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A partial genetic linkage map of slash pine (*Pinus elliottii* Engelm. var. *elliottii*) based on random amplified polymorphic DNAs

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Abstract. A set of 420 random, 10-base, oligonucleotide primers was screened for random amplified polymorphic DNA (RAPD) fragments within a sample of eight megagametophyte DNAs of a single slash pine (*Pinus elliottii* Engelm. var. *elliottii*) tree. The apparently repeatable RAPD fragments were further characterized within a sample of 68 megagametophytes from the same tree. Fragments segregating in a 1:1, present-to-absent, ratio were classified and mapped using multi-point linkage analysis. The analysis revealed 13 linkage groups of at least three loci, ranging in size from 28 to 68 cM, and nine linked pairs of loci. The 22 groups and pairs included 73 RAPD markers and covered a genetic map distance of approximately 782 cM. Genome size estimates, based on linkage data, ranged from 2880 to 3360 cM. Using a 30-cM map scale and including the 24 unlinked markers and the ends of the 13 linkage groups and nine linked pairs, the set of RAPD markers accounts for approximately 2160 cM or 64–75% of the genome. This extent of genomic coverage should allow for the efficient mapping of genes responsible for a reaction to the causal agent of fusiform rust disease, *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme*.

Key words: *Pinus elliottii* var. *elliottii* – Megagametophyte – Random amplified polymorphic DNA (RAPD) – Genetic linkage map – *Cronartium quercuum* f. sp. *fusiforme*

Introduction

Genetic markers have many potential applications in forest genetics and tree breeding research. These range from studies of linkage disequilibrium, genetic diversity, population structure, phylogeny, and mating systems – including natural selection, and paternity and linkage analyses – to quantitative trait loci (QTL) dissection and marker-assisted selection (MAS) (Adams 1981; Soller and Beckmann 1983; Tanksley 1983; Patterson et al. 1988). Some applications require linked markers, while others do not (Weir 1991). In either case genetic maps are beneficial since they allow the proper choice of markers, linked or unlinked, for any particular study. In QTL analyses and MAS applications, linkage maps are required for maximum efficiency, because the marker intervals (i.e., haplotypes) are evaluated for their effect on the expression of the trait-of-interest (Geldermann 1975; Lander and Botstein 1989; Lande and Thompson 1990).

Genetic marker systems and linkage maps in pines are limited. Conkle (1981) reported on linkages between 43 allozyme markers in five pine species (*Pinus* spp.). The linkage maps contained 1–5 linkage groups with 2–10 loci per group. The largest of these maps covered 200 cM. Using two-dimensional polyacrylamide-gel electrophoresis (2D PAGE), Bahrman and Damerval (1989) analyzed the segregation of 119 protein markers in *P. pinaster* Ait. Their linkage analysis placed the markers into 12 linkage groups, with a total genetic distance of 420 cM.

Restriction fragment length polymorphism (RFLP) and RAPD genetic marker systems promise to be many times more powerful at revealing genetic polymorphism than protein-based systems (Botstein et al. 1980; Welsh and McClelland 1990; Williams et al. 1990). RAPDs appear to be well suited for genetic mapping in coniferous tree species. Carlson et al. (1991) readily found heritable variation of RAPD fragments in the progeny of six Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] trees. Most (approximately 70%) of the Mendelian segregating

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fragments occurred in the desirable test cross (A/a × a/a) condition and, in any one cross, half were heterozygous in one parent and haf in the other. Thus, heterozygous loci in each parent could be mapped using a single cross of the two.

An alternative approach to mapping in conifers is to utilize megagametophytes of a single tree as a mapping population (Conkle 1981; Bahrman and Damerval 1989). Megagametophytes, the haploid nutritive tissue in conifer seeds, result from mitotic divisions of a single product of a meiotic division. Haploid tissue is very useful since it also allows dominant markers, such as RAPD fragments, to be scored as co-dominant and their unambiguous assignment of linkage phase in the offspring of phase-unknown parents (Raeder and Broda 1986; Hulbert et al. 1988). Thus, the use of RAPD markers in haploid tissue circumvents many of the problems normally associated with mapping when using individuals from natural populations. Recently, Tulsieram et al. (1992) employed this technique to map 61 RAPD markers in white spruce [*Picea glauca* (Moench) Voss]. Using LOD scores of 4.0 and greater, and recombination distances of 40% and less, 47 markers were mapped to 12 linkage groups, spanning a total genetic distance of 874 cM.

