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A Genetic linkage map of Pinyon pine (*Pinus edulis*) based on amplified fragment length polymorphisms

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Abstract Amplified fragment length polymorphisms (AFLP) were used to rapidly generate a dense linkage map for pinyon pine (*Pinus edulis*). The map population consisted of 40 megagametophytes derived from one tree at Sunset Crater, Arizona. A total of 78 primer combinations, each with three to five selective nucleotides, amplified 542 polymorphic markers. Of these, 33 markers showed significant deviation from the expected Mendelian genotypic segregation ratio of 1 : 1, and 164 showed complete linkage with another marker. This resulted in 338 unique markers mapping to 25 linkage groups, each of which ranged from 2 to 22 markers, averaging 80 centiMorgans (cM) in size and covering 2,012 cM (2,200 cM with the inclusion of 25 cM for each of 7 unlinked markers). Pairwise linkage values gave a genome size estimate of 2,390 cM, suggesting comprehensive coverage of the genome. A search for subsets of primer combinations giving the best map coverage found 10 primer combinations which together marked 72% of the linkage map to within 10 cM; an additional 10 primer combinations increased this percentage to 85%. Our map represents an initial step towards the identification of quantitative trait loci associated with pest resistance and water stress in pinyons and will further allow us to examine introgression rates between *P. edulis* and *P. californiarum*.

Key words AFLP · Linkage map · *Pinus edulis*

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Introduction

Genetic mapping studies are now common in agricultural and tree improvement programs, as illustrated by those for soybean [*Glycine max* (L.) Merril (Apuya et al. 1988; Keim et al. 1990, 1997)] and loblolly pine [*Pinus taeda* L. (Devey et al. 1994)]. For conifers, linkage maps may help circumvent the long generation times which impede artificial selection, through the process of identifying "quantitative trait loci" (QTLs) and application of marker-aided selection of such loci (Strauss et al. 1992). Linkage and QTL studies also provide a novel source of information about past evolution of quantitative traits in wild species, such as *Microseris* (Vlot et al. 1992; van Houten et al. 1994; Homberger and Bachmann 1995), *Helianthus anomalus* (Rieseberg et al. 1993), and *Mimulus* (Lin and Ritland 1996).

The polymerase chain reaction (PCR) has enabled rapid construction of genetic linkage maps for a number of organisms. Until recently, the favorite PCR-based markers have been either simple-sequence repeats (SSRs, Akkaya et al. 1992) or random amplified polymorphic DNAs (RAPDs, Williams et al. 1990). The RAPD technique has been particularly useful for studies of wild plant species, as development costs are minimal, but these markers can be unpredictable in expression when conditions are not precisely controlled. A new type of marker, amplified fragment length polymorphism (AFLP, Vos et al. 1995), has similar development costs but is much more reliable and consistent in expression and should provide a powerful new class of markers for mapping studies (Ballvora et al. 1995; Becker et al. 1995; Meksem et al. 1995; Thomas et al. 1995; Van Eck et al. 1995; Folkertsma et al. 1996; Mackill et al. 1996; Keim et al. 1997). Another advantage of this technique is the simultaneous assay for large numbers of polymorphic loci (approx. 10–50) on a single sequencing gel.

One feature of conifers that facilitates genetic mapping is the presence of the "gametophyte", a haploid tissue in the seed derived from the mother tree. Assay of this tissue directly reveals the products of meiosis (essentially, behaving like a cross to a homozygous testor strain), allowing an efficient mapping of genes (Guries et al. 1978; Conkle 1981; Bahrman and Damerval 1989; Tulsieram et al. 1992; Nelson et al. 1993, 1994). One potential drawback of the AFLP technique is the dominant nature of the markers developed; however, any problems associated with the use of dominant markers for mapping can be overcome using megagametophytes because they allow dominant markers to be scored as co-dominant and linkage phase to be determined in the offspring of phase-unknown parents (Raeder and Broda 1986; Hulbert et al. 1988).

In this paper, we report a linkage map of AFLPs based upon assays of megagametophytes of pinyon pine (*Pinus edulis*). In addition, we present an algorithm for selecting an optimal subset of primer combinations that most evenly spans the genomic map. Our linkage map should facilitate studies of the evolutionary ecology of pinyon pines, in particular, a population which apparently has genetically differentiated along a steep moisture gradient in north-central Arizona. In a 2,000 km^2 cinder field, generated by the recent eruption of Sunset Crater in 1265 (Krutch 1974), evidence supports the genetic divergence of these individuals from those growing in adjacent sandy-loam soils (Mopper et al. 1991). It is apparent that genes for nutrient- and water-stress are the primary candidates for this differentiation (Mopper et al. 1991; Mopper and Whitham 1992; Gehring and Whitham 1994). The development of a linkage map for pinyon pine will enable us to map QTLs associated with adaptations to nutrient- and water-stress (Keim et al. 1990), including insect resistance.

Materials and methods

DNA extraction

DNA was extracted from a mapping population consisting of the megagametophytes of 40 wind-pollinated seeds derived from a single ponderosa pine growing at Sunset Crater National Monument near Flagstaff, Arizona, USA. The extraction procedure was a modification of Wagner et al. (1987). A suitable volume (approx. 1 ml) of chilled (0°–4°C) homogenization buffer (50 m*M* TRIS-HCl pH 8.0, 5 m*M* EDTA, 1.0% PEG, 0.5 m*M* spermidine, 0.1% 2-mercaptoethanol, and 350 m*M* sucrose) was added to desiccated megagametophytes, which were ground using a chilled mortar and pestle. The resulting suspension was placed in 1.5-ml tubes and spun in a pre-cooled centrifuge at 12,000 rpm for 5 min. Pellets were resuspended in 600 µl of chilled wash buffer composed of 50 m*M* TRIS-HCl pH 8.0, 25 m*M* EDTA, 0.5 m*M* spermidine, 0.1% 2-mercaptoethanol, and 350 m*M* sucrose. The following ingredients were then added sequentially, with mixing after each addition: NaCl to a final concentration of 75 m*M*, sodium dodecyl sulfate to a final concentration of 0.75% (wt/vol), and hexadecyltrimethylammonium bromide/0.7 *M* NaCl to a final concentration of 1.0% (wt/vol). Samples were incubated in a water bath at 60°C for 30 min, with frequent mixing, and then CTAB-complexed proteins were extracted with an equal volume of chloroform. After spinning at 12,000 rpm for 5 min, DNA was precipitated by removing excess liquid then adding an equal volume of isopropanol. DNA was pelleted by centrifuging as before, the isopropanol decanted, and the DNA washed in 1 ml of 70% ethanol. Pellets were air-dried prior to dissolving the DNA in TRIS-EDTA (pH 8.0).

