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Genetic mapping of QTLs controlling vegetative propagation in *Eucalyptus grandis* and *E. urophylla* using a pseudo-testcross strategy and RAPD markers

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Abstract We have extended the combined use of the "pseudo-testcross" mapping strategy and RAPD markers to map quantitative trait loci (QTLs) controlling traits related to vegetative propagation in *Eucalyptus*. QTL analyses were performed using two different interval mapping approaches, MAPMAKER-QTL (maximum likelihood) and QTL-STAT (non-linear least squares). A total of ten QTLs were detected for micropropagation response (measured as fresh weight of shoots, FWS), six for stump sprouting ability (measured as # stump sprout cuttings, #Cutt) and four for rooting ability (measured as % rooting of cuttings, %Root). With the exception of three QTLs, both interval-mapping methods yielded similar results in terms of QTL detection. Discrepancies in the most likely QTL location were observed between the two methods. In 75% of the cases the most likely position was in the same, or in an adjacent, interval. Standardized gene substitution effects for the QTLs detected were typically between 0.46 and 2.1 phenotypic standard deviations (σ_P), while differences between the family mean and the favorable QTL genotype were between 0.25 and 1.07 σ_P. Multipoint estimates of the total genetic variation explained by the QTLs (89.0% for FWS, 67.1% for #Cutt, 62.7% for %Root) indicate that a large proportion of the variation in these traits is controlled by a relatively small number of major-effect QTLs. In this cross, *E. grandis* is responsible for most of the inherited variation in the ability to form shoots, while *E. urophylla* contributes most of the ability in rooting. QTL mapping in the pseudo-testcross configuration relies on within-family linkage disequilibrium to establish marker/trait associations. With this approach QTL analysis is possible in any available full-sib family generated from undomesticated and highly heterozygous organisms such as forest trees. QTL mapping on two-generation pedigrees opens the possibility of using already existing families in retrospective QTL analyses to gather the quantitative data necessary for marker-assisted tree breeding.

Key words RAPD · Pseudo-testcross · *Eucalyptus* · QTL · Vegetative propagation

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Introduction

Vegetative propagation is a powerful way to capture the genetic superiority of a selected individual. In clonal propagation, both additive and non-additive sources of genetic variation contribute to the gain, while in sexual propagation the gain is achieved exclusively on the basis of the interfamily component of the genetic variance. Therefore, the full benefit of broad-sense heritability is realized rather than only some portion of the narrow-sense heritability. In practice, however, much of the gain from clonal propagation is due to the greatly increased selection differential

In horticulture, vegetative propagation of desired plant phenotypes has been used successfully for centuries (Hartman and Kester 1983). In forestry, aside from a few genera like *Populus*, *Salix* and *Cryptomeria*, vegetative propagation of "plus" trees has not been used extensively in most operational forest planting programs (Zobel and Talbert 1984). Cuttings from physiologically mature trees of many species are difficult or impossible to root.

Species of the genus *Eucalyptus* constitute the majority of the world's planted hardwood forests and one of the world's main sources of cultivated biomass (Eldridge et al. 1993). In sprouting species such as the eucalypts, the stump sprouts are physiologically juvenile and can in-principle be rooted. Propagation systems based on rooted cuttings have been optimized and implemented at the production level, resulting in outstanding gains in productivity and uniformity (Campinhos and Ikemori 1980; Delwaulle

1985). Currently, the largest operational clonal forestry programs are with species of *Eucalyptus*. In the tropics, such operations yield the highest productivity of woody biomass on earth (Brandao 1984), reaching 100 m^2 per hectare per year when the best clones are used on the best sites.

As an alternative to rooted cuttings, methods of micropropagation have been developed for several species of *Eucalyptus* (DeFossard 1974; Gupta and Mascarenhas 1987). Some progress has been made in the micropropagation of adult selected trees, to mitigate the lack of propagation potential due to maturation. Although in vitro methods are not economically viable for large *Eucalyptus* planting operations, they have been used as an efficient way to rapidly develop a "sprout nursery" to produce the cuttings needed for operational planting (Grattapaglia et al. 1990).

The ability to sprout, root, and respond to tissue culture varies widely both within and particularly across species of *Eucalyptus* (Hartney 1980; Zobel 1993). Variation in rooting ability frequently dictates which trees will be available in a planting operation, severely limiting the use of clonal propagation when particular species are the most desired. For example, *Eucalyptus globulus*, which has some of the best wood properties for cellulose pulp production, roots very poorly. The transfer of vegetative propagation traits by intra- and inter-specific hybridization is an increasingly important objective in many breeding programs. Very little information is available on the genetic basis of such traits. Easy and hard-to-root species of *Eucalyptus* have been identified (Hartney 1980). However, no estimates of genetic parameters such as heritability, or information on the genetic control and architecture of vegetative propagation traits, are available for *Eucalyptus* trees.

Genetic linkage maps of molecular markers offer a powerful tool to investigate the genetic architecture of polygenic traits and to potentially assist in their manipulation through marker-assisted selection and breeding. A number of studies in recent years have used molecular markers to examine the inheritance of quantitative traits. Results to-date strongly support the existence of a few major genes controlling large proportions of the total variation in a wide range of quantitatively inherited traits (reviewed by Stuber 1992; Dudley 1993). These studies have been limited to a few annual crop plants and have been performed using segregating populations derived from crosses between inbred lines. Such populations are not available in trees and will be difficult to obtain for many species due to high genetic load and long generation times. To circumvent this limitation we recently adopted a "two-way pseudo-testcross" approach with RAPD (random amplified polymorphic DNA) markers to construct linkage maps for individual trees of *Eucalyptus* (Grattapaglia and Sederoff 1994). In this report we extend the use of this approach for QTL analysis. We have identified quantitative trait loci (QTLs) controlling significant proportions of the phenotypic variation in traits related to the ability to vegetatively propagate trees in *Eucalyptus grandis* and *E. urophylla*. The results provide the possibility of marker-assisted breeding of these traits in forest-tree improvement programs.