In the present study we have tested the applicability of RAPDs to genetic mapping using megagametophytes of a slash pine tree. Slash pine is native to the southeast USA, but is known world-wide as an important timber-, fiber-, and resin-producing species. Breeding objectives in the USA have emphasized selection for resistance to fusiform rust disease, caused by the obligate biotrophic fungus *Cronartium quercuum* f. sp. *fusiforme*. The durability of the resistance attained is unknown, but some studies have indicated strong adaptation of the fungus to selected host families (Snow et al. 1975). Artificial inoculation studies have suggested the presence of specific genetic factors conditioning a typically resistant response in pine to the development of fusiform rust disease (Griggs and Walkinshaw 1982; Kinloch and Walkinshaw 1991; CD Nelson, RL Doudrick, WL Nance, unpublished data). Identification of linked markers to these genetic factors should greatly expand our knowledge of the genetics of this host-pathogen association. Towards this end, we have identified and mapped over 100 polymorphic RAPD fragments in a tree hypothesized to be homozygous resistant and susceptible at two loci that condition reaction to single-spore-derived cultures of *C. q. fusiforme*.

Materials and methods

DNA extraction

Megagametophytes of slash pine clone 8–7 were dissected from wind-pollinated seeds. Total DNA was prepared from individual megagametophytes with a modified SDS extraction (Geisen 1990; Lee and Taylor 1990; Doudrick et al. 1993). Briefly, the tissue was ground in 200 µl of grinding/lysis buffer (50 mM Tris-HCL pH 7.2, 50 mM EDTA pH 8.0, 3% SDS, 1% 2-mercaptoethanol) and diatomaceous earth with a high speed drill-driven Pellet Pestle* (*Owens-Illinois). The resulting slurry was incubated at 65 °C for 1 h, extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and re-extracted with an equal volume of chloroform:1-octanol (24:1). The nucleic acids were precipitated with an 0.1 final vol of 3.0 M sodium acetate pH 5.2 and 2.5 vol of absolute ethanol, incubated at −70 °C for 30 min, pelleted by centrifugation, rinsed with 70% ethanol and dried under vacuum. The dried nucleic acids were suspended in 100 µl of TE buffer (10 mM Tris-HCL pH 7.6, 0.1 mM EDTA pH 8.0) containing 5 µl of RNAse A (10 mg/ml), incubated at 37 °C for 2 h, and finally re-precipitated and re-suspended in 25 µl of TE buffer. This protocol typically yielded 5 µg of DNA per megagametophyte (range 3–7 µg).

RAPD procedure

RAPD reactions were based on the protocol proposed by Williams et al. (1991), with modifications suggested by R. R. Sederoff (North Carolina State University). The reaction consisted of the following in a 15-µl total volume: either 1.5 or 3.0 ng of template DNA, 5 pmoles of primer DNA (0.33 µM), 3 nmoles of each dNTP (200 µM each) (Promega), 1.5 µl of 10 × Taq DNA polymerase reaction buffer (Boehringer-Mannheim) (100 mM Tris-HCL pH 8.3, 500 mM KCL, 15 mM $MgCl_2$, 0.01% gelatin), and 0.75 U of Taq DNA polymerase (Boehringer-Mannheim). The reactions were loaded in flexible microtitre plates (Becton-Dickinson), overlaid with 50 µl of mineral oil, covered with mylar film and temperature cycled in a programmable temperature cycler (MJ Research PTC-100). The temperature profile was 5 s at 95 °C, 1 min 55 s at 92 °C; followed by 45 cycles of 5 s at 95 °C, 55 s at 92 °C, 1 min at 35 °C, 2 min at 72 °C; followed by 7 min at 72 °C, and ending with an indefinite hold at 4 °C. The completed RAPD reactions were electrophoresed in 2% agarose gels and TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM EDTA, glacial acetic acid to pH 7.2) for approximately 3.5 h at 3 V/cm (150 V), stained with ethidium bromide, washed in water, and visualized and photographed over UV light.