AFLP procedure

We resolved AFLPs according to the methods of Vos et al. (1995) as modified by Travis et al. (1996). This involved two successive PCR reactions: a selective preamplification, involving one or two selective nucleotides, and a more stringent selective restriction fragment amplification (SRFA), with three to five selective nucleotides. Selective preamplifications involving one selective nucleotide (+1 primers) were followed by SRFA reactions with three or four selective nucleotides (+3 and +4 primers), while selective preamplifications involving +2 primers were followed by SRFA reactions using +5 primers. In the +1 preamplification reactions, adenine was the selective nucleotide for both the *Eco*RI- and *Mse*I-primers; in the +2 preamplifications, AC and AG were used as selective nucleotides for these two primers, respectively. In early screens of +3 primers, 10 combinations of selective nucleotides were attempted. The subsequent choice of +4 primers was guided by our observation that +3 primers with the highest GC-content were the most informative. We chose the primers ACAG, AGGC, and AGGG, for binding to *Eco*RI, and for binding to *Mse*I, we chose AGAG, ACGC, and ACGG. Only a single pair of +5 primers was chosen, *Eco*RI-ACGCA and *Mse*I-AGCGA. Loci with unreliable amplification (<95%) were excluded from scoring. Poor amplification did not occur consistently for any 1 megagametophyte.

A total of 116 primer combinations (PCs) were screened for polymorphism. With regard to number of selective nucleotides for the *Eco*RI- and *Mse*-I-primers, respectively, 10 of these PCs were +3/+3, while the two sets of three +4 primers were used in all possible +3/+4 ($n = 48$), +4/+3 ($n = 48$), and +4/+4 ($n = 9$) combinations. The final 2 PCs included a single +5/+4 combination and a single +5/+5 combination.

Our method of selecting primer combination (PCs) will occasionally yield the same AFLP locus for certain pairs of +3/+4 and +4/+3 SRFA reactions. In addition, we expect each of these subsets of redundant markers to be amplified by 1 of the 9 possible +4/+4 reactions. For example, +4/+4 PC *Eco*RI-ACAG/*Mse*I-AGAG will amplify a subset of loci amplified by *Eco*RI-ACA/*Mse*I-AGAG and *Eco*RI-ACAG/*Mse*I-AGA. These redundancies can be identified by their complete linkage in the mapping analysis.

Linkage analysis

Mendelian segregation of loci was verified by chi-square tests for 1 : 1 ratios with α set at 0.05. We performed multipoint linkage analysis on loci showing Mendelian segregation according to the methods of Nelson et al. (1993) using MAPMAKER 3.0b (Lander et al. 1987). Linkage was deemed significant if LOD values were at least 4.0 and loci lay within 25 cM (centiMorgans). Linkage phases were inferred by observed recombination frequencies, e.g., if in assuming coupling the proportion of recombinant gametes was greater than 0.5, then repulsion was inferred (since linkage only with 25 cM was accepted, the probability of phase misidentification was very low).

We constructed framework orders by first excluding from each linkage group those markers lying within approximately 10 cM of each other (precise values were dependent on the number of markers in a group). Remaining loci were added if their best position was at least 10 times more likely than the next best. A permutation procedure was used to test the likelihood of the established order for each

successive set of 5 markers against all possible orders. Also, genome size was estimated according to Hulbert et al. (1988) and Nelson et al. (1993). We obtained three estimates by considering the number of significant pairwise linkages between the 338 unique loci assuming threshold LOD values of 2.0, 3.0, and 4.0, respectively.

Optimal subsets of primer combinations

Different PCs reveal different numbers of loci in different map positions, and it is expected that when many PCs are assayed, some or even many PCs will provide nearly redundant markers. It is thus desirable to identify subsets of PCs that give the best map coverage. For this, we used a simple algorithm which started with all 78 useful PCs. At the first step, single PCs were successively omitted, and for each omission, a measure of uniformity of map coverage by the remaining primer combinations was calculated. After all PCs were considered the PC giving the least reduction in uniformity was omitted, and the entire procedure repeated. Successive iterations give maps with fewer PCs. This procedure is not guaranteed to give the optimal combinations of PCs. As a double check, one might evaluate all combinations of a subset of i PCs selected from a suite of n PCs. In our case, this is computationally impossible, as for example, if one wants to chose $i = 20$ PCs from $n = 78$ PCs, there are over 10^{18} possible combinations!

A number of criteria are possible for the measure of uniformity, such as maximizing the proportion of the map lying with-in 5 cM of a marker, or within 10 cM of a marker. A more objective method is to minimize the probability of double-crossovers between adjacent markers (e.g., the probability that a QTL will be undetected in a backcross). For markers within approximately 20 cM, this is approximately $r(1 - r)$, where r is the recombination rate between adjacent markers. We adopted this more objective criteria, but it gave similar results to the other criteria.

Results

Of the 116 primer combinations screened, 78 were useful in revealing a total of 542 polymorphisms. Of these, 33 showed deviations from 1:1 ratios. Primer combinations that gave the remaining 509 polymorphisms were as follows: one +3/+3 PC (*Eco*RI-AGG/*Mse*I-ACG) yielded 35 polymorphisms; thirty-five +3/+4 PCs successfully yielded polymorphisms ranging in number from 1–13, with a mean of 5.97 ± 0.60 (4.35 ± 0.58 inclusive of unsuccessful PCs); thirty-one +4/+3 PCs successfully yielded polymorphisms ranging in number from 1–29, with a mean of 5.81 ± 1.03 (3.75 ± 0.78); each of the nine +4/+4 PCs successfully yielded polymorphisms ranging in number from 2–14, with a mean of 7.33 ± 1.34; the single +5/+4 PC successfully yielded 9 polymorphisms; and the single +5/+5 PC successfully yielded 8 polymorphisms. Fragment sizes ranged from 50 to 450 base pairs. Table 1 gives the ranking of all successful PCs.

