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Linkage of Random Amplified Polymorphic DNA Markers in *Pinus Halepensis* MILL.

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Abstract

A genetic linkage analysis involving 60 random decamer primers in Aleppo pine (*Pinus halepensis* MILL.), one of the most important Mediterranean conifers, is reported. Five trees originating from five natural Spanish populations and 40 haploid megagametophytes per tree were investigated based on joint segregation and independent assortment. Twenty-two decamers were selected for their stable and repeatable banding patterns and 10 of these produced 24 polymorphic loci that presented Mendelian inheritance. Some degree of segregation distortion was evident in 16% of the loci tested. A total of 155 linkage tests were executed based on LOD-scores and χ^2 contingency tables. Six linkage groups that include 13 loci were detected: $OPA01_{750}$: $OPP04_{1200}$: $OPP04_{930}$, $OPA11_{2500}$: $OPA11_{950}$, $OPA19_{1150}$: $OPA19_{1090}$, $OPN06_{690}$: $OPN06_{420}$, $OPN12_{450}$: $OPN12_{300}$ and $OPP10_{640}$: $OPP10_{600}$. Recombination frequencies were homogeneous across trees and chromosomal interference was found to be negative. Total consensus genetic map length ranged from 175 cM to 225 cM depending on the mapping function used. This is the first linkage study in this species using RAPD markers and single tree megagametophytes.

Key words: Pinus halepensis, RAPD, Mendelian inheritance, linkage test, gene mapping.

1. Introduction

Linkage analysis is the basis for the establishment of a genetic linkage map. Linkage maps are useful for different genetic studies: location of genes controlling important traits in plants, analysis of genetic variation in natural populations, taxonomy, evolution, etc. Traditionally, genetic linkage maps have been based on biochemical markers such as isoenzymes

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(STRAUSS and CONKLE, 1986; ARAVANOPOULOS, 1998) or in DNA markers such as restriction fragment length polymorphisms (RFLPs, DEVEY *et al.,* 1994).

In conifers, the use of RFLPs may be complicated by the large size of the genome, the lack of pedigrees and the long generation times (TULSIERAM *et al.,* 1992). The advent of the random amplified polymorphic DNA markers (RAPDs), provided a novel source of markers for mapping. Nevertheless, due to their dominance, the megagametophyte haploid tissue is preferred as the DNA source; diploid tissue can be employed but with a loss of genetic information. The megagametophyte has a maternal origin and is derived from a single meiotic product (BARTELS, 1971), so it is possible to perform linkage studies in single trees without the need of controlled crosses. RAPD markers have demonstrated their utility in establishing genomic maps in conifers and particularly in pines (NELSON *et al.,* 1993; KUBISIAK *et al.,* 1995; KONDO *et al.,* 2000). The RAPD markers, which may be used in mapping, should be chosen by their repeatability, polymorphism and Mendelian inheritance (GRATTAPAGLIA and SEDEROFF, 1994; PLOMION *et al.,* 1995). Hence, it is very important to select primers that, in addition to being polymorphic, produce clear and intense bands (FURMAN *et al.,* 1997). The first use of RAPD markers to construct a single tree genetic linkage map from megagametophytes was reported by TULSIERAM *et al.,* (1992) in *Picea glauca*.

The objectives of this study were: (a) to investigate the repeatability and inheritance of RAPD markers and (b) to estimate linkage relationships among RAPD markers that were stable, repeatable and presented Mendelian mode of inheritance, using haploid tissue from single tree megagametophytes in Aleppo pine (*Pinus halepensis* MILL.). Aleppo pine is one of the most important low elevation Mediterranean species. It is a fast growing, highly adaptable conifer with considerable drought resistance distributed over 3.5 million Ha in the Mediterranean region. It is an economically important species for which breeding programs are under way.

2. Materials and Methods

2.1 plant material and DNA extraction

Seeds were collected from five single trees each originating from a different unrelated population (*Table 1*). Each tree was identified by a code (11-5, 61-4, 103-3, 141-1, and 183-3) and the seeds were stored at 4 °C until the DNA isolation. After 24 hours in water, the megagametophytes (endosperms) were isolated by removing the coats and the embryo. DNA from 40 megagametophytes of each tree was extracted according to DOYLE and DOYLE (1990).