Material and methods

Plant material

The experimental material consisted of a single controlled cross between two highly heterozygous elite trees. *E. grandis* (clone 44, Coffs Harbor provenance, Australia – selection from a Zimbabwe seed source), as the female parent, was crossed with *E. urophylla* (clone 28 selection from Rio Claro land race, Brazil), used as the male, in 1989 at Aracruz Florestal S.A., Brazil. Sixty-two F_1 individuals of this population had already been used for the construction of genetic linkage maps (Grattapaglia and Sederoff 1994). For the present study, the mapping population was expanded to 122 individuals. Seeds were originally germinated on solid agar containing half-strength MS medium (Murashige and Skoog 1962) under a 14-h photoperiod. The population was immortalized by establishing clonal cultures of the individuals by vegetative propagation in vitro on maintenance medium (half-strength MS medium supplemented with 0.5 mg/l of IBA, indol-butyric acid). Rooted plantlets produced in vitro were transplanted to containers, containing a 1:1:1 mixture of vermiculite, soil and peat moss, under mist irrigation for 2 weeks. After 2 months of growth, two plants per individual were transplanted to large (20-l) pots and managed for fast growth and cutting production.

Experimental designs and traits measured

The following quantitative traits were evaluated: (1) micropropagation response (fresh weight of in vitro micropropagated shoot clumps, FWS); (2) sprouting (number of stump sprout cuttings, #Cutt); (3) adventitious rooting response (percentage of rooted cuttings, % Root). Two plants per individual were used for phenotype evaluations. This clonal replication of the individuals provided some increased precision in trait measurement. Micropropagation response was analyzed by a randomized complete block design with two blocks and two-explant plots. Each plot corresponded to a tube with two explants in it. Explants were stem segments containing one axillary meristem derived from plants grown in maintenance medium. The two basal axillary nodes are the most consistently responsive to the induction of shoot growth in tissue culture (Grattapaglia, unpublished). Therefore, only these explants were used to control this source of inherent physiological variation. Fresh and dry (24 h at 105°C) weight of shoot clumps were determined at 25 days of culture. Because dry and fresh weight were found to be highly correlated traits in our study (r=0.98), QTL analyses were performed only for fresh weight.

For coppice and rooting response, two potted plants per individual were grown for 3 months (approximately 2-cm stem diameter) and then cut back to stimulate dormant buds to sprout. Sprouting was evaluated as the number of operational quality (one node/two leaf) stump sprout cuttings that could be harvested after 60 days following cut back of the plant. Cuttings harvested in this evaluation were put to root to evaluate the adventitious rooting response. The basal 1 cm of the cutting was dipped into a 5000 ppm IBA solution in talc and placed into a rooting medium consisting of a 3:1 mixture of vermiculite and pine bark. A variable number of cuttings was therefore used to evaluate rooting response, and the measurement was recorded in terms of the percent cuttings able to root. The experiment was a randomized complete block design with two blocks (corresponding to the two potted plants) and a variable number of cuttings per plot.

RAPD marker genotyping

DNA extractions, RAPD assay conditions, marker identification and scoring were performed as described elsewhere (Grattapaglia and Sederoff 1994). RAPD markers in the pseudo-testcross mating configuration are present in a heterozygous state in one parent and absent in the other, or vice versa, and segregate 1:1 in the F_1 generation. Two separate sets of linkage data are obtained, one for each par-

ent. A total of 165 markers for *E. grandis* and 166 for *E. urophylla* that segregated accordingly (χ^2 test at α=0.05) were employed in this study. These included 100 and 83 framework markers respectively for *E. grandis* and *E. urophylla*. Markers assigned to the framework map were those that had been previously ordered with a likelihood support ≥1000:1 (Grattapaglia and Sederoff 1994).

Data analysis

Although all the RAPD markers used in this study have been mapped previously (Grattapaglia and Sederoff 1994), genetic maps were calculated from the genotypic data *de novo* and checked for consistency with the previously reported maps. Linkage relationships among markers were determined using MAPMAKER (Lander et al. 1987). To allow the detection of linkage of RAPD markers in repulsion phase, the data set was duplicated and re-coded. LOD 5.0 and maximum θ=0.30 were used as linkage thresholds for grouping markers. The software program GMENDEL (Liu and Knapp 1990), with a threshold *P* value=0.0001 and θ=0.30, was also used for the linkage analysis.

QTL mapping analysis was performed using interval mapping methods implemented by MAPMAKER-QTL (Lander and Botstein 1989) and QTLSTAT (Knapp et al. 1992). QTL analyses were performed on the mean trait value of the F_1 individuals, computed as the average of plot means across the two blocks (stock plants). The genetic analysis was carried out under a backcross model. Separate analyses were performed on each parental linkage map. A LOD score threshold of 1.6 *or* a nominal significance level of *P*=0.01 were used to declare the presence of a linked QTL in the interval. With this stringency, and given the number of markers per chromosome used, a per-chromosome false positive rate of 5% was ensured as estimated numerically by Darvasi et al. 1993. For each LOD peak, the 1.0 LOD support intervals were determined. For all detected QTLs, the percentage of variance explained as estimated by MAPMAKER-QTL and QTLSTAT (as non-linear R^2), and the shifts in trait value in phenotypic standard deviations, were also reported. When linked QTLs with no overlapping 1.0 LOD support intervals were detected, the locus with the highest LOD score was fixed and the chromosome scanned again for the linked effect. Multipoint estimates of the total variation explained by the mapped QTLs were obtained by interval mapping with MAPMAKER-QTL and by multiple linear regression using PROC GLM (SAS 1988).

Results

Linkage map calculations

The majority of the RAPD markers used in this study were previously classified as framework markers, i.e., their order was established with a likelihood support 1000:1 (Grattapaglia and Sederoff 1994). They are identified with bold type on the linkage maps (Figs. 1 and 2). When genotyping with RAPD markers, each arbitrary primer amplifies more than one segregating marker. So, in addition to the target framework markers, accessory markers are obtained by default. Most of the markers used were framework markers (100 of 165 in *E. grandis* and 83 of 166 in *E. urophylla*); however, data for accessory markers were included in the analysis when assembling the linkage maps and carrying out the QTL analysis. A LOD score of 5.0 and a maximum θ=0.30 were set as linkage thresholds for grouping markers. Map distances in centimorgans were calculated using Kosambi's mapping function. Orders of marker loci in each linkage group were established using a matrix correlation method implemented by MAPMAKER. The orders obtained compared very closely to those resulting from simulated annealing performed by GMENDEL. The order for the framework markers (in bold type) in the maps presented conforms to the 1000:1 likelihood support. The locus-order support was relaxed to 100:1 when builiding the maps to include all markers.