The linkage analysis mapped 338 markers to unique positions. We could not reliably map 43 of the 509 markers analyzed, in that alternative placements were not 10 times as less likely, and we omitted an additional 128 markers from the final map due to their complete linkage with other markers. Of the 62 non-segregating groups, 2 included 8 markers, 3 included 7 markers, 1 included 6 markers, 2 included 5 markers, 5 included 4 markers, 19 included 3 markers, and 30 included a pair of markers. We arbitrarily chose 1 marker from each of these clusters for inclusion in the final map (but include all markers in the choice of optimal subsets of PCs). Extremely small recombination distances (<2 cM) among neighboring loci resulted in 83 markers that were mapped to two equally likely positions. Seven of the original markers remained unlinked.

Figure 1 presents a partial genetic linkage map for pinyon pine. This map includes 248 AFLP markers distributed over 25 linkage groups. The number of markers per linkage group ranged from 2 to 22, with a mean of 9.36 ± 1.21. Each group covered a mean distance of 80.47 ± 12.54 cM, with a mean pairwise distance of 9.32 ± 0.42. Overall genome coverage was 2,012.3 cM, which increased to approximately 2,200 cM following the addition of 25 cM for each unlinked marker. Genome size estimates from pairwise linkages ranged from 2.290 cM for LOD scores of 4.0 to 2.490 for LOD scores of 2.0. Thus, the 248 markers in the current map may be assumed to provide a reasonably comprehensive coverage of the pinyon pine genome.

Analysis of variance (ANOVA) was used to determine the efficacy of the +3 primers, categorized according to their relative guanine-cytosine content, in generating polymorphic markers, when used in combination with the two sets of three +4 primers. The restriction site for binding of the +4 primer was crossed with relative GC-content in a two-factor experimental design. Additionally, each of the six +4 primers were nested within their respective binding sites. The data were analyzed in the framework of a general linear model, since the number of PCs in each category was unbalanced (SAS Institute 1990). The ANOVA revealed a significant effect of GC-content on the mean number of primers generated ($F = 6.48$, $df = 2$, $P = 0.0025$), such that the +3 primers with one or two GCs in the two terminal selective nucleotide positions (the first position was fixed as adenine) generated significantly more polymorphic markers than the +3 primers with no guanine or cytosine. In addition, there was a significant effect of the +4 primers nested within binding sites ($F = 6.93$, $df = 4$, $P = 0.0001$), although the number of +4 primers examined was too small to formulate general conclusions. There was no effect of binding site on the mean number of polymorphic markers generated ($F = 0.45$, $df = 1$, $P = 0.5046$), nor were there any detectable interaction effects (binding site X GC-content: $F = 0.08$, $df = 2$, $P = 0.9256$; +4 primer X GC-content: $F = 0.78$, $df = 8$, $P = 0.6220$). Of further note is the potential effect of a statistical outlier on the aforementioned analysis, namely the PC *Eco*RI-AGGC/*Mse*I-ACT which generated 29 polymorphic markers. The removal of this datum from the ANOVA had no effect on outcomes. A list of least squares means is given in Table 2.

Table 1 A ranking of AFLP primer combinations used to generate 509 polymorphic markers from megagametophytes of a single pinyon pine. Primer combinations are described in terms of the selective nucleotides used: the *Eco*RI-primer is given to the left of the backslash; the *Mse*I-primer to the right. Each combination was first ranked for the overall number of polymorphisms generated, and then for the actual number of markers that could reliably be assigned to a unique position within the linkage map (in parentheses)

No polymorphisms	1–4 poymorphisms	5–9 polymorphisms	>9 polymorphisms
ACA/ACA	1	5	10
ACA/AGA	ATC/AGAG (0)	AAT/ACGC (2)	ACG/ACGC (4)
ACC/ACC	AGGC/AAC (0)	ATC/ACGG (2)	AAG/ACGG (5)
ACC/ACG	AGGC/ATT (0)	AGGC/AAA (4)	AGGC/ATC (6)
ACC/AGC	AGGG/ACA (0)	AGGC/ACGC (4)	AGC/ACGG (7)
ACC/AGG	AAA/ACGC (1)	AAC/ACGG (5)	ATG/ACGG (7)
AGG/ACC	AAC/AGAG (1)	AGT/AGAG (5)	AGGG/ACGC (7)
AGG/AGC	ATA/AGAG (1)		
AGG/AGG	ACAG/AGG (1)	6	11
AAT/ACGC	AGGG/AGA (1)	ACC/ACGG (2)	ACT/ACGG (6)
AAA/ACGG		AGA/ACGC (4)	AGGG/AGC (6)
ATT/ACGG	2	AGGG/AGAG (5)	AGGC/ACA (7)
AAA/AGAG	ATA/ACGC (0)	AGGC/ACG (6)	
AAG/AGAG	ATC/ACGC (0)	ACAG/ACGG (6)	12
ACA/AGAG	ACA/ACGC (1)		ACT/ACGC (4)
ACC/AGAG	ACC/ACGC (1)	7	AGGC/AGC (9)
AGA/AGAG	ATG/ACGC (1)	AGG/ACGC (3)	AGGC/AGAG (11)
AGC/AGAG	ACAG/ACT (1)	AAT/ACGG (4)	
AGG/AGAG	AGGG/AGG (1)	AGA/ACGG (4)	13
ATG/AGAG	ACT/AGAG (2)	AGGC/AAG (5)	AAG/ACGC (6)
ATT/AGAG	ACAG/ACC (2)	AGC/ACGC (7)	ACAG/ACG (9)
ACAG/AAA	AGGG/ATA (2)		
ACAG/AAG	AGGG/ATC (2)	8	14
ACA/AAT	AGGG/ATG (2)	ACGCA/AGCGA (2)	AGGG/ACGC (11)
ACAG/ACA	AGGC/ACGC (2)	ATA/ACGG (3)	
ACAG/AGT		AGG/ACGG (4)	>14
ACAG/ATA	3	ACG/AGAG (5)	AGGC/ACT (18)
ACAG/ATG	AGG/ACGC (0)	AGGC/AGA (5)	AGG/ACG (28)
ACAG/ATT	ACAG/AAC (1)	AGT/ACGC (6)	
AGGC/AAT	ACA/AGC (1)	ACAG/AGA (6)	
AGGC/ACC	ACAG/ACGC (1)	AGGC/ATG (6)	
AGGC/AGT	AGGG/ACT (3)	AGGG/AGT (7)	
AGGC/ATA	AGGG/ATT (3)	ACAG/AGAG (7)	
AGGG/AAA			
AGGG/AAG	4	9	
AGGG/AAT	ACAG/ATC (3)	AGT/ACGG (4)	
AGGG/ACC	ACA/ACGG (4)	ACGCA/AGAG (4)	
AGGG/ACG	AGGG/AAC (4)	AAC/ACGC (5)	
		AGGC/AGG (8)	