All primers used in this study were random sequence, 10-base, oligonucleotide primers with C + G contents ranging from 50 to 80%. Two-hundred and twenty were obtained from Operon Technologies (Oligo Sets A, B, C, D, E, F, G, J, W, X, and Y). An additional 200 primers (numbers 101–300) were obtained from J. E. Carlson of the University of British Columbia. All 420 primers were initially screened with a sample of eight megagametophytes of clone 8–7. Primers showing amplification and potential polymorphism were re-screened with the same set of at least four megagametophytes to check for repeatability. Primers producing at least one repeatable polymorphic fragment were chosen for the mapping phase, which involved assaying each primer against a set of 68 megagametophytes of clone 8-7. During the course of the mapping experiments several opportunities arose to inspect replicate reactions of primers and megagametophytes. Fragments showing signs of non-repeatability at these points were discarded from further analysis.

Linkage analysis

Polymorphic fragments were scored as + for present, − −/ + + for absent, and 0 for missing (i.e., failed or unscorable reaction) in the mapping sample. The RAPD fragments were tested for a 1:1 segregation ratio using chi-square tests. All fragments with an α-value greater than 0.05 were classified as

heterozygous genetic markers and used in the linkage analysis. Multi-point linkage analysis was performed with MAPMAKER II version 1.9 (Lander et al. 1987) using a modified backcross data file. The data for each marker were coded naturally (i.e., + for fragment present and − for absent) and inversely (i.e., − for present and + for absent). Prior to linkage analysis, the + and − codes were converted to H (heterozygote) and A (homozygote) allowing MAPMAKER to compute likelihoods and LOD scores for backcross data and to correctly identify linkages between markers in both coupling (HH/AA) and repulsion (HA/AH) phases.

Initial linkage groups were found using all possible two-point recombination estimates with a minimum LOD score of 4.0 and a maximum centiMorgan (cM, Haldane function) distance of 25. Three-point and multi-point analyses were then used to find framework orders for each group. For this we used a LOD of 3.0, a distance of 25 cM, and an exclusion LOD of 3.0. The remaining loci in each group were then placed if a position at least 50 times more likely than the next most likely position could be found. All loci not belonging to a linkage group were then tested for linkage to the framework groups with the linkage criteria relaxed to a LOD of 4.0 and a distance 30 cM. Finally the likelihoods of the ordered linkage groups were tested against the likelihoods of all maps obtainable by permuting the orders of all adjacent triplets.

Genome size estimates were calculated using a method-of-moments estimator, $G(Z) = [M*M-1)/2]*2*X(Z)/K(Z)$ (Hulbert et al. 1988; method 3 in Chakravarti et al. 1991), where for a given LOD score, Z, G(Z) is the genome size in cM, M is the number of markers analyzed, X(Z) = the maximum cM distance between linked markers, and K(Z) is the number of linkages. Two-point linkages at LOD values of 2.0, 3.0 and 4.0 between the 104 distinct loci were determined and used to provide three different estimates. The maximum of these estimates was used in further calculations of genome coverage.

Results

For the 420 primers screened, 66 were initially found useful in revealing polymorphisms between megagametophytes of clone 8-7. With these 66 primers, 156 fragments were scored from approximately two-thirds of the mapping population. Thirty-three of these fragments were classified as unreliable, and omitted from further analysis. Chi-square analysis indicated that 12 of the remaining 123 fragments were not segregating 1:1 for presence-to-absence. The 111 RAPD fragments that were apparently segregating 1:1 are listed in Table 1. These fragments were produced by 64 primers. Thirty-three primers produced one polymorphic fragment, twenty produced two, seven produced three, three produced four, and one produced five. Fragments sizes ranged from 275 to 1650 base pairs. Fig. 1 and 2 show a representative sample of stained gels from the screening and mapping phases, respectively.

Five pairs and one triplet-set of markers did not recombine within the mapping population. Three of the pairs were produced by the same primers and the fragments were of similar size. Different primers were involved with the other two pairs and the triplet. Omitting one marker of the pair and two markers of the triplet resulted in 104 unique loci suitable for linkage analysis and genome size estimation.