The framework marker orders of these maps (based on 122 meioses) are well conserved when compared to our previously published maps (based on 62 meioses). In *E. grandis*, there were four cases involving a switch in the order of two adjacent markers: Z18_1630 and R16_820 on group 8; R16_730 and N7_1322 on group 6; P8_1350 and R15_1650 on group 2; and U19_800 and R20_1080 on group 4. There were three cases of a single marker being out of the original order: marker X15_600 on group 5; marker N13_533 on group 7; and marker Y15_740 on group 9. In *E. urophylla*, there were three cases of a switch in the order of adjacent markers: markers B7_1549 and A18_509 on group 3; marker pairs G5_304 and G2_1444, and K12_631 and P8_570, on group 11. One triplet of markers was inverted in orientation (markers X4_300, T12_1500 and Z16_1480) on group 5. There were five cases of a single marker out of order: marker L17_560 on group 2; Z16_344 on group 6; K3_290 on group 8; X17_2100 on group 9; and M4_1027 on group 10.

In *E. urophylla* the original marker linkage grouping remained the same. In *E. grandis*, however, one case of breakage and two cases of mergers of previously reported linkage groups were observed. Groups 8 and 12 merged into one. Group 11 was split in two (designated 11a and 11b) which in turn were linked to groups 9 and 13. Although the total number of linkage groups was reduced from 14 to 12, it is still one more than the expected number based on the haploid number of chromosomes in *Eucalyptus grandis* (*n*=11). The mergers of groups 8 and 12 and groups 11 and 13 had been suggested previously (Grattapaglia and Sederoff 1994). However, LOD scores for linkages were below the adopted threshold for grouping (LOD< 5.0). This study, involving a larger number of meioses, increased the power and the precision of the linkage analysis and resulted in more-likely linkage groups for *E. grandis*. Significant LOD scores for linkage among several markers in the merged groups were observed. For group 11, the increased sample size apparently resolved a case of spurious linkage that had kept the group together originally.

Quantitative traits

The two parents of the original cross used in this QTL experiment could not be evaluated for the vegetative propagation traits. Therefore, the trait values for the F_1 progeny individuals cannot be compared to the parental generation. It is known, however, that stump sprout cuttings of *E. urophylla* typically root at significantly higher percentages than *E. grandis* and that both species have similar sprouting ability and response to tissue culture (Grattapaglia et

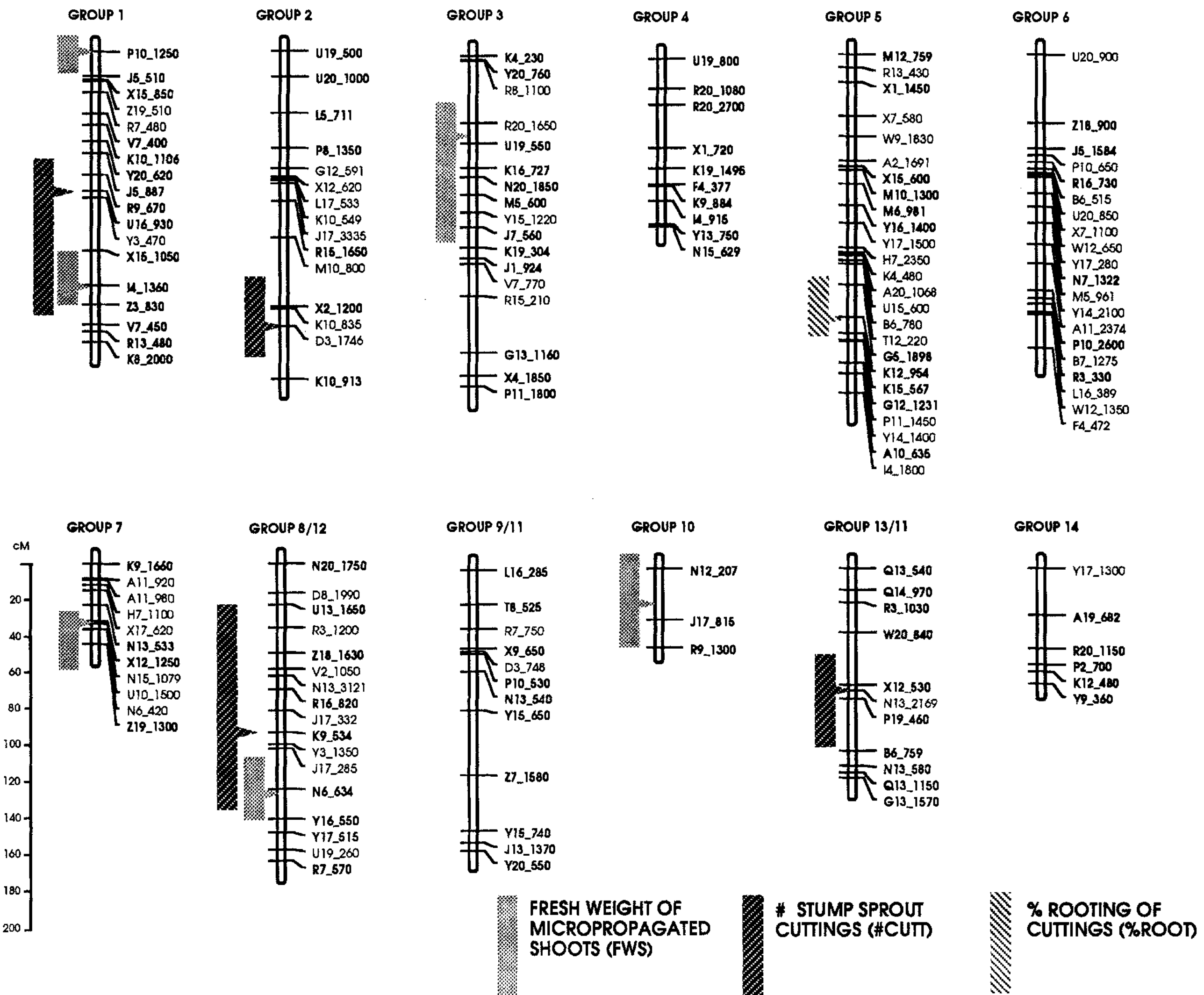

Fig. 1 Quantitative trait locus (QTL) map of traits related to vegetative propagation response in *E. grandis* clone 44. Linkage maps of RAPD markers were constructed using MAPMAKER (LOD 5.0 θ=0.30) and GMENDEL (*P* value=0.0001 θ=0.30). RAPD markers in *bold type* were classified as framework markers (ordered with log-likelihood support$\geq$1000:1) while the remaining markers were ordered with support$\geq$100:1. *Bars* to the left of linkage groups correspond to the 1.0 LOD support intervals for the location of the QTL (i.e., the interval over which the QTL position is at most 10-times less likely than the most likely position). *Arrows* indicate the most likely position (highest LOD peak) estimated with MAPMAKER-QTL

al. 1987; F. Bertolucci, unpublished results). Interspecific F_1 hybrids typically display intermediate behavior for these traits at the family mean level. However, because the individuals crossed are highly heterozygous, the F_1 is genetically heterogeneous, and a significant level of genetic variation exists. This genetic variation was explored in the QTL mapping experiment. Extreme phenotypes with trait values greater than two phenotypic standard deviations from the mean were observed for all three traits and with values less than the mean for all traits with the exception of %Root (Fig. 3).

