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AFLP genetic maps of *Eucalyptus globulus* and *E. tereticornis*

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Abstract Amplified fragment length polymorphism (AFLP) analysis is a rapid and efficient technique for detecting large numbers of DNA markers in eucalypts. We have used AFLP markers in a two-way pseudo-testcross strategy to generate genetic maps of two clones of different *Eucalyptus* species (*E. tereticornis* and *E. globulus*). Of 606 polymorphic fragments scored, 487 segregated in a 1:1 ratio, corresponding to DNA polymorphisms heterozygous in one parent and null in the other. In the maternal *E. tereticornis* map, 268 markers were ordered in 14 linkage groups (919 cM); the paternal *E. globulus* map had 200 markers in 16 linkage groups (967 cM). Results from PGRI software were compared with MAPMAKER. The average density of markers was approximately 1 per 3.9 cM. Framework markers were ordered with an average confidence level of 90%, covering 80–100% of the estimated *Eucalyptus* genome size. In order to investigate the homologies between the *E. tereticornis* and the *E. globulus* genetic linkage maps, we included 19 markers segregating 3:1 in the analysis. Some homeologous linkage groups were recognized. The linkage data developed in these maps will be used to detect loci controlling commercially important traits.

Key words Genetic map · Linkage · *Eucalyptus* · AFLP marker

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Introduction

The development of polymerase chain reaction (PCR)-based genetic marker techniques has dramatically changed the pace of genetic mapping studies of forest species. PCR-based methods reduce the cost of identifying genetic markers and allow large-scale genotyping of individuals at many loci (Ferreira and Grattapaglia 1995). Molecular markers can be used in fundamental genetic research and tree improvement activities that include population management, marker-assisted breeding and marker-assisted selection.

Eucalypts are widely used for plantation forestry in tropical and subtropical regions of the world (Eldridge et al. 1994). They are predominantly outcrossing, highly heterogeneous and genetically diverse (Moran and Bell 1983). Most *Eucalyptus* breeding programs are based on recurrent selection and/or interspecific hybridization. Linkage disequilibrium, generated by hybridization, and the possibility of capturing non-additive genetic variance through clonal propagation greatly enhance the possibility of using genetic linkage maps for accelerating breeding through marker-assisted selection and recombination (Grattapaglia and Sederoff 1994).

Map construction in highly heterozygous plant species can make use of informative genetic markers that segregate 1:1 for the presence or absence of a DNA fragment in an F_1 progeny set (Ritter et al. 1990). Highly informative dominant polymorphic markers that are heterozygous in one parent and null in the other are chosen. Two sets of markers can be obtained, one for each parent (Grattapaglia and Sederoff 1994).

The AFLP (Amplified Fragment Length Polymorphism) assay is a powerful novel technique for genetic fingerprinting, genome mapping and genetic variability studies (Zabeau and Vos 1993; Vos et al. 1995; Maughan et al. 1996; Maheswaran et al. 1997). In the

AFLP analysis, a large number of loci are sampled in high-resolution sequencing gels. This allows the construction of high-density genetic maps for individual trees with a small number of oligonucleotide primers and minute amounts of DNA. AFLP markers are typically dominant, but codominant markers that vary in size and intensity can also be identified. The AFLP technique is based on the selective amplification of genomic restriction fragments using PCR. Prior to PCR amplification, genomic DNA is digested by two restriction enzymes, and oligonucleotide adapters are ligated to the restriction fragments to generate template DNA for subsequent PCR. Addition of extra nucleotides at the 3′ end of the PCR primers complementary to the adapter sequences allows the selective amplification of only a subset of the restriction fragments. AFLP fingerprints consist predominantly of unique fragments, thus sampling genomic variation mostly in coding and low-copy regions. Most AFLP fragments correspond to unique positions on the genome and, hence, can be exploited as landmarks in genetic and physical mapping (Thomas et al. 1995; Vos et al. 1995; Meksem et al. 1995).

Molecular marker-based linkage maps have been useful for identifying and localizing important genes controlling both qualitatively and quantitatively inherited traits in a wide range of plant species (Tanksley et al. 1989). Early assumptions about the genetic architecture of quantitative traits have been challenged with data indicating that major gene effects are not rare in forest trees (Grattapaglia et al. 1996; O'Malley et al. 1996). The ability to quickly map individual trees and the incorporation of molecular marker technologies into existing breeding programs can increase the efficiency of breeding and selection programs. We report here on the application of AFLP markers to genome mapping of a clone of *E. tereticornis* and a clone of *E. globulus*, which are part of an ongoing genetic improvement program.

Materials and methods

Plant material

The starting mapping population consisted of 91 F_1 progeny from a controlled cross between an *E. tereticornis* (clone TT Esc 87/90) seed parent and an *E. globulus* (clone GB MJ 6/90) pollen parent.

DNA extraction

Total genomic DNA was isolated from frozen leaf tissue following the Doyle and Doyle (1987) protocol, modified by the addition of 1% polyvinylpyrrolidone (avg. mol wt 40,000) and 1% 2-mercaptoethanol to the extraction buffer (Grattapaglia and Sederoff 1994). DNA concentration was estimated by comparison of the fluorescence intensities of ethidium bromide-stained samples to those of λ DNA standards, on 0.8% agarose gels.

AFLP protocol

The AFLP method was performed essentially as described in Vos et al. 1995, with minor adaptations for eucalypt DNA.

Template preparation

In 0.5 ml eppendorf tubes, 250 ng of genomic DNA from the parents and each of their 91 progeny was simultaneously digested for 3 h at 37°C with 12 U of *Eco*RI and 8 U *Mse*I (Vos et al. 1995). To ensure complete digestion, we visually assessed each individual sample on an 0.8% agarose gel and compared it to a 1 kb DNA size marker (Gibco-BRL). To each restriction digest, 10 µl of ligation mixture was added, as in Vos et al. (1995). Ligation reactions were incubated at 37°C for 2.5 h and then held at 4°C overnight. They were subsequently, diluted five- to tenfold, in sterile distilled water to adjust the samples to equivalent DNA concentrations.