Using two-point data 60 markers were classified into 13 linkage groups with linkage criteria of LOD 4.0 and distance 25 cM. Three-point analyses were then performed for each group. Orders of markers that were consistent for all three-point tests with LOD 3.0, distance 25 cM, and an exclusion threshold LOD of 3.0, were taken as framework orders. This resulted in the positioning of 47 markers within the 13 groups. Potential positions for the remaining 13 markers within the groups

Table 1. Linkage group association of 111 RAPDs in slash pine clone 8-7

Group A	Group B	Group C	Group D	Group E
195 1500	114 1200	116 0375	159 0550	154 0550
225 0450	133 0750	124 0750	213 1300	E14 1050
254 0900	266 1200	254 0800	219 0275	W06 0350
B08 0800	E09 0525	295 0325	A11 0550	X11 0500
G03 1150	G02 0650	297 0300	E17 0450	
X04 0450	G09 0750	B05 0450	G09 0500	
		B05 0475		
		E09 1175		
		E12 0500		
		E12 0600		

Group F	Group G	Group H	Group I	Group J
112 0475	106 0700	269 0450	203 0470	241 0600
159 1000	116 0875	A11 1250	213 0900	270 1200
219 0600	119 0575	G12 0475	266 0600	E14 0875
241 0700	133 0600	X04 0775		E19 0400
268 1650	G12 1100			F05 0475
E19 0525	G14 0600			
F07 0900				
W06 0575				

Group K	Group L	Group K	Linked pairs
159 0400	190 0600	254 0350	119 0800 – 255 0275
285 0625	213 0725	G12 1200	213 0950 – E19 0850
A16 0550	239 0650	W07 0700	A09 1300 – C06 0300
			C05 1600 – E09 0800
			B16 0700 – G10 0475
			159 0625 – C05 0900
			195 0750 – B11 0600
			F08 1000 – W07 0350
			193 0400 – B09 0450
			114 0570 – 114 0580
			116 0675 – A08 0350

Not linked

114 1250	167 1000	A16 0600	C04 0650	G05 0800
129 1000	268 1300	A16 0850	C06 0750	G13 0550
129 1100	269 1000	B04 1300	C06 1000	
146 0700	270 0400	B08 0850	C06 1200	
159 0350	A11 0525	B17 0575	D15 0700	

Note: RAPDs are denoted by the primer used and the approximate length (in bp) of the polymorphic fragment