The frequency distributions of phenotypes for the three traits did not differ significantly from normality as assessed by the Shapiro-Wilk statistic calculated using PROC UNIVARIATE (SAS 1988). Mean, standard deviation, and sample size for each trait are also presented. Sample sizes for phenotypic measurements were slightly less than the total number of individuals genotyped, as a result of a loss of individuals either due to contamination in the tissue culture experiment or to a loss of plants in the greenhouse trials. One individual that did not sprout enough to yield operational cuttings, but remained alive following the cut back of the stock plant, was included in the analysis for #Cutt with zero trait value. This individual, for which no cuttings were available for establishing the rooting evaluation, was not included in the rooting measurements. Therefore, the sample size dropped from n=97 to n=96 when measuring %Root. There were two individuals that on the average had less than five cuttings per plant. Because %Root estimated for these individuals was based on such a small sample of cuttings, QTL analyses were performed both including and excluding them. No significant differences were observed in the results (data not shown), and therefore those individuals were kept in the data set.

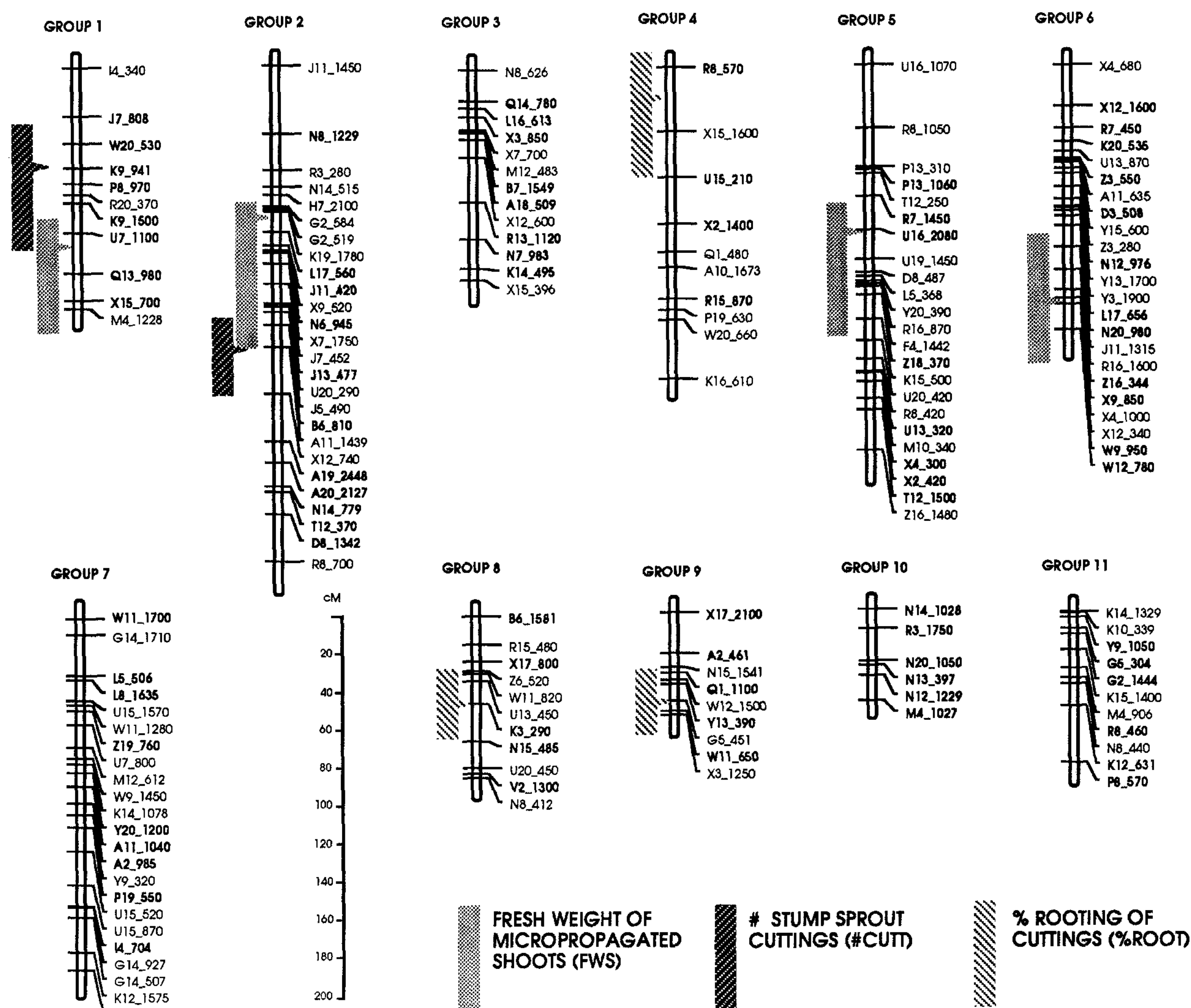

Fig. 2 Quantitative trait locus (QTL) map of traits related to vegetative propagation response in *E. urophylla* clone 28. Linkage maps of RAPD markers were constructed using MAPMAKER (LOD 5.0 θ=0.30) and GMENDEL (*P* value=0.0001 θ=0.30). RAPD markers in *bold type* were classified as framework markers (ordered with log-likelihood support≥1000:1) while the remaining markers were ordered with support≥100:1. *Bars* to the left of linkage groups correspond to the 1.0 LOD support intervals for the location of the QTL (i.e., the interval over which the QTL position is at most 10-times less likely than the most likely position). *Arrows* indicate the most likely position (highest LOD peak) estimated with MAPMAKER-QTL

The majority of individuals had an average of six or more cuttings per plant. Therefore the estimated %Root corresponds to the average percent rooting across the two plants, based on an average of six or more cuttings each, i.e., a total of 12 or more cuttings per individual genotype.

For all traits, the analyses were performed on the untransformed phenotypic data. When using MAPMAKER-QTL the data were log transformed to more closely fit a normal distribution and the analyses repeated. However, the analyses of log-transformed data did not alter any of the results. Therefore all the results presented are for untransformed data.