Selective pre-amplification of primary template DNA

One sixteenth of the total pool of genomic restriction fragments was selectively enriched through PCR amplification. The sequence used to prime synthesis from the *Eco*RI end, with one additional 3′ nucleotide (*Eco* + 1 primer) was: 5′-GAC TGC GTA CCA ATT C(A). The sequence of the selective Mse + 1 primer was: 5′-GAT GAG TCC TGA GTA A(C). An alternative *Mse* + 1 primer was also tested: 5′-GAT GAG TCC TGA GTA A(A). Pre-amplification reactions (60 µl) were performed in 96-well polypropylene plates (Vos et al. 1995), using a MJ Research Programmable Thermal Controller with a heated lid. Thermocycling conditions were adjusted to promote selectivity: 28 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 60 s (Cervera et al. 1996). No mineral oil was used in the reactions. A 4 µl aliquot was removed from each sample following thermocycling and run on an 0.8% agarose gel to determine whether pre-amplification was successful. The remaining volume was diluted 5–20 fold in water, according to the intensity of the smear, to adjust the samples to equivalent DNA concentrations (Cervera et al. 1996).

Selective restriction fragment amplification

A screening of 12 primer combinations with two to six additional 3′ nucleotides (complementary to both the *Eco* and *Mse* adapters) was carried out to determine the most appropriate number of selective nucleotides for the eucalypt AFLP analyses. The primer complementary to the *Eco*RI adapter was end-labeled with γ-[^{33}P]-ATP as in Vos et al. (1995). Final amplifications were performed in 96-well PVC plates, overlaid with 50 µl of mineral oil and cycled in a MJ Research Programmable Thermal Controller (12 cycles of 94°C for 30 s, 60°C for 30 s decreasing by 0.7°C/cycle, 72°C for 60 s; followed by 22 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s). Each DNA sample was tested beforehand in two independent final amplifications to check the reproducibility of the reactions.

Separation of labeled fragments and autoradiography

Previously warmed 1 × TBE buffer was used to pre-run 0.4 mm-thick 5% polyacrylamide denaturing gels in BioRad (38 × 50 cm) sequencing apparatus, for 15 min. Reaction products were mixed with an equal volume (20 µl) of formamide dye (Vos et al. 1995), denatured (90°C, 3 min) and rapidly cooled to 4°C. All reactions (3 µl) from a microtitre plate were loaded onto a single sequencing gel. Samples were flanked by a [^{33}P]-labeled 10 bp DNA ladder and run at 70 W until the bromophenol blue dye reached the end of the

glass plate. Gels were transferred to 3 MM (Whatman) filter paper, covered with Saran wrap, dried under vacuum (80°C, 20 min) and exposed to standard X-ray film without intensifying screens (average of 3 days) at room temperature.

Data analysis

Mapping data were obtained by visual scoring of autoradiograms, independently by two readers. Only clear, unambiguous bands were scored. Goodness of fit of observed-to-expected allelic ratios was analyzed using a χ^2 test. Markers were defined as polymorphic fragments that did not significantly depart from Mendelian ratios at the $\alpha = 0.01$ level. Markers segregating 1 : 1 were used for the construction of both linkage maps. DNA polymorphisms that showed segregation-ratio distortion (statistically significant differences in the proportion of alleles) were analyzed separately. The data for the 3 : 1 segregating markers were later integrated into the analysis to compare the female and male linkage maps.

Map construction

Heterozygous genetic markers present in one parent but not in the other were used to construct separate genetic linkage maps for the female (*E. tereticornis*) and male (*E. globulus*) parents using the two-way pseudo-testcross strategy (Grattapaglia and Sederoff 1994). Markers linked with recombination frequency $\theta \leqslant 0.25$ and a linkage significance value $\alpha \leqslant 0.00001$ were assigned to linkage groups. The PGRI program constructs confidence intervals for the estimated locus order using a bootstrap resampling method with all markers (Liu 1998). This gives a measure of the quality of the locus order. The software program MAPMAKER (Mapmaker Macintosh v2.0) was used for comparison. The data set was duplicated and recoded to allow the detection of linkage of markers in repulsion phase. A LOD score of 5.0 and maximum $\theta = 0.25$ were set as linkage thresholds for grouping markers. Map distances in centi-Morgans were calculated using Kosambi's mapping function.

Results

Inheritance of AFLP markers in a *Eucalyptus* F_1 interspecific family

Screening procedure to choose primers

Pre-amplification reactions using A as the selective nucleotide in both primers did not work as well as those using A for the *Eco* primer and C for the *Mse* primer. Adding selective nucleotides to the AFLP primers reduced the number of observable bands approximately fourfold with each additional nucleotide and yielded fingerprints that were subsets of the originals, as expected. *Eco* and *Mse* primers with +3 selective nucleotides gave the maximum number of readable bands per reaction (Fig. 1).

Reproducibility

Of the 30 primer combinations tested (in 93 individuals), 20% gave rise to complex profiles that were difficult to read; these were discarded. Seven individuals were identified as pollen contaminants (absence of monomorphic bands from the male parent) and were also eliminated. In the 35,551 total data points, 0.8% were missing data and 6.9% were non-reproducible. Eleven samples accounted for the majority of the non-reproducible profiles and were discarded, thereby decreasing the proportion of non-reproducible data points to 1.9%. The two readers scored 1.4% of the data points differently. A 0.2% error was detected in data entry.