2.2 DNA amplification

The DNA amplification by polymerase chain reaction (PCR) was carried out in a Perkin Elmer 9600 Thermocycler. The profile for the amplification consisted of: (a) one cycle of 2 minutes at 94 °C, 1 minute at 42 °C and 2 minutes at 72 °C, (b) 4 cycles of 45 seconds at 94 °C, 1 minute at 42 °C and 2 minutes at 72 °C, (c) 40 cycles of 30 seconds at 94 °C, 45 seconds at 36 °C and 1 minute and 30 seconds at 72 °C and (d) a cycle of 8 minutes at 72 °C. The first four cycles employed an annealing temperature of 42 °C in order to select bands (WOLFF, 1994) and to facilitate repetitiveness of the results. The reaction mixture volume was 25 µl and contained: *Taq* DNA polymerase (Pharmacia) 0,5 Units, KCl 50 mM, $MgCl_2$ 2,5 mM, Tris-HCl pH 9 10 mM, DNA sample 25 ng, primer 200 mM, dNTPs 200 mM of each. The reactions were visualised under UV light, after running in 1,5 % agarose gels in 1 x TAE buffer and ethidium bromide staining.

Table 1. – Location of natural Spanish populations from which single trees were selected to study linkage analysis of RAPD markers.

Population	*Latitude*	*Longitude*	*Altitude (m)*	*Tree code*
Cabanellas	42º14'08''N	2º47'24''E	210	11-5
Zuera	41º55'00''N	0º55'40''W	575	61-4
Villa de Ves	39º10'44''N	1º14'52''W	850	103-3
Ricote	38º08'33''W	11º25'50''W	780	141-1
S'Avall	39º17'14''N	3º02'52''E	10	183-3

2.3 primer screening

A total of 60 primers (random decamers) that belong to the Kits A, N and P of Operon Technologies were screened for satisfactory amplification products with DNA from 9 seeds (2 seeds from tree 11-5, 1 from 61-4, 2 from 141-1, 2 from 103-3, 1 from 172-3 and 2 from 183-3). In order to choose primers for polymorphic products the selected primers were employed to amplify DNA from 6 seeds of each tree (probability of type-I error in heterozygote detection p=0.03). To analyse the Mendelian inheritance 40 megagametophytes from each tree were tested with the polymorphic primers. The distribution of the observed "progeny" genotypes was compared with the expected distribution based on the "parental" genotypes. Mendelian inheritance was examined by testing the "goodness of fit" to the expected ratios. The chi-square test was used. The consistency of the results over different trees was tested by computing heterogeneity chi-square (SOKAL and ROHLF, 1981).

The RAPD markers were named according to the convention used by PARAN *et al.* (1991). The primer name and a subscript indicating the size of the fragment starting from the most anodal banding phenotype and progressing sequentially in the cathodal direction, were employed. For example, the first primer in Operon Kit A yielding a RAPD fragment with a size of 750bp would be termed "$OPA\text{-}1_{750}$".

2.4 estimation of linkage, recombination fractions and mapping distances

Forty meiotic products (megagametophytes) in each of five *Pinus halepensis* trees were employed. In total 24 RAPD loci were scored (*Table 2*). The data were arranged as a pseudo-testcross in order to test linkage. The five trees were converted to five fictitious full-sib families. The genotype of the fictitious maternal parent of the family was inferred from the segregation observed among megagametophytes. A fictitious paternal parent that was homozygous at all loci of a heterozygous progeny was also created. The use of trees (families) originating from unrelated populations permitted the investigation of the stability of locus segregation and linkage, as well as the potential development of a consensus linkage map. All of the RAPD loci used and the *P. halepensis* trees employed, were also employed in a population genetics study (GOMEZ *et al.,* 1999). Care was taken to ensure that these loci were the same (in terms of RAPD bands molecular weight) across populations and trees studied, a prerequisite for the consideration of a consensus linkage map.