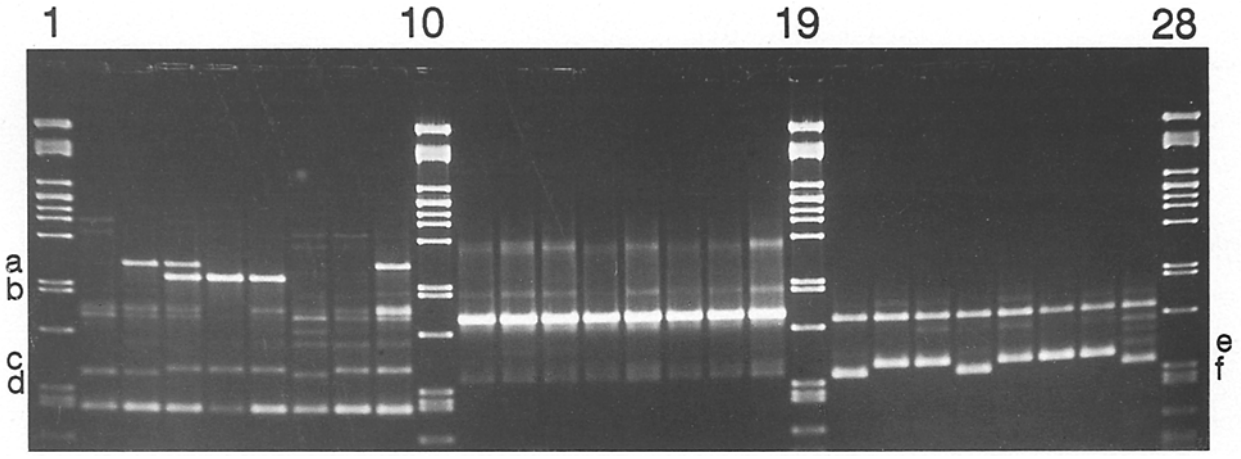

Fig. 1. Visualization of RAPDs produced by three 10-base oligonucleotide primers, 114, 235, and E12. Eight megagametophyte template DNAs from clone 8-7 were amplified with each primer. Primer 114 is in *lanes 2–9*, primer 235 is in *lanes 11–18*, and primer E12 is in *lanes 20–27*. Four RAPD fragments can be visualized with primer 114 (*a, b, c, and d*), none with 235, and two with E12 (*e and f*). The lower molecular weight RAPD fragments produced by primer 114 (*c and d*) and the RAPD fragments produced by primer E12 (*e and f*) appear to be co-dominant markers (observed as mutually exclusive fragments). The larger molecular weight RAPD fragments produced by primer 114 (*a and b*) are independent, dominant markers. Molecular weight size standard, lambda phage DNA (0.6 μg, New England Biolabs)/*Pst*I (I.B.I.) digest, is in *lanes 1, 10, 19, 28*

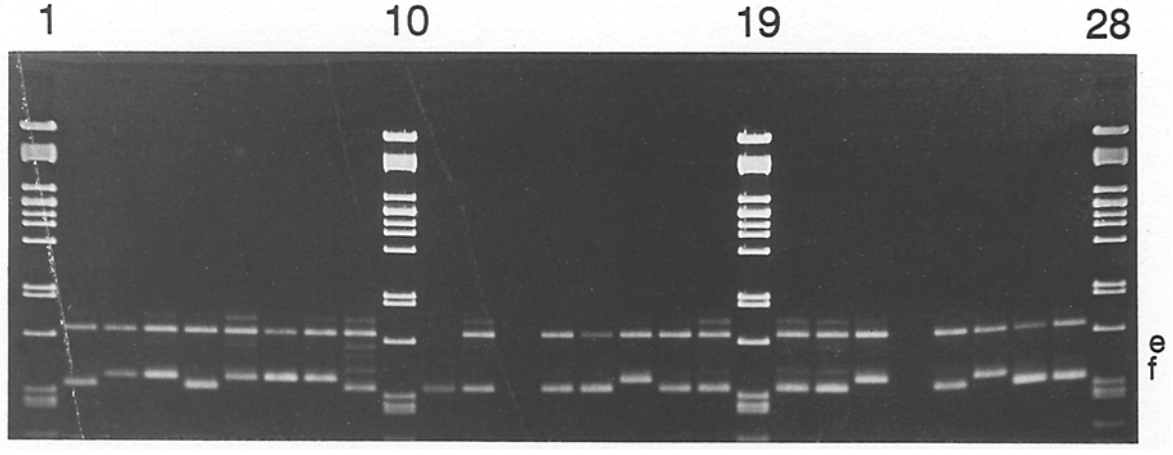

Fig. 2. Visualization of RAPDs produced by 10-base oligonucleotide primer E12. Primer E12 was used to amplify 24 megagametophyte templates from clone 8-7. The first eight megagametophytes (*lanes 2–9*) are the same eight used in the screening-phase gel shown in Fig. 1. *Lanes 13 and 23* are failed reactions. Molecular weight size standards are the same as in Fig. 1

were then tested. Positions that were 50 times more likely than the next best position were accepted. Six markers were positioned, while seven markers were found to have two equally-likely positions. The remaining 44 markers were then tested for linkage to the 13 groups with linkage criteria of LOD 4.0 and distance 30 cM. Two markers met these criteria. Three- and multi-point analyses were then used to order the enlarged groups. Two-point analysis of the remaining 42 loci resulted in nine pairs of linked markers with LOD 4.0 and distance 25 cM, and 24 unlinked markers.

Figure 3 presents the partial genetic linkage map of slash pine clone 8-7. Thirteen linkage groups consist of three or more RAPD markers and nine groups contain linked pairs of markers only. These 22 linkage groups and pairs contain 73 mapped loci and cover approximately 782 cM of map distance. Using a 30 cM map scale, i.e., adding 15 cM for each end of each group and 30 cM for each unlinked marker, we can account for 2160 cM with all 104 loci. The weighted-average distance between markers within the 22 linkage groups is 15.2 cM (15.6 cM in the groups and 14.0 in the pairs). Estimates of genome size were 2880, 3040, and 3360 cM for LOD scores of 2.0, 3.0, and 4.0, respectively. Thus the current set of 104 loci is apparently distributed over approximately two-thirds of the genome.