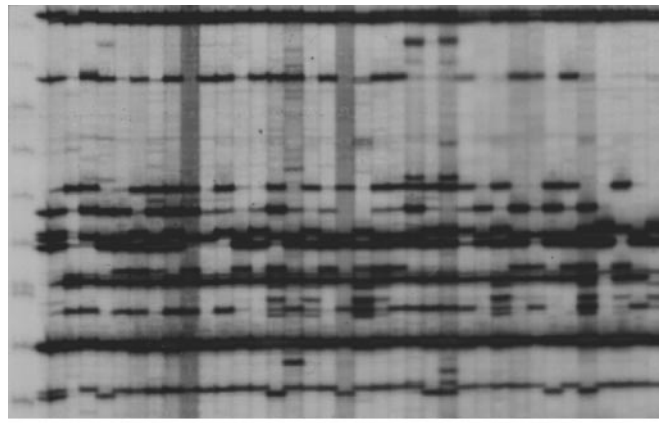


Fig. 1 Detail of an autoradiogram showing the segregation of alleles revealed by AFLP markers. A 10 bp DNA ladder is arrayed on the *left side* of the *Eucalyptus* lanes. The *first lane* is the female parent (*E. tereticornis*), the second lane is the male parent (*E. globulus*), followed by the F_1 progeny

Segregation analysis

With our standard reaction conditions, an average of 64 restriction fragments were detected per lane, 39% of which were polymorphic in this F_1 progeny set. The size of the AFLP fragments in these experiments ranged from approximately 40 to 400 bp. A total of 606 polymorphic fragments were scored in 24 gels (Table 1). As expected for this genus, the levels of polymorphism detected were very high. Some variation was evident in the number of polymorphic fragments revealed by different primer combinations, suggesting that prescreening could be useful. Of the 487 markers segregating 1 : 1 (in 73 individuals), 285 were heterozygous in the female parent (*E. tereticornis*) and 202 in the male parent (*E. globulus*). Approximately 2% of the polymorphisms involved small size differences, suggesting codominant marker inheritance. Intensity differences (putative codominant loci where only a single allele is observed) were discernible for approximately 16% of the markers. This information was not used because accurate scoring was difficult. Markers segregating 3 : 1 were also scored. Approximately 15% of the bands displayed skewed segregation ratios (Table 2).

Table 1 Numbers of AFLP amplification products generated with 24 different primer combinations. *Eco* +3 and *Mse* +3: 3′ end selective nucleotides of the primers complementary to the *Eco* and *Mse* adapter, respectively

Primer combination *Eco* +3	*Mse* +3	Total number of polymorphic bands
AAA	CCG	15
AAA	CGA	41
AAC	CCC	23
AAC	CCT	26
AAG	CCG	13
AAG	CCT	30
AAG	CGG	20
AAG	CTG	46
ACA	CAC	22
ACA	CAG	26
ACA	CCA	37
ACA	CCC	23
ACA	CCT	22
ACA	CGC	13
ACA	CGG	20
ACA	CTC	36
ACC	CCA	24
ACG	CCA	18
ACT	CCA	26
AGC	CCG	7
ATC	CCA	26
ATG	CCA	39
ATT	CCA	32
ATT	CCT	21

Table 2 Average numbers of segregation types of AFLP amplification products over the primer combinations tested

Total bands scored	Bands segregating 1:1*	Bands segregating 3:1*	Bands showing segregation ratio distortion*
606	487	32	87
100%	80%	5%	15%

* Significant at $\alpha = 0.01$ level

Heterozygosity

Minimum estimates for the average heterozygosity of the *E. tereticornis* and *E. globulus* clones was 30.5% and 22.4%, respectively, based on the number of polymorphisms relative to the total number of bands per individual.