Suspect linkage groups were investigated with the LINKEM (VOWDEN *et al.,* 1995) software. This program, employs the -loglikelihood ratio test (LOD-score) for linkage, as well as contingency table χ^2 tests. In double heterozygotes, unbalanced segregation in one pair of alleles does not affect the χ^2 analysis of joint segregation (BAILEY, 1961), but does not permit the use of the likelihood ratio linkage test. Therefore in such cases

Table 2. – Single locus segregation of polymorphic RAPD markers in megagametophytes of *P. halepensis* individuals and (2 tests to determine their Mendelian inheritance.

Locus	Tree	Observed Progeny		Expected Progeny		X^2 (df)	$P>X^2$
		±	=	±	=		
$OPA01_{750}$	61-4	19	21	20	20	0.100 (1)	0.750
$OPA01_{520}$	61-4	24	16	20	20	1.600 (1)	0.210
$OPA07_{700}$	141-1	20	20	20	20	0.000 (1)	1.000
	183-3	18	22	20	20	0.400 (1)	0.530
	Pooled	38	42	40	40	0.200 (1)	0.50<p<0.70
	Heterogeneity					0.200 (1)	0.50<p<0.70
$OPA075_{510}$	141-1	15	25	20	20	2.500 (1)	0.110
$OPA11_{2500}$	183-3	21	19	20	20	0.100 (1)	0.750
$OPA11_{950}$	103-3	18	22	20	20	0.400 (1)	0.530
	183-3	21	19	20	20	0.100 (1)	0.750
	Pooled	39	41	20	20	0.050 (1)	0.80<p<0.90
	Heterogeneity					0.450 (1)	0.50<p<0.70
$OPA11_{680}$	183-3	17	23	20	20	0.900 (1)	0.340
$OPA18_{780}$	61-4	22	18	20	20	0.400 (1)	0.530
	103-3	20	20	20	20	0.000 (1)	1.000
	Pooled	42	38	40	40	0.200 (1)	0.50<p<0.70
	Heterogeneity					0.200 (1)	0.50<p<0.70
$OPA19_{1150}$	61-4	30	10	20	20	10.000 (1)	0.002
	141-1	21	19	20	20	0.100 (1)	0.750
	Pooled	51	29	40	40	6.050 (1)	0.01<p<0.02
	Heterogeneity					4.050 (1)	0.025<p<0.05
$OPA19_{1090}$	61-4	36	4	20	20	25.60 (1)	<0.001
	103-3	17	23	20	20	0.900 (1)	0.340
	141-1	17	23	20	20	0.900 (1)	0.340
	Pooled	70	50	60	60	3.333 (1)	0.05<p<0.10
	Heterogeneity					24.067 (1)	<0.001
$OPA19_{530}$	103-3	20	20	20	20	0.000 (1)	1.000
	141-1	18	22	20	20	0.400 (1)	0.530
	Pooled	38	42	40	40	0.200 (1)	0.50<p<0.70
	Heterogeneity					0.200 (1)	0.50<p<0.70
$OPN06_{690}$	11-5	18	22	20	20	0.400 (1)	0.530
$OPN06_{440}$	11-5	17	23	20	20	0.900 (1)	0.340
	141-1	17	23	20	20	0.900 (1)	0.340
	Pooled	34	46	40	40	1.800 (1)	0.10<p<0.20
	Heterogeneity					0.000 (1)	1.000
$OPN06_{420}$	11-5	21	19	20	20	0.100 (1)	0.750
$OPN12_{450}$	103-3	18	22	20	20	0.400 (1)	0.530
$OPN12_{300}$	103-3	18	22	20	20	0.400 (1)	0.530
$OPP01_{600}$	11-5	23	17	20	20	0.900 (1)	0.340
	61-4	15	25	20	20	2.500 (1)	0.110
	141-1	16	24	20	20	1.600 (1)	0.210
	Pooled	54	66	60	60	1.200 (1)	0.20<p<0.25
	Heterogeneity					3.800 (1)	0.05<p<0.10
$OPP04_{1200}$	61-4	25	15	20	20	2.500 (1)	0.110
	103-3	25	15	20	20	2.500 (1)	0.110
	183-3	22	18	20	20	0.400 (1)	0.530
	Pooled	72	48	60	60	4.800 (1)	0.025<p<0.05
	Heterogeneity					0.600 (1)	0.30<p<0.50
$OPP04_{930}$	11-5	24	16	20	20	1.600 (1)	0.210
	61-4	18	22	20	20	0.400 (1)	0.530
	183-3	22	18	20	20	0.400 (1)	0.530
	Pooled	64	56	60	60	0.530 (1)	0.30<p<0.50
	Heterogeneity					1.870 (1)	0.10<p<0.20
$OPP04_{690}$	11-5	20	20	20	20	0.000 (1)	1.000
	183-3	26	14	20	20	3.600 (1)	0.060
	Pooled	46	34	40	40	1.800 (1)	0.10<p<0.20
	Heterogeneity					1.800 (1)	0.10<p<0.20
$OPP04_{400}$	11-5	38	2	20	20	32.400 (1)	<0.001
	183-3	20	20	20	20	0.000 (1)	1.000
	Pooled	58	22	40	40	16.200 (1)	<0.001
	Heterogeneity					16.200 (1)	<0.001
$OPP10_{1000}$	103-3	20	20	20	20	0.000 (1)	1.000
$OPP10_{640}$	11-5	37	3	20	20	28.900 (1)	<0.001
	61-4	20	20	20	20	0.000 (1)	1.000
	103-3	36	4	20	20	25.600 (1)	<0.001
	Pooled	56	24	40	40	12.800 (1)	<0.001
	Heterogeneity					41.700 (1)	<0.001
$OPP10_{600}$	11-5	4	36	20	20	25.600 (1)	<0.001
	61-4	17	23	20	20	0.900 (1)	0.340
	Pooled	21	59	40	40	18.050 (1)	<0.001
	Heterogeneity					8.450 (1)	0.005<p<0.001