Figure 2 shows the results of the procedure to determine optimal subsets of PCs. Figure 2a plots the proportion of the marker map (not the actual map) that lies within X cM of a marker, for X = 2.5, 5, 10, and 20. If all 71 PCs are included, about 50% of the map lies within 2.5 cM, while over 95% of the map lies within 10 cM. As shown by the slight decline from 71 to 35 markers, this figure shows that at least half of the PCs can be removed without significant loss of map resolution. This occurs despite a one-third reduction in the number of marker loci (Fig. 2b). More dramatic declines of resolution occur by the time only 20 PCs are left, and the declines became very rapid with 10 PCs or fewer. Overall, the figure suggests that between 10 and 20 PCs (Fig. 2a), which together account for 100–150 individual loci (Fig. 2b), would be most efficient for future surveys for QTL variation. Up to 72% map coverage was achieved with an average distance of 10 cM between adjacent markers using 10 primer combinations; an additional 10 primer combinations increased this percentage to 85%. Table 3 gives the 20 most informative PCs, in rank order, as determined by this analysis.

Discussion

We have constructed the first reported linkage map for a conifer using AFLP technology. Our map provides

Fig. 1 Genetic linkage map constructed from 40 megagametophytes extracted from the seeds of a single pinyon pine growing at Sunset Crater, Arizona. Linkage groups are indicated by *letters A* through *Y*; marker names are given to the *right* of each marker. The phase of each marker (+ or −) precedes its *Eco*RI/*Mse*I primer designation, which is represented by the three to five selective nucleotides used and followed by the fragment size in base pairs.