Map construction

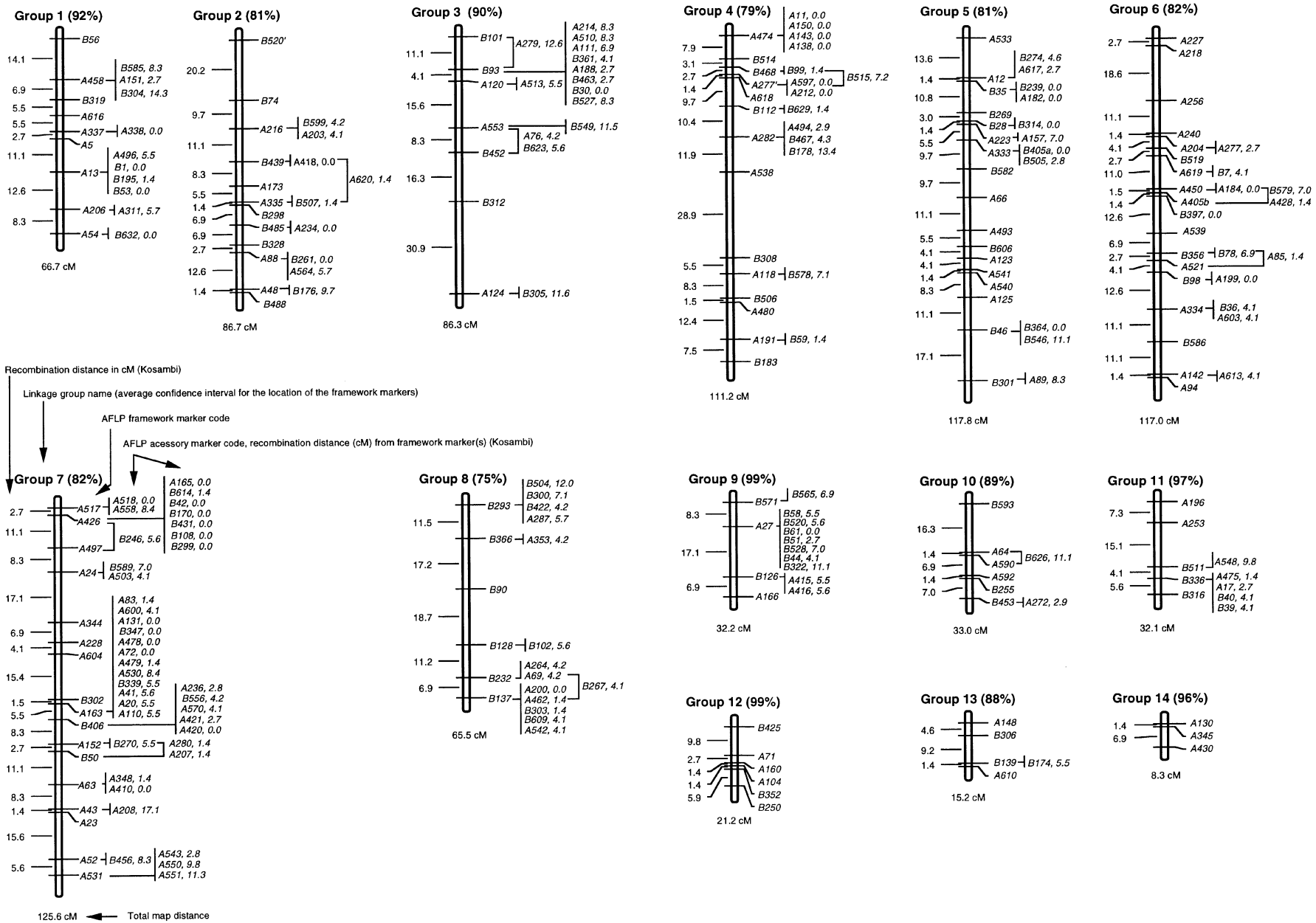
Linkage analysis

Overall, linkages were robust at $\theta \leqslant 0.25$ and $0.000001 \leqslant \alpha \leqslant 0.001$ (equivalent to a LOD score range of 3.0–6.0). For *E. tereticornis*, 8 major (66–126 cM) and 6 minor (8–33 cM) linkage groups were formed at a threshold of $\theta = 0.25$ and $\theta = 0.00001$. There were 9 unlinked markers (3%). For *E. globulus*, 10 major (46–122 cM) and 6 minor (1–28 cM) linkage groups were formed at a threshold of $\theta = 0.25$ and $\alpha = 0.00001$. Only 2 putative markers remained unlinked (1%). An additional 8 markers were removed from the *E. tereticornis* map as they could not be ordered with equal confidence in PGRI (Liu 1998) and MAPMAKER (Mapmaker Macintosh v2.0). Linkage analysis was also carried out with the terminal markers of the established linkage groups together with the unlinked markers. For *E. tereticornis* at $\theta \leqslant 0.3$ and $\alpha \leqslant 0.001$, linkage groups 1 and 13 merge. For *E. globulus* at $\theta \leqslant 0.35$ and $P \leqslant 0.01$, linkage groups 11 and 12 merge.

Statistical support for gene order

A framework map was constructed for the AFLP marker data based on a 90% confidence level for correct order using a PGRI bootstrap algorithm (Liu 1998). Markers that could not be placed on the map with a 90% confidence level were designated as accessory markers and were positioned on the map next to the closest framework marker. The same data were analyzed with MAPMAKER (Mapmaker Macintosh v2.0). The Multipoint First Order command (a matrix correlation procedure) was used for preliminary locus ordering. Framework map hypotheses were constructed independently. A subset of framework markers was ordered with an interval support $\geqslant 3$ (Keats et al. 1991). Approximately 50% of these markers were also chosen as framework markers in PGRI. The PGRI considers all possible orders with all markers in the linkage groups (using the simulated annealing algorithm). The approach to resolve framework maps in MAPMAKER is somewhat arbitrary. Approximately 12% of the markers displayed differences in locus ordering with nearby markers (less than 5 cM apart), in the two programs.

Fig. 2 Genetic linkage map of *E. tereticornis* (clone TT Esc 87/90). Linkage relationships of 285 AFLP markers were established at a threshold of maximum $\theta = 0.25$ and $P = 0.00001$ (PGRI software, Liu 1998). One hundred and twenty-eight framework markers could be ordered in 14 linkage groups covering 919 cM of map distance, together with 140 accessory markers [shown adjacent to the closest(s) framework marker(s)]. Nine markers remained unlinked (*B117*, *B332*, *B440*, *B437*, *B512*, *B580*, *B584*, *?A601*, *B612*). AFLP markers that could not be ordered with equal confidence in PGRI and MAPMAKER were eliminated from the analysis (*A145*, *A218*, *A240r*, *B275*, *A276*, *B447*, *B524*, *B631*)


Group 1 (92%)
66.7 cM
Group 2 (81%)
86.7 cM
Group 3 (90%)
86.3 cM
Group 4 (79%)
111.2 cM
Group 5 (81%)
117.8 cM
Group 6 (82%)
117.0 cM
Recombination distance in cM (Kosambi)
Linkage group name (average confidence interval for the location of the framework markers)
AFLP framework marker code
AFLP acessory marker code, recombination distance (cM) from framework marker(s) (Kosambi)
Group 7 (82%)
125.6 cM
Total map distance
Group 8 (75%)
65.5 cM
Group 9 (99%)
32.2 cM
Group 10 (89%)
33.0 cM
Group 11 (97%)
32.1 cM
Group 12 (99%)
21.2 cM
Group 13 (88%)
15.2 cM
Group 14 (96%)
8.3 cM

Genomic maps

In *E. tereticornis*, 128 markers were placed on a framework map defining 919 cM of total map distance (1,144 cM if 25 cM intervals surrounding unlinked markers were included) (Fig. 2). The linkage groups had an average length of 65 cM. The average spacing of *E. tereticornis* framework markers was 8 cM. In *E. globulus*, 120 markers were placed on a framework map covering 967 cM (1,017 cM if 25 cM intervals surrounding unlinked markers were included) (Fig. 3). The linkage groups had an average length of 61 cM. The average spacing of *E. globulus* framework markers was 9 cM.