linkage were investigated only by the contingency table χ^2 test approach. The predetermined threshold level for LOD was set to 1.80, a decision based on the number of possible locus pair tests and the observation that higher levels appear to provide a very conservative test for linkage (GERBER and RODOLFE, 1994). Furthermore it was decided not to consider linkage when: (a) the recombination fraction was θ>0.30, and (b) the value θ = 0.50 was included in the confidence interval of the recombination fraction.

When a locus pair segregated in more than one family (tree) then the test for linkage and the estimation of recombination frequencies was conducted from individual trees, as well as jointly from all available trees. Pooling data from different trees produces more robust tests when compared to estimates of the total χ^2 value across trees due to the reduction in the degrees of freedom, nevertheless because of the uncertainty of correct parental phase there is a small chance that pooling may mask true association among locus pairs. Homogeneity of recombination were based on the following statistics: (a) homogeneity of LOD- scores according to MORTON (1965) and BEAVIS and GRANT (1991):

$$X_r^2 = (2\ln 10) \sum_{i=1}^{N} [z_i(\theta) - z_p(\theta)]$$

where z_i and z_P are the respective LOD scores, given the maximum likelihood estimates of recombination for the ith tree and for data pooled from all N trees, and (b) homogeneity of recombination frequencies according to BAILEY (1961):

$$X_\theta^2 = \sum_{i=1}^{N} \frac{n_i(\theta_i - \theta_w)^2}{\theta_w(1-\theta_w)}$$

where N is the number of trees, n_I is the number of megagametophytes of the ith tree, θ_i is the recombination frequency in the ith family, and θ_w is the weighted average recombination frequency according to COLQUHOUN (1971).

In gene mapping, methods of statistical analysis are employed to deduce relationships among the physical phenomena of chiasma formation, crossing over, and the actual distance between two genes. If n loci belong to the same linkage group the $n(n$-1)/2 recombination fractions θ can be reduced to n-1 map intervals w by a suitable mapping function w = f(θ), which is the *sine qua non* for a linkage map. The function suggested by KOSAMBI (1944), with a standard error according to OWEN (1950) and the function suggested by HALDANE (1919) were calculated. In order to estimate the impact of chiasma interference, the coefficient of coincidence (C) and its variance was calculated according to BAILEY (1961).