Phenotypic correlations estimated among traits were not significant at α=0.05. These were: FWS×#Cutt r=0.17; FWS×%Root r=0.004; #Cutt×%Root r=0.07.

QTL analysis

QTL analyses were performed using two different interval mapping methods. MAPMAKER-QTL (MMQ) was used to estimate QTL parameters and test statistics at 2-cM intervals within every marker bracket and to select the most probable location for the QTL as the location that maximized the likelihood ratio. QTLSTAT (QST) employs non-linear least-squares to estimate the QTL genotype means and tests the hypothesis of "no QTL" versus the hypothesis of "one QTL" for every marker bracket. The QTL is therefore assigned to a particular marker interval directly, without trying to estimate the most likely position within that interval.

With a few exceptions (see below) the results of the two analyses agreed closely. A total of ten putative QTLs were

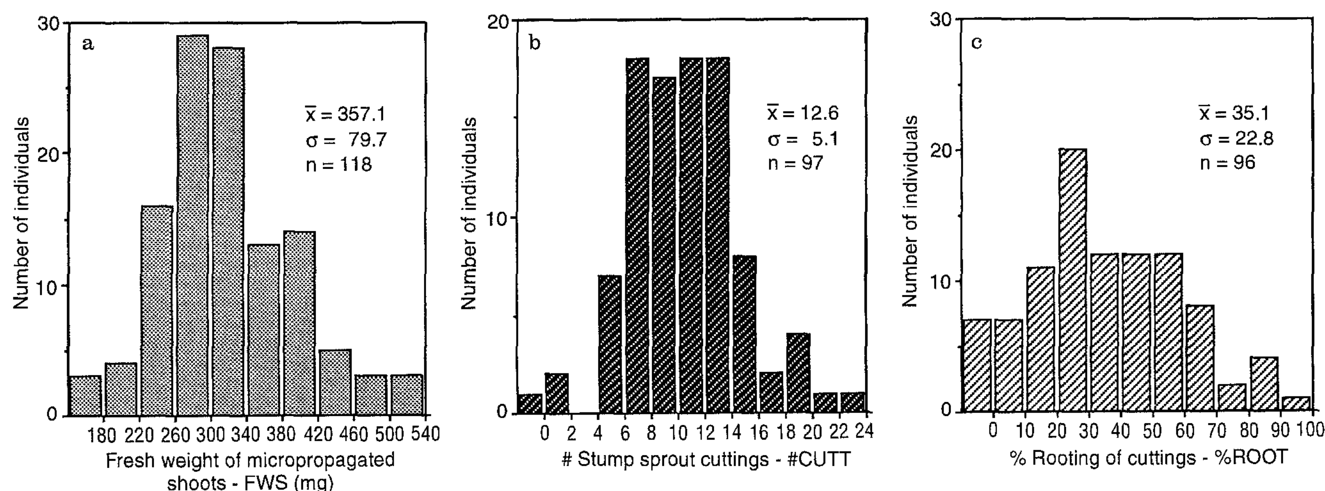

Fig. 3a–c Frequency distributions for vegetative propagation traits in the interspecific F_1 family used for QTL mapping. **a** Fresh weight of micropropagated shoots (FWS) in mg; **b** Number of stump sprout cuttings (#Cutt); **c** Percent rooted cuttings (%). Mean ($\bar{x}$), standard deviation (σ), and sample size (*n*) used in the QTL analysis are indicated beside the histograms

Table 1 Locations and magnitudes of effect of QTLs controlling traits related to vegetative propagation response in *E. grandis* clone 44, as determined by interval mapping analysis using MAPMAKER-QTL

Trait[a]	Linkage group	Marker interval	QTL[b] position	LOD peak	1.0 LOD support[c] interval	% Var[d] expl.
FWS	1	P10_1250 – J5_510	0.0	2.1	Off end – P10_1250 – 8.0	9.6
	1	I4_1360 – Z3_830	0.0	2.3	16.0 – I4_1360 – Z3_830	9.1
	3	R20_1650 – U19_550	8.0	1.3*	8.0 – R20_1650 – J7_560–8.0	6.0
	7	N15_1079 – U10_1500	0.0	1.9	6.0 – X12_1250 – Off end	7.5
	10	N12_207 – J17_815	18.0	2.2	Off end – J17_815 – 12.0	11.7
	8/12	N6_634 – Y16_550	4.0	2.7	16.0 – N6_534 – Y16_550	12.3
#Cutt	1	R9_670 – U16_930	0.0	1.5*	4.0 – J5_887 – Z3_830 – 4.0	7.4
	2	D3_1746 – K10_913	0.0	1.6	20.0 – X2_1200 – D3_1746 – 14.0	7.3
	8/12	K9_534 – Y3_1350	0.0	1.7	10.0 – R3_1200 – N6_634 – 14.0	7.8
	13/11	N13_2169 – P19_460	0.0	2.4	14.0 – X12_530 – P19_460 – 24.0	10.8
%Root	5	K12_954 – K15_567	0.0	1.8	4.0 – G5_1898 – K15_567 – 2.0	8.5

* Significant in the QTLSTAT analysis ($P \leq 0.01$)
[a] FWS, fresh weight of micropropagated shoot clumps; #Cutt, number of operational stump sprout cuttings; %Rott, percent adventitious rooting of cuttings
[b] Most likely QTL position corresponding to LOD peak, as estimated by MAPMAKER-QTL; cM distance from leftmost marker of interval
[c] Interval over which the position of the QTL is at most 10-times less likely than the most likely position estimated by MAPMAKER-QTL; from left to right: cM distance from the left, marker segment, and cM distance to the right; Off end=off the end of linkage group
[d] Percent of the phenotypic variation explained, as estimated by MAPMAKER-QTL

detected for micropropagation response (FWS), six for sprouting ability (#Cutt), and four for rooting ability (%Root) (Tables 1–4). In three instances, a genomic region was declared significant based on one analysis but did not reach the significant threshold for the other. In *E. grandis* a QTL was declared on group 3 based on a P=0.003, when the LOD peak was only 1.3. On group 1 a QTL was declared following a P=0.009 when the LOD score was only 1.5. In *E. urophylla*, on the other hand, a QTL was declared based on a LOD peak of 1.6 but the P value from QST was 0.014. Typically, LOD scores or Wald statistics above the significant thresholds adopted were observed along stretches involving more than one marker interval. When two linked LOD peaks were observed, with an overlapping LOD 1.0 support interval, only one QTL was declared in the region at the location with the highest peak. When the LOD 1.0 supports did not overlap, a procedure suggested by Lander and Botstein (1989) was adopted. The position of one QTL was fixed and the chromosome scanned again for a linked QTL effect. Such a procedure was applied to *E. grandis* in two regions: on linkage group 1 for FWS and group 5 for %Root. Only on linkage group 1 for FWS, after controlling for each peak, did sufficient evidence remain (Δ LOD=2.9) to declare two linked peaks (Fig. 1, Tables 1 and 2).