The position of 15 markers segregating 1 : 1, obtained with a different pre-amplification selective nucleotide (A/A), was assessed. Two remained unlinked; the other 13 mapped to positions ranging from 2.6 to 40.2 cM away from existing markers (resulting from the A/C pre-amplification).

The positions of fragments showing segregation-ratio distortion were assessed after establishing gene order for the other markers. In the female parent, 70 distorted fragments mapped to 8 different linkage groups interspersed among the other markers. The inclusion of these fragments would result in the merger of linkage groups 2 and 14 and linkage groups 10 and 12. In this latter group, 24 distorted fragments mapped close together. In the male parent, 41 distorted fragments were dispersed in 7 different linkage groups. The inclusion of fragments showing segregation-ratio distortion would result in a 20% increase of map length for *E. tereticornis* and a 14% increase for *E. globulus*.

Map comparison

Marker loci that are heterozygous in both parents should yield a 3 : 1 segregation ratio. In order to identify homologies between the *E. tereticornis* and the *E. globulus* genetic linkage maps, we included 19 markers segregating 3 : 1 in the analysis. These markers did not show segregation-ratio distortion ($\alpha = 0.05$) and were tightly linked with existing 1 : 1 segregating markers in both maps ($0.0002 \leqslant \theta \leqslant 0.17924$). Fifteen revealed straightforward homologies between 9 linkage groups in *E. tereticornis* and 10 linkage groups in *E. globulus* (established between each 3 : 1 marker and several close 1 : 1 markers on both maps). Four, located on *E. tereticornis* linkage group 7, mapped to 3 different *E. globulus* linkage groups (Fig. 4). Marked differences in the map lengths of the matched linkage groups were discernible. The results of the 3 : 1 segregating markers do not contradict results obtained with the linkage analysis of the end markers or the information resulting from the introduction of the putative markers showing segregation-ratio distortion (Fig. 4).

Discussion

Quality of AFLP markers

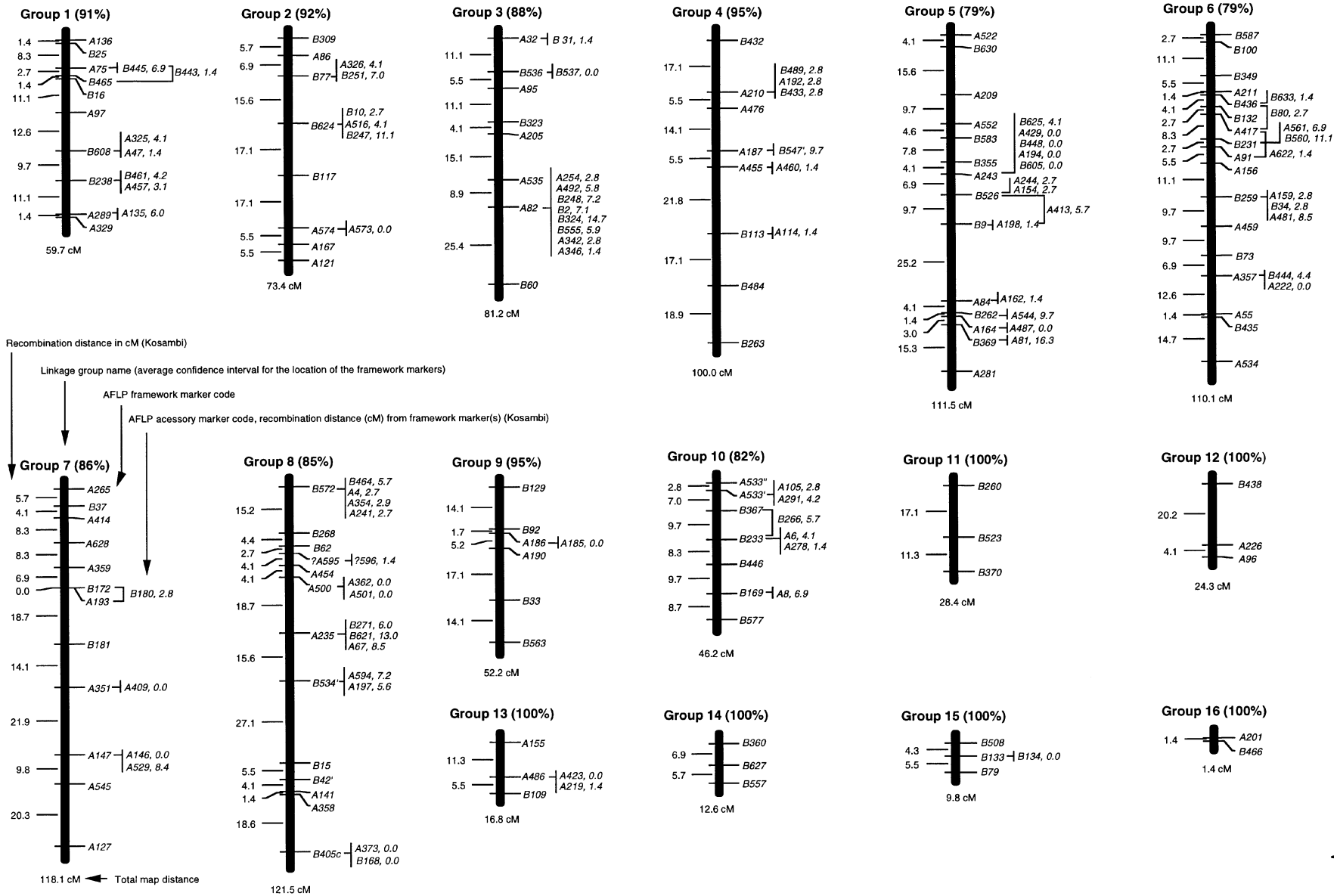
The AFLP technique is robust and reliable for mapping in eucalypts. The method involves several selective amplification steps, is relatively insensitive to the template DNA concentration and uses primer sequences of at least 16 nucleotides, at stringent reaction conditions, for primer annealing. Incomplete digestion of the DNA will cause problems in AFLP fingerprinting because partial digestion fragments will create inconsistent banding patterns (Vos et al. 1995). Eleven samples were dropped from the analysis due to poor DNA quality.