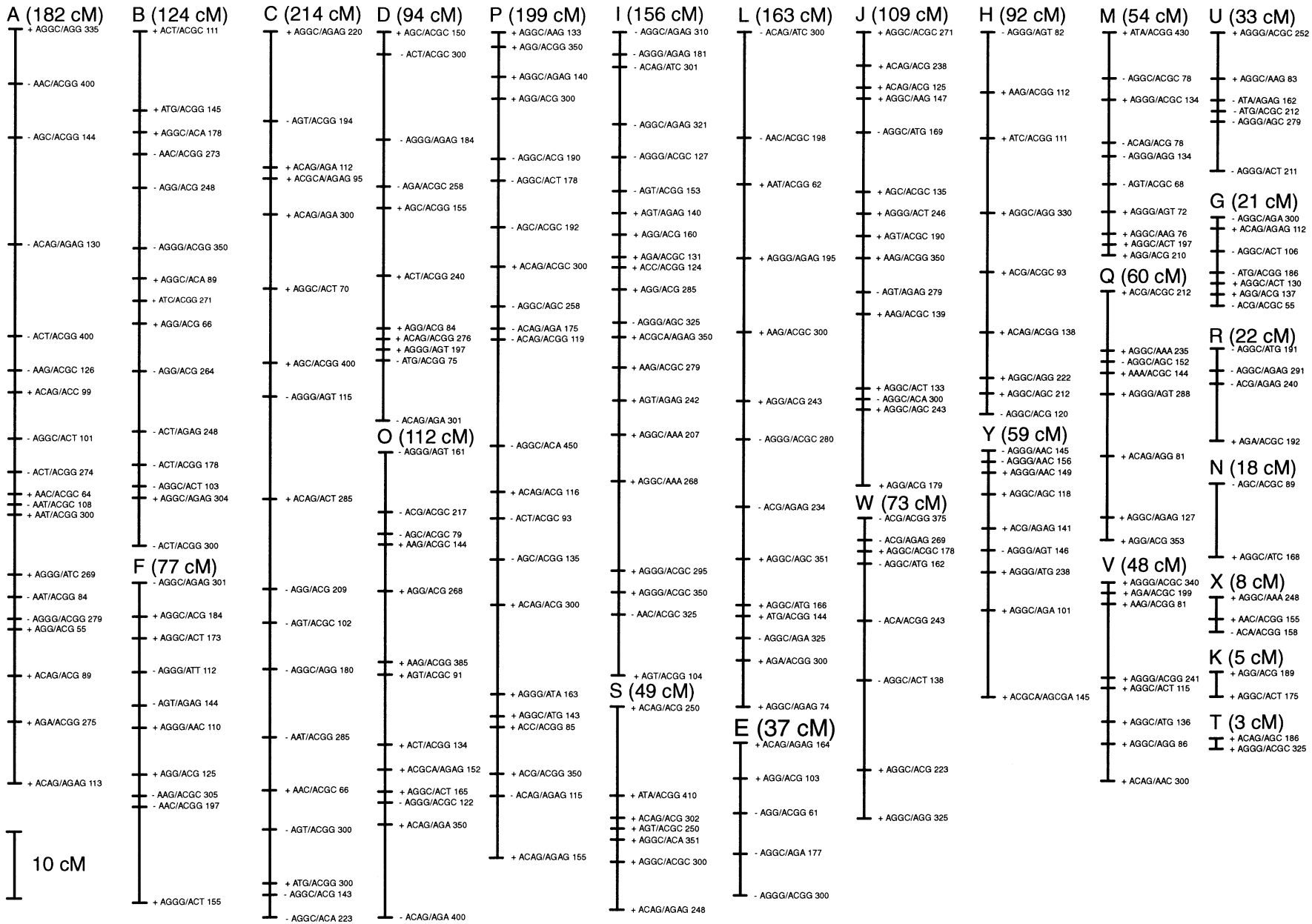
A (182 cM)
+ AGGC/AGG 335
- AAC/ACGG 400
- AGC/ACGG 144
- ACAG/AGAG 130
- ACT/ACGG 400
- AAG/ACGC 126
+ ACAG/ACC 99
- AGGC/ACT 101
- ACT/ACGG 274
+ AAC/ACGC 64
- AAT/ACGC 108
+ AAT/ACGG 300
+ AGGG/ATC 269
- AAT/ACGG 84
- AGGG/ACGG 279
+ AGG/ACG 55
+ ACAG/ACG 89
+ AGA/ACGG 275
+ ACAG/AGAG 113
10 cM
B (124 cM)
+ ACT/ACGC 111
+ ATG/ACGG 145
+ AGGC/ACA 178
- AAC/ACGG 273
- AGG/ACG 248
- AGGG/ACGG 350
+ AGGC/ACA 89
+ ATC/ACGG 271
+ AGG/ACG 66
- AGG/ACG 264
- ACT/AGAG 248
- ACT/ACGG 178
- AGGC/ACT 103
+ AGGC/AGAG 304
- ACT/ACGG 300
F (77 cM)
- AGGC/AGAG 301
+ AGGC/ACG 184
+ AGGC/ACT 173
- AGGG/ATT 112
- AGT/AGAG 144
+ AGGG/AAC 110
+ AGG/ACG 125
- AAG/ACGC 305
- AAC/ACGG 197
+ AGGG/ACT 155
C (214 cM)
+ AGGC/AGAG 220
- AGT/ACGG 194
+ ACAG/AGA 112
+ ACGCA/AGAG 95
+ ACAG/AGA 300
+ AGGC/ACT 70
+ AGC/ACGG 400
- AGGG/AGT 115
- AGG/ACG 209
- AGT/ACGC 102
- AGGC/AGG 180
+ ACAG/ACT 285
- AAT/ACGG 285
+ AAC/ACGC 66
- AGT/ACGG 300
+ ATG/ACGG 300
- AGGC/ACG 143
- AGGC/ACA 223
D (94 cM)
+ AGC/ACGC 150
- ACT/ACGC 300
- AGGG/AGAG 184
- AGA/ACGC 258
+ AGC/ACGG 155
+ ACT/ACGG 240
+ AGG/ACG 84
+ ACAG/ACGG 276
+ AGGG/AGT 197
- ATG/ACGG 75
- ACAG/AGA 301
O (112 cM)
- AGGG/AGT 161
- ACG/ACGC 217
- AGC/ACGC 79
+ AAG/ACGC 144
+ AGG/ACG 268
+ AAG/ACGG 385
+ AGT/ACGC 91
+ ACT/ACGG 134
+ ACGCA/AGAG 152
+ AGGC/ACT 165
- AGGG/ACGC 122
+ ACAG/AGA 350
- ACAG/AGA 400
P (199 cM)
+ AGGC/AAG 133
+ AGG/ACGG 350
+ AGGC/AGAG 140
+ AGG/ACG 300
- AGGC/ACG 190
- AGGC/ACT 178
- AGC/ACGC 192
+ ACAG/ACGC 300
- AGGC/AGC 258
- ACAG/AGA 175
- ACAG/ACGG 119
- AGGC/ACA 450
+ ACAG/ACG 116
- ACT/ACGC 93
- AGC/ACGG 135
+ ACAG/ACG 300
+ AGGG/ATA 163
+ AGGC/ATG 143
+ ACC/ACGG 85
+ ACG/ACGG 350
- ACAG/AGAG 115
+ ACAG/AGAG 155
I (156 cM)
- AGGC/AGAG 310
- AGGG/AGAG 181
- ACAG/ATC 301
- AGGC/AGAG 321
- AGGG/ACGC 127
- AGT/ACGG 153
+ AGT/AGAG 140
+ AGG/ACG 160
+ AGA/ACGC 131
+ ACC/ACGG 124
+ AGG/ACG 285
- AGGG/AGC 325
+ ACGCA/AGAG 350
+ AAG/ACGC 279
+ AGT/AGAG 242
+ AGGC/AAA 207
+ AGGC/AAA 268
+ AGGG/ACGC 295
+ AGGG/ACGC 350
- AAC/ACGC 325
+ AGT/ACGG 104
S (49 cM)
+ ACAG/ACG 250
+ ATA/ACGG 410
+ ACAG/ACG 302
+ AGT/ACGC 250
+ AGGC/ACA 351
+ AGGC/ACGC 300
+ ACAG/AGAG 248
L (163 cM)
- ACAG/ATC 300
- AAC/ACGC 198
+ AAT/ACGG 62
+ AGGG/AGAG 195
+ AAG/ACGC 300
+ AGG/ACG 243
- AGGG/ACGC 280
- ACG/AGAG 234
+ AGGC/AGC 351
+ AGGC/ATG 166
+ ATG/ACGG 144
- AGGC/AGA 325
+ AGA/ACGG 300
+ AGGC/AGAG 74
E (37 cM)
+ ACAG/AGAG 164
+ AGG/ACG 103
- AGG/ACGG 61
- AGGC/AGA 177
- AGGG/ACGG 300
J (109 cM)
+ AGGC/ACGC 271
+ ACAG/ACG 238
+ ACAG/ACG 125
+ AGGC/AAG 147
- AGGC/ATG 169
+ AGC/ACGC 135
+ AGGG/ACT 246
+ AGT/ACGC 190
+ AAG/ACGG 350
- AGT/AGAG 279
+ AAG/ACGC 139
+ AGGC/ACT 133
- AGGC/ACA 300
+ AGGC/AGC 243
+ AGG/ACG 179
W (73 cM)
- ACG/ACGG 375
- ACG/AGAG 269
+ AGGC/ACGC 178
- AGGC/ATG 162
- ACA/ACGG 243
- AGGC/ACT 138
+ AGGC/ACG 223
+ AGGC/AGG 325
H (92 cM)
- AGGG/AGT 82
+ AAG/ACGG 112
+ ATC/ACGG 111
+ AGGC/AGG 330
+ ACG/ACGC 93
+ ACAG/ACGG 138
+ AGGC/AGG 222
+ AGGC/AGC 212
- AGGC/ACG 120
Y (59 cM)
- AGGG/AAC 145
- AGGG/AAC 156
+ AGGG/AAC 149
+ AGGC/AGC 118
+ ACG/AGAG 141
- AGGG/AGT 146
+ AGGG/ATG 238
+ AGGC/AGA 101
+ ACGCA/AGCGA 145
M (54 cM)
+ ATA/ACGG 430
- AGGC/ACGC 78
+ AGGG/ACGC 134
- ACAG/ACG 78
- AGGG/AGG 134
- AGT/ACGC 68
+ AGGG/AGT 72
+ AGGC/AAG 76
+ AGGC/ACT 197
+ AGG/ACG 210
Q (60 cM)
+ ACG/ACGC 212
+ AGGC/AAA 235
- AGGC/AGC 152
+ AAA/ACGC 144
+ AGGG/AGT 288
+ ACAG/AGG 81
+ AGGC/AGAG 127
+ AGG/ACG 353
V (48 cM)
+ AGGG/ACGC 340
+ AGA/ACGC 199
+ AAG/ACGG 81
+ AGGG/ACGG 241
+ AGGC/ACT 115
+ AGGC/ATG 136
+ AGGC/AGG 86
+ ACAG/AAC 300
U (33 cM)
+ AGGG/ACGC 252
+ AGGC/AAG 83
- ATA/AGAG 162
- ATG/ACGC 212
- AGGG/AGC 279
- AGGG/ACT 211
G (21 cM)
- AGGC/AGA 300
+ ACAG/AGAG 112
- AGGC/ACT 106
- ATG/ACGG 186
+ AGGC/ACT 130
+ AGG/ACG 137
- ACG/ACGC 55
R (22 cM)
- AGGC/ATG 191
- AGGC/AGAG 291
- ACG/AGAG 240
+ AGA/ACGC 192
N (18 cM)
- AGC/ACGC 89
+ AGGC/ATC 168
X (8 cM)
+ AGGC/AAA 248
+ AAC/ACGG 155
- ACA/ACGG 158
K (5 cM)
+ AGG/ACG 189
+ AGGC/ACT 175
T (3 cM)
+ ACAG/AGC 186
+ AGGG/ACGC 325