3. Results and Discussion

3.1 screening, identification, polymorphisms and inheritance of RAPD markers

Sixty oligonucleotide primers were screened in order to select those that produced clear and intense bands. In total, 8 primers (12.9 %) failed to amplify and 22 (36.7 %) were selected by their clear and repeatable banding pattern. These 22 primers were then used in PCR amplifications in order to investigate for scorable polymorphisms among the megagametophytes of single trees. Amplification products varied ranging from three to nine bands. Out of 22 primers, 10 (45.5 %) were found to reveal variation. A total of 24 polymorphic stable and repeatable RAPD bands were identified (2.4 polymorphic bands/primer). For the investigation of Mendelian inheritance, 44 chi-square tests were conducted. The segregation ratio of these 24 RAPD polymorphisms was in agreement with the expected 1:1 segregation in megagametophytes (*Table 2*). Nevertheless, significant segregation distortion was observed in seven cases (15.9 %). Segregation distortion did not appear to be random across trees. Two trees (141-1, 183-3) did not present any distortion in 15 loci. On the contrary, one tree (11-5), showed segregation distortion in 40 % of the loci studied, while

Table 3. – Number of analysed trees (above the diagonal) and total number of megagametophytes from each pair of polymorphic loci (below the diagonal).

	A01	*A01*	*A07*	*A07*	*A11*	*A11*	*A11*	*A18*	*A19*	*A19*	*A19*	*N06*	*N06*	*N06*	*N12*	*N12*	*P01*	*P04*	*P04*	*P04*	*P04*	*P10*	*P10*	*P10*
	750	*520*	*700*	*510*	*2500*	*950*	*680*	*780*	*1150*	*1090*	*530*	*690*	*440*	*420*	*450*	*300*	*600*	*1200*	*930*	*690*	*400*	*1000*	*640*	*600*
OPA01$_{750}$		2	0	0	0	0	0	1	1	2	0	0	0	0	0	0	2	1	1	0	0	1	2	2
OPA01$_{520}$	80		0	0	0	0	0	1	1	2	0	0	0	0	0	0	2	1	1	0	0	1	2	2
OPA07$_{700}$	0	0		1	1	1	1	0	1	1	1	0	1	0	0	0	1	1	1	1	1	0	0	0
OPA07$_{510}$	0	0	40		0	0	0	0	1	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0
OPA11$_{2500}$	0	0	40	0		1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0
OPA11$_{950}$	0	0	40	0	40		1	1	0	1	1	0	0	0	1	1	0	2	1	1	1	1	1	0
OPA11$_{680}$	0	0	40	0	40	40		0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0
OPA18$_{780}$	40	40	0	0	0	40	0		1	2	1	0	0	0	1	1	1	2	1	0	0	1	2	1
OPA19$_{1150}$	40	40	40	40	0	0	0	40		2	1	0	1	0	0	0	2	1	1	0	0	0	1	1
OPA19$_{1090}$	80	80	40	40	0	40	0	80	80		2	0	1	0	1	1	3	2	1	0	0	2	3	2
OPA19$_{530}$	0	0	40	40	0	40	0	40	40	80		0	1	0	1	1	1	1	0	0	0	1	1	0
OPN06$_{690}$	0	0	0	0	0	0	0	0	0	0	0		1	1	0	0	1	1	1	1	1	0	1	1
OPN06$_{440}$	0	0	40	40	0	0	0	0	40	40	40	40		1	0	0	2	1	1	1	1	0	1	1
OPN06$_{420}$	0	0	0	0	0	0	0	0	0	0	0	40	40		0	0	1	1	1	1	1	0	1	1
OPN12$_{450}$	0	0	0	0	0	40	0	40	0	40	40	0	0	0		1	0	1	0	0	0	1	1	0
OPN12$_{300}$	0	0	0	0	0	40	0	40	0	40	40	0	0	0	40		0	1	0	0	0	1	1	0
OPP01$_{600}$	80	80	40	40	0	0	0	40	80	120	40	40	80	40	0	0		2	2	1	1	1	3	3
OPP04$_{1200}$	40	40	40	0	40	80	40	80	40	80	40	40	40	40	40	40	80		3	2	2	1	3	2
OPP04$_{930}$	40	40	40	0	40	40	40	40	40	40	0	40	40	40	0	0	80	120		2	2	0	2	2
OPP04$_{690}$	0	0	40	0	40	40	40	0	0	0	0	40	40	40	0	0	40	80	80		2	0	1	1
OPP04$_{400}$	0	0	40	0	40	40	40	0	0	0	0	40	40	40	0	0	40	80	80	80		0	1	1
OPP10$_{1000}$	40	40	0	0	0	40	0	40	0	80	40	0	0	0	40	40	40	40	0	0	0		2	1
OPP10$_{640}$	80	80	0	0	0	40	0	80	40	120	40	40	40	40	40	40	120	120	80	40	40	80		3
OPP10$_{600}$	80	80	0	0	0	0	0	40	40	80	0	40	40	40	0	0	120	80	80	40	40	40	120	