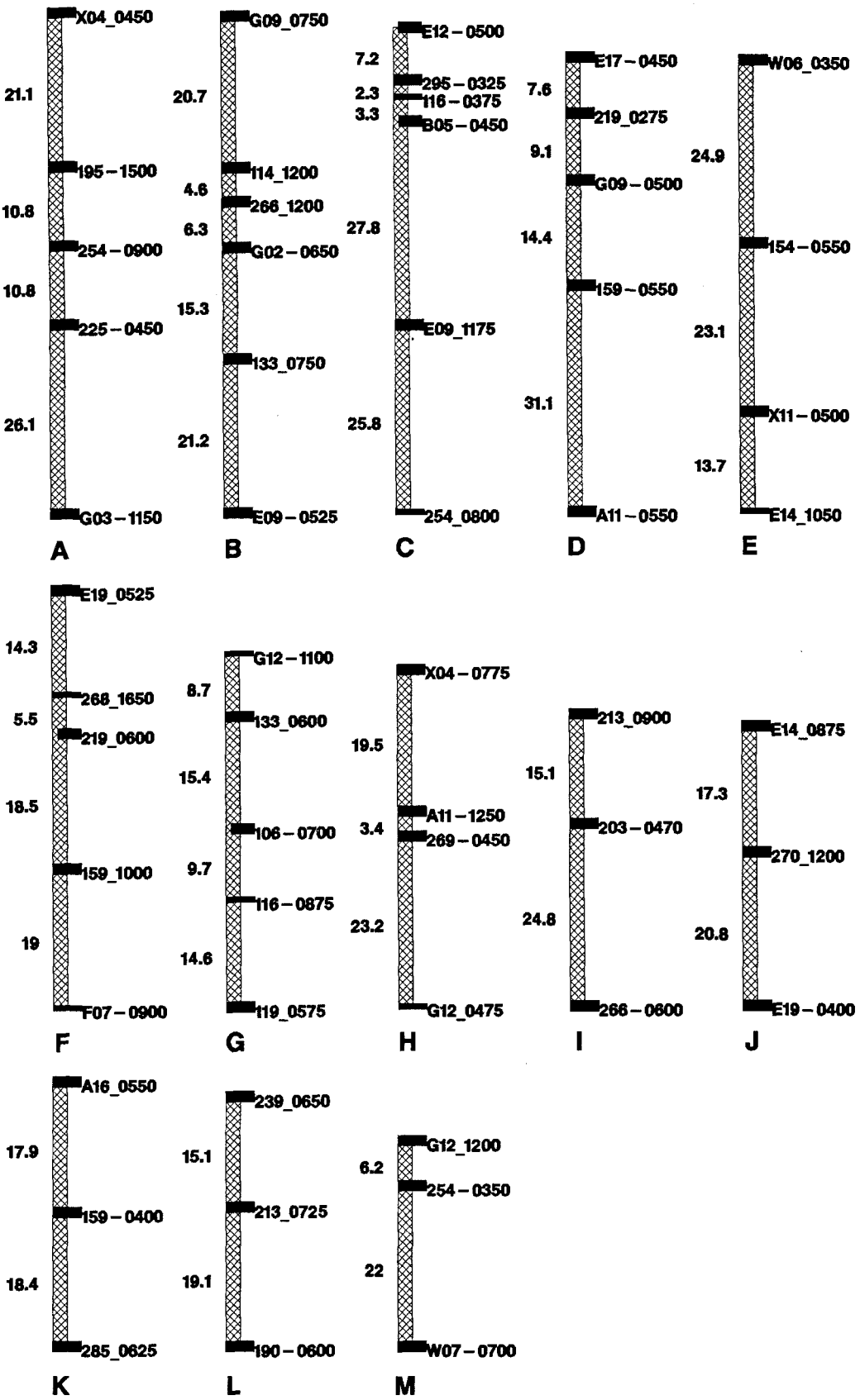

Fig. 3. Partial RAPD genetic linkage map of slash pine clone 8-7. Linkage groups with three or more loci are indicated by letters *A through M*. Marker names are given of the right-hand side of the linkage groups and Haldane centimorgan distances are given on the left-hand side. The marker names contain the primer ID and the approximate length in base pairs of the RAPD fragment. The symbols (– or _) separating the primer and fragment in the marker names indicate phase relationships between linked markers, i.e., – – and _ _ markers are in coupling (– –/+ + or + +/– –). Framework loci (LOD = 3.0, distance <25 cM) are marked with *thick crossbars*. Loci for which alternative marker(s) (i.e., 0% recombination) were found are indicated with a *short crossbar*. Linked loci for which a position could not be determined (at LOD > 1.7) are not included