Table 2 Locations and properties of QTLs controlling traits related to vegetative propagation response in *E. grandis* clone 44 as determined by least-squares interval mapping analysis using QTLSTAT

Trait[a]	Linkage group	Marker interval	*P* value	Wald[b] statistics	R^{2c}	Genotype means ± SD[d]		Δ_1[e]	Δ_2[f]
						(+)	(–)		
FWS	1	K10_1106 – Y20_620	0.0001	15.7	0.17	269.8 ± 25.4	438.5 ± 23.8	2.10	1.02
	1	X15_1050 – I4_1360	0.002	10.1	0.10	329.1 ± 13.1	374.4 ± 14.3	0.57	0.22
	3	R20_1650 – U19_550	0.003	9.2	0.09	379.3 ± 14.4	335.1 ± 13.4	0.55	0.28
	7	X12_1250 – N15_1079	0.0007	11.9	0.11	376.8 ± 12.4	334.1 ± 14.7	0.54	0.25
	10	N12_207 – J17_815	0.003	8.6	0.13	378.1 ± 12.9	334.8 ± 14.6	0.55	0.26
	8/12	Y16_550 – Y17_515	0.0002	14.1	0.11	383.4 ± 14.4	335.9 ± 12.6	0.60	0.33
#Cutt	1	J5_887 – R9_670	0.009	7.1	0.13	11.4 ± 1.0	14.2 ± 1.0	0.55	0.32
	2	M10_800 – X2_1200	0.01	6.5	0.11	13.4 ± 1.3	11.1 ± 1.2	0.45	0.15
	8/12	R3_1200 – Z18_1630	0.001	11.0	0.24	14.4 ± 1.0	10.9 ± 1.0	0.70	0.36
	13/11	X12_530 – N13_2169	0.003	9.5	0.14	13.2 ± 1.0	10.0 ± 1.0	0.63	0.12
%Root	5	K12_954 – K15_567	0.005	8.3	0.10	27.6 ± 5.2	41.4 ± 4.4	0.61	0.27

* Significant in the MAPMAKER-QTL analysis (LOD ≥ 1.6)
[a] FWS, fresh weight of micropropagated shoot clumps; #Cutt, number of operational stump sprout cuttings; %Root, percent rooting of cuttings
[b] A Wald statistics of 10.0 is approximately equal to *P*=0.001
[c] Percent of the phenotypic variation explained, estimated as the non-linear regression R^2 (SS_{marker}/SS_{total}) using QTLSTAT
[d] Estimates of genotype means for the alternative RAPD marker-linked QTL alleles; (+) presence of the RAPD band, (–) absence of the RAPD band
[e] Difference between alternative QTL genotypes expressed in phenotypic standard deviations
[f] Difference between the favorable QTL genotype and the population mean expressed in phenotypic standard deviations

Table 3 Locations and magnitudes of effect of QTLs controlling traits related to vegetative propagation response in *E. urophylla* clone 28, as determined by interval mapping analysis using MAPMAKER-QTL

Trait[a]	Linkage group	Marker interval	QTL[b] position	LOD peak	1.0 LOD Support[c] interval	% Var[d] expl.
FWS	1	Q13_980 – X15_700	8.0	1.6	18.0 – Q13_980 – Off end	6.8
	2	K19_1780 – L17_560	4.0	2.6	2.0 – G2_584 – A11_1439	10.2
	5	U16_2080 – U19_1450	0.0	2.0	16.0 – U16_2080 – K15_500 – 6.0	8.0
	6	X12_340 – W9_950	2.0	1.6	2.0 – Z16_344 – Off end	10.7
#Cutt	1	W20_530 – K9_941	10.0	1.7	10.0 – W20_530 – U7_1100 – 4.0	8.9
	2	A11_1439 – X12_740	0.0	2.0	4.0 – B6_810 – X12_740	9.2
%Root	4	R8_570 – X15_1600	14.0	5.8	Off end – X15_1600 – 20.0	21.0
	8	K3_290 – N15_485	0.0	1.9	2.0 – U13_450 – K3_290 – 18.0	8.6
	9	G5_451 – W11_650	0.0	1.7	W12_1500 – Off end	7.3

[a] FWS, fresh weight of micropropagated shoot clumps; #Cutt, number of operational stump sprout cuttings; %Root, percent rooting of cuttings
[b] Most likely QTL position corresponding to LOD peak, as estimated by MAPMAKER-QTL; cM distance from leftmost marker of interval
[c] Interval over which the position of the QTL is at most 10-times less likely than the most likely position estimated by MAPMAKER-QTL; from left to right: cM distance from the left, marker segment, and cM distance to the right; Off end=off the end of linkage group
[d] Percent of the phenotypic variation explained, as estimated by MAPMAKER-QTL

In the QTL summaries we report the marker interval where either the highest LOD score estimated by MMQ, or Wald statistic by QST, were observed. For MMQ we also locate the 1.0 LOD support interval (Tables 1–4). The results of both analyses generally agree. However, the marker bracket where the highest LOD score was located generally did not correspond exactly to the one with the highest Wald statistics. For a total of 20 QTL regions, in only eight (40%) did the marker bracket with the highest LOD score correspond to the bracket with the peak. Frequently (7 in 20, 35%) we found that the marker brackets where the LOD and Wald peaks were detected were adjacent, rather than overlapping, and shared a common marker. This was more common for *E. grandis* than *E. urophylla*. For example, QTLs for FWS on groups 1, 7 and 8/12 and for #Cutt on groups 1 and 13/11b in *E. grandis* were located to different but adjacent intervals in the two analyses (Tables 1 and 2). Note that in the majority of these cases the most likely position estimated by MMQ was exactly at the shared marker, i.e., QTL position 0.0 cM. Finally we also found cases of non-adjacent, but nearby, marker intervals (5 in 20, 25%). Different, but nearby, marker intervals were located for QTLs controlling #Cutt on groups 2 and 8/12 for *E. grandis*, FWS on group 5 and