Table 4. – Linkage groups, LOD values, associated probabilities, recombination fractions (θ), standard errors (s) and associated confidence intervals (CI) for each tree and jointly, Kosambi mapping distances (*D*) and associated standard errors (s_D) in cM.

Loci	*Tree*	*LOD*	P_{LOD}	θ	*s*	*CI*	*D*	s_D
$OPN06_{690}$:$OPN06_{420}$	11-5	1.82	0.002	0.280	0.071	(0.15-0.42)	31.64	10.34
$OPP04_{1200}$:$OPP04_{930}$	11-5+61-4+183-3	6.82	<0.0001	0.250	0.040	(0.18-0.33)	27.47	5.33
$OPA01_{750}$:$OPP04_{1200}$	61-4	-	-	0.250	0.068	(0.13-0.40)	27.47	9.07
$OPA01_{750}$:$OPP04_{930}$	61-4	1.82	0.002	0.280	0.071	(0.15-0.42)	31.64	10.34
$OPP10_{640}$:$OPP10_{600}$	61-4	2.78	0.0002	0.230	0.066	(0.12-0.37)	24.87	8.37
$OPN12_{450}$:$OPN12_{300}$	103-3	2.27	0.0006	0.250	0.068	(0.13-0.40)	27.47	9.07
$OPA19_{1150}$:$OPA19_{1090}$	141-1	2.27	0.0006	0.250	0.068	(0.13-0.40)	27.47	9.07
$OPA11_{2500}$:$OPA11_{950}$	183-3	1.82	0.002	0.280	0.071	(0.15-0.42)	31.64	10.34

the percentage was 20 % for tree 61-4 and 10 % for tree 103-3. Polymorphisms that showed significant departures from 1:1 are often found in RFLPs, RAPDs and isoenzymes, in crops and forest trees (TULSIERAM *et al.,* 1991; ARAVANOPOULOS *et al.,* 1994), but the causes of this phenomenon are not well known. Segregation distortion may be due to chance (for instance due to sampling error), or may be due to linkage to other loci that can distort segregation evidently due to some form of prezygotic or post-zygotic selection (ARAVANOPOULOS *et al.,* 1994). Heterogeneity in the segregation patterns of a particular locus across different trees was observed in 38 % of the 14 cases that could be investigated (*Table 2*). The majority of the trees studied were not different from each other with respect to the genotypic frequency distributions that their megagametophytes produce and the heterogeneity was mainly induced by the significant segregation distortion concentrated in trees 11-5 and 61-4 (*Table 2*). In general, results show that a high percentage of RAPD markers (84.1 %) comply with Mendelian inheritance expectations. These markers are now available for other studies that would include RAPD analysis.

3.2 estimation of linkage parameters

There were 276 possible two-locus combinations, which can be formed using the 24 loci employed, from which 151 (55 %) could be tested. The number of trees (families) employed and the total number of genotypes used in the linkage analysis is presented in *Table 3*. In individual linkage tests the number of megagametophytes used ranged from 40 to 120. Every two-locus combination was tested in an average of 1.3 trees and by employing an average of 52 megagametophytes. Significant segregation distortion was observed for seven loci in three tress, resulting in the exclusion of seven locus pair combinations in two trees from the analysis.