Table 2 Least squares means (±standard error) of +3/+4 and +4/+3 primer combinations categorized by the restriction site for binding of the +4 primer (*Eco*RI and *Mse*I), the sequence of the +4 primer, and the number of guanine-cytosine residues present in the two terminal nucleotides of the +3 primer. Superscripts represent statistically-equivalent subgroups within each category, determined on the basis of pairwise *t*-tests at an alpha-level of 0.05

GC-conent	*Eco*RI (3.50 ± 0.63[a])			*Mse*I (4.08 ± 0.63[b])		
	ACAG (2.29±1.08[a])	AGGC (5.83±1.08[b])	AGGG (2.38±1.08[a])	AGAG (1.12±1.08[c])	ACGG (6.38±1.08[d])	ACGC (4.75±1.08[d])
0 (1.45±0.84[a])	0.00±2.06	1.50±2.06	1.25±2.06	0.25±2.06	3.75±2.06	2.00±2.06
1 (4.92±0.59[b])	2.12±1.45	9.25±1.45	2.62±1.45	1.12±1.45	7.62±1.45	6.75±1.45
2 5.00±0.84[b])	4.75±2.06	6.75±2.06	3.25±2.06	2.00±2.06	7.75±2.06	5.50±2.06

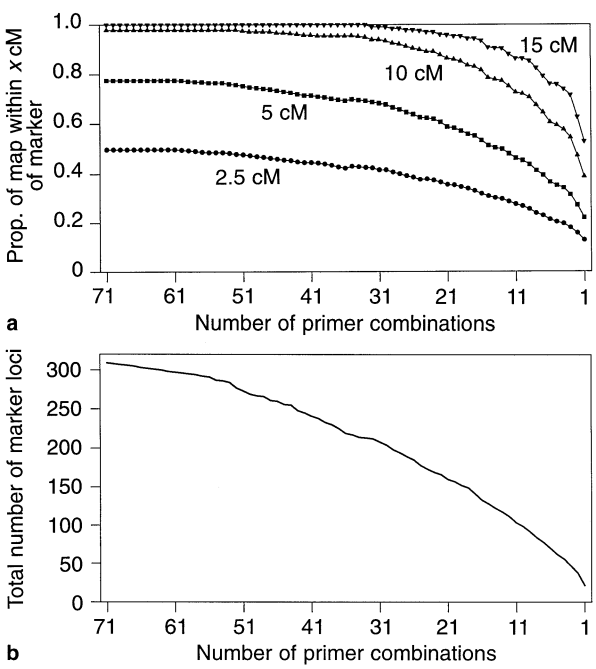

Fig. 2a, b Results of the search for optimal subsets of primer combinations, starting with all 71 PCs, with successive reductions down to a single PC. **a** Proportion of map lying within a certain distance *X*, for $X = 2.5$, 5, 10 and 15 cM. **b** Number of marker loci revealed by the corresponding subset of PCs selected in **a**

Table 3 The best 20 primers that provide most uniform coverage of the linkage map, ranked in order of increasing importance, and the map coverage jointly provided by all markers of a given rank and higher. For example, the first 10 primers together span 72% of the marker map to within 10 cm

Rank	Primer combination	Proportion of map spanned to within			
		2.5 cM	5 cM	10 cM	15 cM
1	AGGC/AAG	0.129	0.222	0.389	0.530
2	AGGC/ACT	0.158	0.273	0.473	0.627
3	ACAG/ACG	0.180	0.315	0.545	0.715
4	ACT/ACGG	0.197	0.343	0.577	0.740
5	ACAG/AGAG	0.204	0.353	0.594	0.758
6	ACAG/AGA	0.214	0.367	0.606	0.761
7	AAG/ACGC	0.230	0.395	0.645	0.793
8	AAT/ACGG	0.240	0.414	0.674	0.826
9	AGGG/ACGC	0.256	0.438	0.706	0.855
10	AAC/ACGC	0.268	0.454	0.720	0.864
11	AGGC/ACG	0.276	0.463	0.726	0.865
12	AAG/ACGG	0.287	0.481	0.751	0.882
13	ACG/ACGC	0.297	0.499	0.773	0.905
14	AGGG/ACGG	0.302	0.505	0.779	0.906
15	AGGC/AGAG	0.309	0.514	0.786	0.909
16	AGC/ACGG	0.319	0.534	0.817	0.936
17	AGGC/ACA	0.331	0.552	0.831	0.945
18	ATG/ACGG	0.340	0.559	0.836	0.945
19	AGGG/AGAG	0.347	0.572	0.853	0.954
20	AGGC/AGG	0.354	0.583	0.860	0.954