The high yield of information achieved with AFLP markers makes it an extremely efficient tool for the analysis of informative testcross configurations, compared to other anonymous marker systems (e.g. randomly amplified polymorphic DNAs, RAPDs), despite the increase in technical complexity. AFLP markers detect more point mutations per reaction than RAPDs or restriction fragment length polymorphisms (RFLPs). The AFLP assay is sensitive to single base changes that cause the loss or gain of restriction sites, many kinds of rearrangements and changes in the DNA sequence recognized by the selective nucleotides. The AFLP method generally detects only one allele at a locus (i.e. presence or absence of restriction fragments rather than length differences alone). Dominant AFLP markers are readily analyzed in progeny from controlled crosses using the pseudo-testcross model. We obtained an average of 20 markers segregating 1 : 1 per primer combination, approximately 11 times more than the number of RAPD markers per primer reported for a different interspecific cross in *Eucalyptus* (Grattapaglia and Sederoff 1994). With two sequencing apparatus, 4 AFLP gels containing 100 samples each can be run by one person, in 1 workday. The marker data for a map can be acquired in 2 weeks instead of the 2 months needed for one person to construct equivalent parental linkage maps using RAPDs (Grattapaglia and Sederoff 1994).

Map construction

Estimates of total genome map size have been calculated for other eucalypt species of the subgenus

Fig. 3 Genetic linkage map of *E. globulus* (clone GB MJ6/90). Linkage relationships of 200 AFLP markers were established at a threshold of maximum $\theta = 0.25$ and $P = 0.00001$ (PGRI software, Liu 1998). One hundred and twenty framework markers could be ordered in 16 linkage groups covering 967 cM of map distance, together with 80 accessory markers [shown adjacent to the closest(s) framework marker(s)]. Two markers remained unlinked (*B296*, *B310*)


Group 1 (91%)
1.4 A136
8.3 B25
2.7 A75 B445, 6.9 B443, 1.4
1.4 B465
11.1 B16
A97
12.6 A325, 4.1
B608 A47, 1.4
9.7
B238 B461, 4.2 A457, 3.1
11.1
1.4 A289 A135, 6.0
A329
59.7 cM
Group 2 (92%)
5.7 B309
6.9 A86
B77 A326, 4.1 B251, 7.0
15.6
B624 B10, 2.7 A516, 4.1 B247, 11.1
17.1
B117
17.1
5.5 A574 A573, 0.0
5.5 A167
A121
73.4 cM
Group 3 (88%)
11.1 A32 B 31, 1.4
B536 B537, 0.0
5.5 A95
11.1
4.1 B323
A205
15.1
A535 A254, 2.8 A492, 5.8 B248, 7.2 B2, 7.1 B324, 14.7 B555, 5.9 A342, 2.8 A346, 1.4
8.9
A82
25.4 B60
81.2 cM
Group 4 (95%)
17.1 B432
B489, 2.8 A192, 2.8 B433, 2.8
5.5 A210
A476
14.1
5.5 A187 B547', 9.7
A455 A460, 1.4
21.8
B113 A114, 1.4
17.1
B484
18.9
B263
100.0 cM
Group 5 (79%)
4.1 A522
B630
15.6
A209
9.7
4.6 A552 B625, 4.1 A429, 0.0 B448, 0.0 A194, 0.0 B605, 0.0
B583
7.8
4.1 B355
6.9 A243 A244, 2.7 A154, 2.7
B526 A413, 5.7
9.7
B9 A198, 1.4
25.2
4.1 A84 A162, 1.4
1.4 B262 A544, 9.7
3.0 A164 A487, 0.0
15.3 B369 A81, 16.3
A281
111.5 cM
Group 6 (79%)
2.7 B587
B100
11.1
B349
5.5
1.4 A211 B633, 1.4
4.1 B436 B80, 2.7
2.7 B132
8.3 A417 A561, 6.9 B560, 11.1
2.7 B231
5.5 A91 A622, 1.4
A156
11.1
B259 A159, 2.8 B34, 2.8 A481, 8.5
9.7
A459
9.7
B73
6.9
A357 B444, 4.4 A222, 0.0
12.6
1.4 A55
B435
14.7
A534
110.1 cM
Recombination distance in cM (Kosambi)
Linkage group name (average confidence interval for the location of the framework markers)
AFLP framework marker code
AFLP acessory marker code, recombination distance (cM) from framework marker(s) (Kosambi)
Group 7 (86%)
5.7 A265
4.1 B37
A414
8.3
8.3 A628
A359
6.9
0.0 B172 B180, 2.8
A193
18.7
B181
14.1
A351 A409, 0.0
21.9
A147 A146, 0.0 A529, 8.4
9.8
A545
20.3
A127
118.1 cM
Total map distance
Group 8 (85%)
B572 B464, 5.7 A4, 2.7 A354, 2.9 A241, 2.7
15.2
B268
4.4
B62
2.7 ?A595 ?596, 1.4
4.1 A454
4.1 A500 A362, 0.0 A501, 0.0
18.7
A235 B271, 6.0 B621, 13.0 A67, 8.5
15.6
B534'' A594, 7.2 A197, 5.6
27.1
B15
5.5
B42'
4.1
1.4 A141
A358
18.6
B405c A373, 0.0 B168, 0.0
121.5 cM
Group 9 (95%)
B129
14.1
B92
1.7 A186 A185, 0.0
5.2 A190
17.1
B33
14.1
B563
52.2 cM
Group 13 (100%)
A155
11.3
A486 A423, 0.0 A219, 1.4
5.5 B109
16.8 cM
Group 10 (82%)
2.8 A533''
7.0 A533' A105, 2.8 A291, 4.2
B367
9.7 B266, 5.7
B233 A6, 4.1 A278, 1.4
8.3
B446
9.7
B169 A8, 6.9
8.7
B577
46.2 cM
Group 14 (100%)
B360
6.9
B627
5.7
B557
12.6 cM
Group 11 (100%)
B260
17.1
B523
11.3
B370
28.4 cM
Group 15 (100%)
4.3 B508
B133 B134, 0.0
5.5
B79
9.8 cM
Group 12 (100%)
B438
20.2
A226
4.1 A96
24.3 cM
Group 16 (100%)
1.4 A201
B466
1.4 cM