The potential pairs of linked loci are presented in *Table 4*. Eight pairs of loci were found to be linked forming six linkage groups and involving a total of 13 loci. Therefore 54 % of the available markers were mapped. Recombination frequencies for the $OPP04_{1200}$:$OPP04_{930}$ locus pair were calculated from individual trees (11-5, 6-4 and 183-3), as well as from pooled data. Homogeneity of recombination across trees was inferred from both tests employed (X_r^2 = 0.53, 0.490<p<0.520; X_θ^2 = 2.799, 0.050<p<0.100). The absence of recombination frequency heterogeneity and the examination of expected parental phase, which indicated that it was the same across trees (data not shown), prompted the use of the pooled data set results.

One linkage group involved three loci ($OPA01_{750}$, $OPP04_{1200}$, $OPP04_{930}$) and the rest two loci ($OPA11_{2500}$: $OPA11_{950}$; $OPA19_{1150}$: $OPA19_{1090}$; $OPN06_{690}$: $OPN06_{420}$; $OPN12_{450}$: $OPN12_{300}$; $OPP10_{640}$: $OPP10_{600}$). The pair $OPP04_{1200}$: $OPP04_{400}$ was found linked in tree 183-3, but not linked in tree 11-5. The pooled data set indicated weak linkage (r=0.390, p=0.030) and the group was excluded from further consideration. The rest of the loci were not linked as may be expected by genome size and the random sampling of markers. The KOSAMBI mapping distances associated with the inferred groups are also depicted in *Table 4*. The consensus total map length spans to about 175 cM (average marker spacing of the map: 13.5 cM, average size of a linkage group: 29.12 cM). If the HALDANE mapping function is employed, then the above value is increased to 225 cM. Thus the current set of 13 loci is apparently distributed over approximately 8-11 % of the genome. This is a conservative estimate based on the assumption that a conifer genome expands to some 20 M (ECHT and NELSON, 1997). It has been estimated that some 200 to 300 primers would be needed in order to cover a 20-30 M map (NELSON *et al.,* 1993). For example, KUBISIAK *et al.* (1995) screened 288 primers to account for 2392 cM in *P. palustris;* ECHT and NELSON (1997) 288 primers for 2242 cM in *P. strobus*; NELSON *et al.* (1993) 420 primers for 2160 cM in *P. elliottii*; PLOMION *et al.* (1995) 520 primers for 1380 cM in *P. pinaster*; LI and YEH (2001) 110 primers for 2287 cM in *P. contorta*; and KONDO *et al.* (2000) 208 primers for 1870 cM in *P. thunbergii.* Further options for high-density mapping include the increase of number and type of genetic markers as well as sample size, and the use of bulk segregant analysis, which would facilitate the discovery of polymorphic and potentially linked markers.

The presence of a group with three consecutively linked loci permitted the estimation of interference parameters. The average estimate of the coefficient was C=1.760 (s_c=0.328) and reflected an overall negative interference. Similar interference levels have been reported in other conifers as well (STRAUSS and CONKLE, 1986).

This is the first linkage analysis reported in this species using RAPD markers and single tree megagametophytes. The importance of these results is founded on the use of a genetic map in the study of population biology, evolutionary studies, and its potential application in molecular breeding and marker-assisted selection. Linked markers can form reference points, which will assist in further gene mapping. Evidently the genetic map will assist in the identification of implicated genomic areas in the natural or artificial selection process.

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Construction of Genome Map for *Eucalyptus camaldulensis* DEHN

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Abstract

A linkage genome map for *Eucalyptus camaldulensis* DEHN was constructed using segregation data from a 92 full-sib progeny. The linkage map was based on Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP) and nuclear Microsatellite (SSR) markers. Marker data were analyzed for linkage using mapping software JOINMAP version 2.0. Linkage analysis resulted in 168 markers covering 1236 cM of the genome. The number of linkage groups determined was equivalent the haploid number of chromosomes (n=11) of *Eucalyptus*. This map can be used to define regions of the genome that are associated with important traits in *E. camaldulensis* and other Eucalyptus species such as wood and oil quality.

Key words: Eucalyptus camaldulensis, genome map, linkage, molecular, SSR, RFLP, River red gum

Introduction

Eucalyptus camaldulensis DEHN is one of several *Eucalyptus* native from Australia, hardy, fast-growing evergreen tree,

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