comprehensive coverage of the *Pinus edulis* genome, estimated at approximately 2,500 cM on the basis of 338 mapped markers distributed over 25 linkage groups. Ninety of these markers either mapped to two equally likely positions on either side of a closely linked marker within their respective linkage group ($n = 83$), or remained unlinked ($n = 7$) and are therefore not shown in Fig. 1. An additional 204 AFLP markers were omitted from the linkage analysis due to non-Mendelian segregation patterns ($n = 33$), failure to map unambiguously to a specific region within a linkage group ($n = 43$), or failure to recombine with 1 or more markers ($n = 128$). Marker saturation of this map exceeds 1 marker per 10 cM; we estimate that a doubling of this level of saturation over 95% of the pinyon genome, i.e., 1 marker per 5 cM, will require the mapping of an additional 346 markers (Lange and Boehnke 1982). We further note that, even with 1 marker per 5 cM on average, there will still be significant gaps in the marker coverage.

Recombinational sizes of conifers have been estimated at near 2,500 cM for a variety of species (Nelson et al. 1993, 1994; Devey et al. 1994; Gerber and Rodophe 1994). Our estimate of an overall recombination

distance of 2,290–2,490 cM in pinyon pine adds further support to these findings. The consistency of this value with expectations also suggests that our data did not suffer from a high frequency of mistyping, which is known to result in multi-point linkages that are generally longer than two-point linkages and inflate estimates of genome size (Lin and Ritland 1996). At any rate, we expect our estimate of genome size to be accurate given the fact that beyond 40 markers, estimates nearly reach asymptotic efficiencies (Lin and Ritland 1996).

An unfortunate consequence associated with the large genome of pinyon pine was an excessive number of amplified fragments resulting from standard $+3/+3$ SRFA reactions during AFLP analysis. Increased amplification frequency is known to bring about a decline in the quality of AFLP patterns due to reduced intensity of the radioisotope used as a signal when spread across a large number of largely-monomorphic fragments (Keim et al. 1997). In order to overcome this difficulty, we developed more restrictive PCR primers by increasing the number of selective nucleotides per PC by one to four bases. Although the relationship between the number of selective nucleotides used and the number of bands produced is not linear for higher plants, Vos et al. (1995) demonstrated that for organisms with simple genomes, such as viruses, the addition of each successive selective nucleotide decreases the number of bands produced by a factor of four. Mackill et al. (1996) found similar results in an AFLP-mapping study of rice (*Oryza sativa*). A second method of reducing the amount of amplification product from AFLP reactions was described by Keim et al. (1997) for soybean (*Glycine max*). Because soybean is characterized by an unusually high A + T content (Zhu et al. 1994), the largest number of fragments are produced by using AT-rich PCs, although there is a concomitant loss of band resolution. We suspect the existence of a similar phenomenon in pinyon pine on the basis of our finding that a significantly greater number of polymorphisms were resolvable, on average, for GC-rich PCs than for AT-rich primers.

The large number of markers which failed to recombine in our study was not initially surprising in light of the fact that a known subset of the PCs used were expected to yield redundant loci, as discussed earlier. However, closer examination of the data revealed that only 5 of 128 redundancies could be explained on this basis. Of the remaining 123 markers, 4 of these appeared to represent codominants because they were in repulsion phase with a second non-segregating marker produced by the same PC. This represents a 1.5% incidence of codominance, which is relatively low compared to the 3% typically reported in other studies utilizing RAPD and AFLP markers (Grattapaglia and Sederoff 1994; Becker et al. 1995). In addition, 16 pairs of redundant markers, each produced by a like combination of primers and displaying only slight variation in size, were suggestive of gene duplications. Finally, 3 pairs of non-segregating markers were found to be produced by non-redundant PCs and yet were within 2 bp of the same size, suggesting amplification by 2 or more PCs via non-specific primer binding. However, given that fragments were scored over a 400-bp range and that there were 272 pairs of non-segregating markers, similarly sized pairs would be expected on the basis of chance alone in 3.40 separate instances, which may be a more parsimonious explanation for the latter result.

The remaining completely linked (redundant) markers which could not be attributed to primer redundancy were likely the result of the relatively small mapping population. A mapping population of size 48 was initially planned, as it fit into the 96-well thermocycler, but the number was reduced to 40 due to poor DNA of some samples. With 40 meiotic products, lack of detectable recombination is expected to occur with probability $(1 - r)^{40}$ for the true recombination rate r. At $r = 0.01$ this probability is 10%, which is quite small, and for r up to 0.10, this probability decreases by about 1% for each additional centiMorgan. Thus, there seems to be excessive clustering ($r < 0.01$). Clustering of markers on linkage maps is a common phenomenon (Tanksley et al. 1992; Vallejos et al. 1992; Reiter et al. 1992; Weissenbach et al. 1992; Giese et al. 1994; Grattapaglia and Sederoff 1994; Keim et al. 1997). Interestingly, it has been proposed that these clusters may represented sites of suppressed recombination in heterochromatin (Tanksley et al. 1992; Keim et al. 1997).

The number of linkage groups formed by our analysis exceeds the chromosome number ($N = 12$) known for pines. However, gaps in the linkage map, resulting in 2 or more linkage groups per chromosome, are common even with large numbers of markers. An early map constructed on the basis of isozyme data by Guries et al. (1978) yielded 5 linkage groups constructed from 10 polymorphic loci in pitch pine (*P. rigida*), while Bahrman and Damerval (1989) detected 12 linkage groups consisting of 119 two-dimensional protein markers in maritime pine (*P. pinaster*), and Nelson et al. (1994) detected 22 linkage groups using 188 RAPD markers in longleaf pine (*P. palustris*). In fact, Guries et al. (1978) predicted 30 independent linkage groups in pines on the basis of approximately 2.5 chiasma per chromosome and a haploid number of 12. In light of this, our result of 338 AFLP markers distributed over 25 linkage groups is not surprising.