Table 4 Locations and properties of QTLs controlling traits related to vegetative propagation response in *E. urophylla* clone 28 as determined by least squares interval mapping analysis using QTLSTAT

Trait[a]	Linkage group	Marker interval	P value	Wald[b] statistics	R^{2c}	Genotype means ± SD[d]		Δ_1[e]	Δ_2[f]
						(+)	(–)		
FWS	1	X15_700 – M4_1228	0.014*	6.0	0.09	341.2 ± 13.4	377.8 ± 13.6	0.46	0.26
	2	K19_1780 – L17_560	0.0005	12.7	0.11	382.6 ± 13.4	329.2 ± 13.6	0.67	0.32
	5	K15_500 – U20_420	0.0006	12.4	0.18	442.3 ± 24.3	291.0 ± 20.2	1.90	1.07
	6	X12_340 – W9_950	0.004	8.6	0.15	340.4 ± 13.0	385.8 ± 15.3	0.57	0.36
#Cutt	1	W20_530 – K9_941	0.01	6.5	0.08	11.3 ± 1.2	13.9 ± 1.0	0.52	0.26
	2	X7_1750 – J7_452	0.001	11.3	0.38	16.8 ± 1.7	8.8 ± 1.5	1.58	0.83
%Root	4	R8_570 – X15_1600	0.0000	25.4	0.28	48.3 ± 4.2	23.4 ± 4.7	1.09	0.58
	8	K3_290 – N15_485	0.007	7.4	0.11	40.8 ± 4.5	28.7 ± 5.0	0.53	0.25
	9	Y13_390 – G5_451	0.004	8.8	0.10	42.4 ± 4.4	29.0 ± 4.9	0.59	0.32

* Significant in the MAPMAKER-QTL analysis (LOD≥1.6)
[a] FWS, fresh weight of micropropagated shoot clumps; #Cutt, number of operational stump sprout cuttings; %Root, percent rooting of cuttings
[b] A Wald statistics of 10.0 is approximately equal to P=0.001
[c] Percent of the phenotypic variation explained, estimated as the non-linear regression $R^2(SS_{marker}/SS_{total})$ using QTLSTAT
[d] Estimates of genotype means for the alternative RAPD marker-linked QTL alleles; (+) presence of the RAPD band; (–) absence of the RAPD band
[e] Difference between alternative QTL genotypes expressed in phenotypic standard deviations
[f] Difference between the favorable QTL genotype and the population mean expressed in phenotypic standard deviations

Table 5 Summary of the pseudo-testcross QTL analysis for traits related to vegetative propagation response: multipoint estimates of the % phenotypic variation explained by the mapped QTL [obtained by interval mapping using MAPMAKER-QTL and linear models using PROC GLM (SAS)], repeatabilities, and estimates of % genetic variation

Item	FWS		#Cutt		%Root	
	E. grandis	*E. urophylla*	*E. grandis*	*E. urophylla*	*E. grandis*	*E. urophylla*
# Putative QTL mapped	6	4	4	2	1	3
% Phenotypic variation Multipoint MAPMAKER-QTL	41.6	25.2	22.9	14.7	8.5	26.3
% Phenotypic variation Multipoint PROC GLM (SAS)	32.2	15.4	13.5	4.2	7.9	28.5
Total % phenotypic variation Multipoint MAPMAKER-QTL	52.5		28.2		32.6	
Total % phenotypic variation Multipoint PROC GLM (SAS)	46.8		17.4		33.0	
Repeatability[a]	0.59		0.42		0.52	
% Genetic variation[b]	89.0		67.1		62.7	

[a] Estimated as the ratio between the variance between individual genotypes and the total variance (between and within genotypes)
[b] Estimated as the ratio between the total phenotypic variation estimated by multipoint interval mapping (MMQ) and the trait repeatability

#Cutt on group 2 in *E. urophylla*. It is important to point out that in both cases, i.e., adjacent or nearby, the peak intervals determined by QST were within the 1.0 LOD support interval estimated by MMQ.

A somewhat different case of disagreement was found in *E. grandis*. On group 1, LOD peaks in two intervals were detected for FWS using MMQ. Although one interval (P10_1250 – J5_510) was also significant with QST (Wald=13.1), the highest Wald-statistics peak detected in the region was in a nearby interval (Wald=15.7 in interval K10_1106 – Y20_620, Table 2). In this case, the nearby interval detected with QST was not included in the 1.0 LOD support interval estimated by MMQ. Although MMQ provided evidence for two linked effects, such a statement should be viewed with caution in view of the disagreement of the two analyses regarding the exact position of one of the effects. A larger sample size or a different analytical approach (Zeng 1994) could help resolve this issue.

Estimates of the proportion of phenotypic variation explained by each QTL were obtained from MMQ (Tables 1 and 3). Such proportions were also estimated with QST as the coefficient of determination (R^2) (Tables 2 and 4) for

the single-locus model from the least-squares analysis of variance table by dividing the Type-III Sum of Squares for the QTL genotype by the total Sum of Squares. With one exception (see below) the two estimates proportionally agree. However, the proportions estimated as R^2 values are always larger than the estimates of % variation explained by MMQ. For FWS, individual QTLs explained between 6.8 and 12.3% of the variation, while the corresponding R^2values ranged from 0.09 to 0.18, i.e., between 9 and 18% of the variation was explained by each single QTL model. For %Root, MMQ estimates were from 7.3 to 21% and between 10 and 28% for QST. For #Cutt, MMQ estimates were between 7.3 and 10.8% while QST estimated the same individual effects between 8 and 38%. A major discrepancy was observed for a QTL controlling #Cutt on group 2 in *E. urophylla*. While the MMQ estimate was 9.2%, the R^2 corresponded to 38% of the variation (Tables 3 and 4). Knapp et al. (1992) pointed out that estimates of R^2 obtained from non-simultaneous single-locus models can be significantly inflated by sampling bias. Therefore, the estimates of the proportion of variation explained by single-locus models should be viewed with caution.

Simultaneous multilocus estimates of the total proportion of phenotypic variation explained by the joint action of the putative QTLs mapped for each parental tree were obtained by multipoint interval mapping with MMQ and by multiple linear regression using PROC GLM (SAS 1988) (Table 5). Note that the simple arithmetic sum of the individual effects estimated (Tables 1–4) would always be significantly larger than the multipoint estimates. For example, if we summed all the effects for FWS in *E. grandis* we would have 56.2%, which is larger than the 41.6% estimated by multipoint interval mapping. Similarly, in *E. urophylla* we would find 35.7% compared to 25.2% by multipoint QTL mapping. Estimates of the total% phenotypic variation explained by the joint action of all putative QTLs mapped in both parents were also obtained by multipoint interval mapping and multiple linear regression (Table 5). The estimates obtained by linear regression are generally smaller than those obtained by interval mapping. This was also observed in a maize QTL mapping experiment (Doebley and Stec 1993).