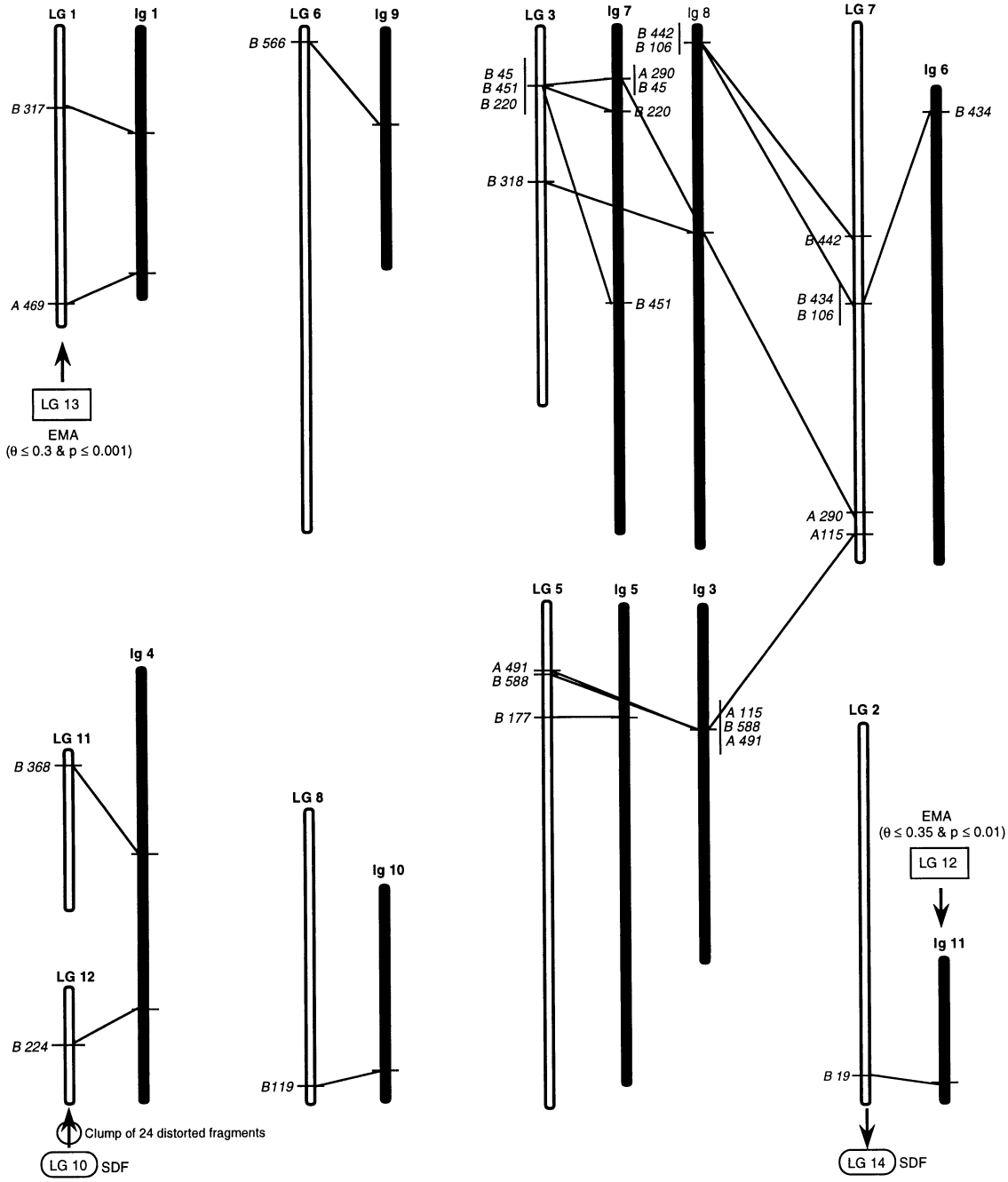


Fig. 4 Reduced scale comparison of the paired *E. tereticornis* (open lines) and *E. globulus* (*dark lines*) linkage groups, resulting from the analyses of 19 markers segregating 3 : 1. Results obtained with the linkage analysis of the end markers (*EMA*) and the information resulting from the analysis of the segregation ratio distortion fragments (*SDF*) ($\theta \leqslant 0.25$ and $P \leqslant 0.00001$) were also included

Symphyomyrtus (Grattapaglia and Sederoff 1994; Verhaegen and Plomion 1996; Byrne et al. 1995): *E. grandis* (1,620 cM, 1,483 cM), *E. urophylla* (1,156 cM, 1,332 cM), *E. nitens* (1,500 cM). This study has described 14 linkage groups for *E. tereticornis* (with a total framework map length of 919 cM) and 16 linkage groups for *E. globulus* (with a total framework map length of 967 cM). There is little difference in map size (total recombination frequency) between the female (*E. tereticornis*) and the male parent (*E. globulus*). For these species $2n = 2x = 22$ chromosomes (Eldridge et al. 1994). This set of AFLP markers could provide a map coverage range of 80–100%, assuming that an unmapped locus located within 10 cM of the terminal markers on the linkage groups and the unlinked loci could be detected (1,289 cM for *E. tereticornis* and 1,307 cM for *E. globulus*).