Recent attempts to generate densely populated linkage maps in conifers have relied primarily on the use of RAPD markers (Carlson et al. 1991; Nelson et al. 1993, 1994), so it may be informative to compare the efficacy of RAPD versus AFLP for the rapid generation of polymorphic markers. Perhaps the single, most difficult aspect of mapping from RAPD markers is the necessity of pre-screening primers for their ability to generate

polymorphisms. For example, Carlson et al. (1991) found that, on average, 1 set of polymorphisms was revealed for every 3 primers screened in Douglas fir (*Pseudotsuga menziesii*), while Nelson et al. (1993, 1994) found an even lower polymorphism rate in slash pine (*P. elliottii*) – 66 of 420 primers yielded 156 polymorphic markers – and longleaf pine – 128 of 576 primers yielded 200 polymorphic markers. Although this process may be partially automated to boost efficiency to more than 750 RAPD reactions per day following the pre-screening stage (Nelson et al. 1994), and each RAPD primer may be expected to yield an average of up to 2 polymorphisms, we were able to process nearly 200 AFLP reactions per day for an average of 4.30 ± 0.50 polymorphisms, inclusive of unsuccessful combinations, and an extreme upper limit of 35 polymorphisms. In addition, RAPD patterns may not be reproducible, particularly among labs (Penner et al. 1993), while the reliability of AFLP patterns was established while the technique was still in the development stage (Vos et al. 1995), and since that time has been supported through published research (Becker et al. 1995). Finally, RAPD may suffer from a fairly high rate of reaction failure, on the order of 5% (Nelson et al. 1994), while we detected a failure rate of just 2.1% per AFLP PC, inclusive of partial lane failures, or 1.8% per marker. For these reasons, we believe AFLP will become an increasingly important tool in the construction of linkage maps in both cultivated and wild plants, as well as for the purpose of QTL mapping in both basic and applied research.

Implications for studies of pinyon pine

Evidence indicates that the population of pinyon pine in Sunset Crater, Arizona has become genetically differentiated from adjacent populations in response to extreme nutrient- and water-stress (Mopper et al. 1991; Mopper and Whitham 1992; Gehring and Whitham 1994). A greater than twofold reduction in soil moisture (Mopper et al. 1991; Gehring and Whitham 1994), nitrates (Mopper et al. 1991), and phosphates (Gehring and Whitham 1994) has apparently led to a significant increase in the frequency of heterozygotes for glycerate dehydrogenase (GLY) on the cinder field, perhaps as a result of a higher level of metabolic efficiency associated with heterosis (Mopper et al. 1991). In addition, Cobb et al. (1994) found a significant increase in SS homozygotes at the GLY locus between juvenile and adult trees at Sunset Crater, further suggesting an association with water stress.

Evidence also suggests that the stressful conditions at Sunset Crater have reduced the ability of trees to resist defoliation by several conspicuous insect herbivores, although the existence of both susceptible and resistant individuals in this population suggests that genetic variation for resistance is present. Whitham and Mopper (1985) have documented the effects of a stemboring moth, *Dioryctria albovitela*, on susceptible individuals growing at Sunset Crater in the form of high shoot mortality, reduced trunk growth and cone production, and altered tree architecture. A genetic basis for resistance to the moth is supported by a consistent long-term pattern of resistance within individuals, and, more directly, by isozyme frequency data. Mopper et al. (1991) found that resistant trees were heterozygous at the peroxidase (PER) and isocitrate dehydrogenase (IDH) locus at a significantly greater frequency than susceptible trees. The Sunset Crater pinyons also vary in their susceptibility to a Diprionid sawfly, *Neodiprion edulicolis*. Mopper et al. (1990) performed reciprocal transfer experiments between trees resistant and susceptible to *Dioryctria* and found increased egg and larval mortality, and decreased densities of *Neodiprion* on *Dioryctria*-resistant trees. A third insect herbivore known to preferentially attack the Sunset Crater pinyons is a scale insect, *Matsucoccus acalyptus*. Reciprocal transfer experiments similar to those performed for *Neodiprion* demonstrated a decrease in survival on originally uninfested trees and a significantly lower density of adults (Cobb and Whitham 1993; Del Vecchio et al. 1993).

An additional consequence of nutrient- and water-stress at Sunset Crater is evidenced by the altered rate of ectomycorrizal colonization characteristic of individuals in this population. Specifically, a two fold increase in ectomycorrhizal colonization was found among trees growing within the cinder field, a pattern which was seen to hold during the reciprocal transplantation of seedlings between cinder and sandy-loam soils (Gehring and Whitham 1994). This suggests the possibility of a genetic basis for variation in fungal associations which may underlie the evolution of insect resistance since it was found that trees susceptible to *Dioryctria* possess 33% fewer ectomycorrhizae (Gehring and Whitham 1991), while scale-susceptible trees suffer a 28% reduction in ectomycorrhizal colonizational (Del Vecchio et al. 1993). It has been hypothesized that the reduced ability of poorly colonized pinyons to resist attack, if genetic, could result from insufficient nutrient uptake necessary for the defense of above-ground tissues (Gehring and Whitham 1994).

The pinyon AFLP map, when used in conjunction with an optimal subset of PCs, will allow studies of QTLs underlying these adaptive traits. Besides the identification of location and gene effect (additive, dominant, epistatic), one might also track changes in gene frequencies within and among pinyon populations by assaying for tightly linked markers. Although large physical distances are known to separate linked markers in conifers (Meagher et al. 1988), methods now exist for chromosome landing based on bulk segregant analysis using AFLP markers (Ballvora et al. 1995; Meksem et al. 1995; Thomas et al. 1995; Cnops et al. 1996) and should ultimately provide us with the ability

to construct allele-specific probes for QTL of particular interest.

Finally, it may be possible to examine rates of recombination between *Pinus californiarum* and *P. edulis* and to examine select linkage groups for evidence of introgression from *P. californiarum* to *P. edulis*, since both morphological (Lanner 1974; Bailey 1987; Malusa 1992; Lanner and Phillips 1992; Christensen et al. 1995) and molecular data (Lahood and Keim, unpublished data) support the existence of extensive hybridization between these two species. On the basis of evidence from chloroplast DNA, we speculate that genes from dry-adapted *P. californiarum* populations associated with adaptations to water stress have begun to introgess preferentially into the Sunset Crater *P. edulis* population. Our AFLP map will make such studies a reality.

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