molecular marker and field data we identified two regions on the longleaf pine genome and two regions on the slash pine genome that putatively influence EHG. The fact that RAPD markers segregating in a 1:I Mendelian fashion provide linkage information for only a single parent (Grattapaglia et al. 1992) precluded our ability to determine whether the genomic regions identified in each parental species are located on homologous chromosomes.

Another drawback associated with the dominant nature of RAPD markers is when the null allele is associated with the allele of interest. For example, the null allele for slash pine marker 452_I700 was associated with increased height growth. If we were to select for the null allele at this locus in our BCC populations we might erroneously be selecting for the null allele inherited from longleaf pine, which may or may not be associated with the allele of interest for our trait. Some ways around this potential problem might be to convert these RAPD markers into codominant sequence characterized amplified region markers, or to perform bulked

segregant analysis on these markers to try to saturate these regions with other types of codominant markers such as microsatellites or restriction fragment length polymorphisms. Codominant markers might allow us to select for parent-specific alleles and could potentially bypass the problems associated with the null RAPD allele.

It should be stated that these results are only preliminary. The power of these analyses was limited by the rather small number of progeny available. These results have, however, brought to our attention several regions in longleaf pine and slash pine that should be given further attention in our BC1 populations.

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