Despite the large numbers of markers mapped, they are sparse in a few regions of the genome and dense in others. This has also been reported for other species and marker systems (Kesseli et al. 1994; Grattapaglia and Sederoff 1994; Becker et al. 1995; van Eck et al. 1995). Specific biological differences in the levels or location of DNA polymorphisms, rates of recombination, variation in copy number of specific genomic sequences (van Eck et al. 1995) or sampling error are factors that could affect the distribution of markers.

The proportion of unlinked markers (2%) is similar to that reported in other linkage mapping studies with eucalypts (Grattapaglia and Sederoff 1994; Byrne et al. 1995). Either they are artifacts (segregating in Mendelian ratios by chance) or they sample parts of the genome where there are few other markers. In the latter case, they would be very valuable.

The transmission of genetic markers and chromosomes from one generation to the other could be distorted by lethal genes or chromosomal rearrangements that alter segregation ratios (Causse et al. 1994; Foolad et al. 1995). Some DNA markers (5% at the $\alpha = 0.05$ level) will have distorted segregation ratios by chance. The proportion of segregation-ratio distorted fragments in this study was 15%. A non-significant distortion ratio (4%) was reported earlier for an F_1 resulting from a cross between *Eucalyptus grandis* and *E. urophylla* (Grattapaglia and Sederoff 1994). Byrne et al. (1995) observed an 8% rate in an F_2 outbred population of *E. nitens*. Verhaegen and Plomion (1996) identified an 8% rate in an F_1 interspecific *E. grandis* × *E. urophylla* progeny set. We found a cluster of 24 linked distorted fragments (4%) connecting linkage groups 10 and 12 in *E. tereticornis*. The clustering of loci showing segregation-ratio distortion in eucalypts has also been reported by others (Byrne et al. 1995; Verhaegen and Plomion 1996). Most of the remaining 11% of the distorted fragments were dispersed in different linkage groups, suggesting that this group results from statistical effects rather than biological ones. *E. tereticornis* and *E. globulus* belong to the same subgenus (*Symphyomyrtus*) but different sections (*Exertaria* and *Maidenaria*, respectively). Speciation is often accompanied by chromosomal rearrangements. Polymorphic bands that have distorted segregation ratios may still be useful even though there is not an exact correspondence between expected and observed inheritance of the band.

Framework maps are important tools by which to scan the genome for quantitative trait loci (QTL). The usefulness of a genomic map depends on the accurate determination of locus order (Plomion et al. 1995). The "ordering problem" is difficult because of the large number of possible locus orders (n!/2 for n loci) and because customary likelihood ratio tests cannot be carried out (Ott 1991). A number of mapping programs are available for locus ordering. The framework map approach (Keats et al. 1991) has been used as a guideline to evaluate gene order. However, the interval support criterion for gene order does not consider the complete data set. Interval support relies on the comparison of the likelihood of alternative permutations of only 3 adjacent markers. Different framework maps can be produced for the same data set because the locus dropping strategy is subjective. The bootstrapping jackknifing approach can analyze data of the entire linkage group. Resampling analysis allows the evaluation of alternative gene orders. The most likely gene order is indicated by a maximum in the matrix of the sum of the diagonal elements of map location and probability (Liu 1998). The mean probability that a marker is located at its corresponding genome position (after resampling) is expressed as the percentage of correct order (PCO). The magnitude of the PCO value depends upon the number of informative recombination events and the quality of the data (Liu 1998). In practice, this method increases the objectivity of framework map construction.

Map comparison

Homeologous linkage groups in *E. tereticornis* and *E. globulus* were recognized using markers that were heterozygous in both parents. These markers represent 5% of the AFLP markers in this study. This number is about three times larger than that found by Grattapaglia and Sederoff (1994) in an interspecific cross between *E. grandis* and *E. urophylla* using RAPD markers. There is an indication of synteny in four regions that are connected by 2 or more markers. The *E. tereticornis* linkage group 7 shows homology to differently paired *E. globulus* linkage groups (numbers 6, 7 and 8), which is inconsistent with the other proposed homologies. If the marker information is correct (is limited to 1 linkage group and is well supported by linkage to neighboring markers), it suggests the presence of chromosomal rearrangements within species, in *Eucalyptus*. Alternatively, there is error in *E. globulus* linkage group 7. Error could be due to non-homologous fragments with identical mobilities, paralogous rather than orthologous fragments, multiple gene-copies and/or pseudogenes (Dowling et al. 1996) or statistical error (Plomion et al. 1995). Further work is needed to determine the exact cause.

Genomic analysis in eucalypts

Genomic mapping applied to plant breeding provides a new approach to the identification of genes that control commercially important traits (Dudley 1993). The identification and manipulation of QTLs specific to individual trees with desirable traits is the first step to implementing marker-assisted breeding. AFLPs have the potential to improve the efficiency of genetic

map construction and to create high-resolution maps around target loci, thereby facilitating gene isolation (Cnops et al. 1996). The progressive accumulation of individual linkage maps with common markers will lead to a unified map where regions associated with trait expression will be identified. Integrated linkage maps will spur the use of molecular techniques for improving breeding strategies. Also, the comparison of genomic maps from different species or genera can provide insights into plant evolution and genome structure (Hohmann et al. 1995).

We plan to use the linkage data developed in these maps to detect loci controlling quantitative trait loci in *E. globulus* and *E. tereticornis*. For this purpose, we need a high degree of confidence in the marker order. A larger set of progeny from the mapped parents has been obtained, cloned and planted in different field trials. These populations will be assessed for quantitative traits.

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