Table 2. Expected primer screening and mapping efforts required to complete a 30-cM RAPD genetic linkage map with various levels of coverage for slash pine clone 8-7

Coverage	RAPD markers		Primers to	
	Total	Needed	Screen	Map
Current	104	–	–	–
0.70	168	64	258	39
0.80	224	120	484	74
0.90	321	217	875	134
0.95	418	314	1266	193

The minimum number of randomly distributed markers required to cover a porportion, p, of a genome of size k at a maximum distance (2*c) between markers is given by Lange and Boehnke (1982) as follows:

$$n = [[\log(1-p)]/\log[1-(2*c)/k]] * 1.25.$$

For QTL analyses and MAS applications, c is recommended to be approximately 15 cM (Beckmann and Soller 1983). Using our maximum genome size estimate and our observed rates of polymorphic loci per primer screened (104/420 = 0.248) and mapped (104/64 = 1.625), we can estimate the effort required to complete a 30 cM map of clone 8-7. Table 2 presents these results in terms of the number of additional primers necessary to screen and map for various levels of genomic coverage.

Discussion

In this study we have identified 104 Mendelian segregating loci in a slash pine clone using RAPD markers. Linkage analysis grouped the loci into 13 linkage groups of at least three loci and nine pairs of linked loci. The 73 mapped and 24 unlinked loci represent 2160 cM of genetic distance or approximately two-thirds of the slash pine genome. The placement of seven additional loci, apparently linked to five groups, could not be determined with high confidence. Efforts to place these loci will require assaying additional megagametophytes for these RAPD fragments. In addition, increasing the number of assayable markers within these linkage groups could further the chance of placing these markers. Based on our results to date, obtaining a 30 cM map over 80% of the genome will require the screening of an additional 484 primers and the mapping of 120 fragments (produced by 74 primers, see Table 2).

Six of the one-hundred and four loci can be scored with alternative RAPD markers, and three of these appear to exist as co-dominant alleles of the same locus. In these three cases the marker fragments, similar in size, are produced by the same primer, and, in all individuals assayed, only one or the other fragment appears (see Fig. 2, primer E12). Markers of this nature should be more generally useful in applications to diploid material, where the typical RAPD markers display dominance. Application of dominant RAPD markers to diploid material will require that the parents exist in a test-cross situation. In our case, only individuals homozygous for fragment-absent alleles will be informative in matings with clone 8-7. However, Carlson et al. (1991) have recently shown this to be the case for 50% of the RAPD markers between any two of six Douglas-fir parents tested. Strategies for converting these dominant markers to co-dominant, including conversion of RAPD fragments to RFLP probes and conversion of RAPD primers to unique, sequence-tagged-site (STS) primers, have yet to be explored in conifers.

We are particularly interested in clone 8-7 and four other slash pine trees that had previously been crossed in a five-tree complete diallel design (Griggs and Walkinshaw 1982). Progeny of these parents typically display qualitative responses to inoculation by single-spore cultures of *C. q. fusiforme*, suggestive of a complementary gene-for-gene interaction system (Kinloch and Walkinshaw 1991). Our long-range goal is to completely characterize this host-pathogen association by identifying and locating the interacting genes in both organisms (Nance et al. 1991). Towards this end we have recently completed a partial genetic linkage map of a single-spore culture of *C. q. fusiforme* (RL Doudrick, CD Nelson, WL Nance, in review), and have cloned approximately 400 progeny of the slash pine diallel cross, as well as having inoculated these clones with the mapped and a second single-spore culture. In addition to gaining fundamental knowledge of the genetics of this host-pathogen association, we anticipate that linked markers in the pine and the fungus will provide the tools necessary for tree breeders and pathologists to monitor breeding populations for resistance genes and fungal populations for avirulence genes.

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