As more than one measurement of the traits were made on each individual genotype, we were able to partition the total phenotypic variance into the variance within and the variance between individual genotypes. Repeatability for each trait was estimated as the ratio of the variance between individuals and the total variance (Table 5). As pointed out by Falconer (1989), the repeatability sets an upper limit to the degree of genetic determination and to the heritability. By weighting the multipoint interval mapping estimates (MMQ) of the total phenotypic variation obtained by the estimates of repeatabilities (heritabilities) we arrived at an upper-limit estimate of the proportion of genetic variation explained by the QTLs mapped (Table 5).

Least-square means of the alternative QTL genotypes and their associated standard deviations were estimated with QST (Tables 2 and 4). Note that for all the putative QTLs detected, variances of the alternative QTL genotype classes were generally equal and close in value across QTLs within traits. Higher values of the within-QTL class variances were observed in two QTLs for FWS: on group 1 in *E. grandis* and group 5 in *E. urophylla*. In both cases, these higher variances corresponded to QTLs where large differences in mean-trait value between the two alternative QTL genotypes (Δ_1) were observed. In our QTL mapping experiment, these differences ranged from 0.46 to 2.1 phenotypic standard deviations (σ_p) (Tables 2 and 4). Estimates of $\Delta_1$were between 0.5 and 0.7 for the majority (65%) of the QTLs detected. A potentially more interesting estimate from the breeding standpoint is the difference in mean-trait value between the family mean and the favorable QTL genotype (Δ_2) (Tables 2 and 4). These differences ranged from 0.25 to 1.07 σ_p and typical values were between 0.2 and 0.4 σ_p.

The QTL detected with the highest LOD score (5.8) was for %Root in *E. urophylla*. The closest linked marker (R8_570) is shown segregating in a sample of F_1 individuals (Fig. 4). The effect of the substitution of this linked RAPD marker resulted in the doubling of the rooting percentage (from 23.4 to 48.3%) or an increase in 12% rooting above the family mean (Δ_2=0.58 σ_p) (Table 4). QTLs of large effects for FWS were detected for both parents (on group 1 for *E. grandis* and group 5 for *E. urophylla*), and in both cases the difference between the favorable allele and the family mean were estimated to be above 1.0 σ_p (Tables 2 and 4). For #Cutt, the QTL detected with the highest LOD score was on group 13/11b, with the most likely position estimated to be exactly at marker N13_2169 (Fig. 5). However, in spite of the high LOD score and percent variation explained (10.8%) as estimated by MMQ, the effective contribution of this region to the trait does not result in a significant shift from the family mean. The difference between the alternative QTL genotypes (Δ_1) is 0.63 σ_p; however the difference between the favorable QTL genotype and the family mean (Δ_2) is only 0.12 σ_p. It seems that estimates of Δ_1 can be misleading. Even though a significant shift in average trait value is caused by the allelic substitution at the QTL, the final trait value can still be within the average of the family. Estimates of Δ_2 seem to be more useful than both Δ_1 and % variance explained as indicators of the relative importance of the QTL detected, as they translate into more meaningful values from the standpoint of marker-assisted selection.

In both species, there were two cases where the LOD 1.0 support interval of QTLs detected for FWS and #Cutt overlapped: on groups 1 and 8/12 in *E. grandis* and groups 1 and 2 in *E. urophylla*. Both traits involve multiple shoot formation from dormant buds, differing in the fact that, in FWS shoot formation is stimulated in vitro with the action of cytokinin, while in #Cutt it relies exclusively on the intrinsic physiological ability to break the dormancy of resting juvenile buds. It seems reasonable to suggest that these two traits – although not significantly correlated in this experiment – should share some common QTLs. Although pleiotropic gene action might be a possibility for these QTLs, we cannot at this point distinguish between pleiotropy and tight linkage of different QTLs.

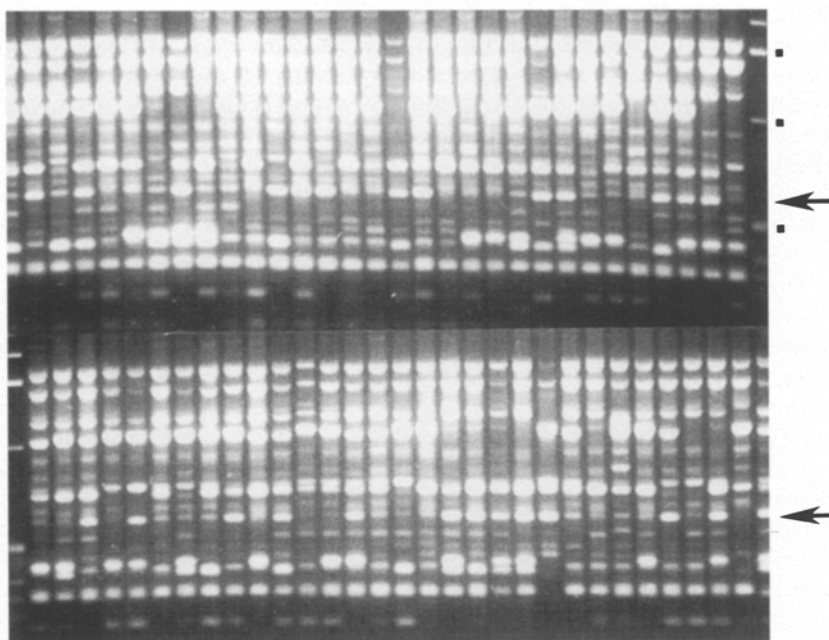

Fig. 4 Segregation of RAPD marker R8_570 from *E. urophylla* clone 28. R8_570 was found to co-segregate in coupling with a QTL controlling the % rooting of cuttings (LOD 5.8, P=0.000, Tables 3 and 4). Single-locus estimates of the proportion of the phenotypic variance explained by this QTL were 21% (MAPMAKER-QTL) and 28% (QTLSTAT). *Last lane* in top panel and *first lane* in bottom panel are 1-kb ladder size standards. From left to right, top panel shows the RAPD profile for 31 F_1 progeny; bottom panel shows an additional 29 progeny and the two parents, *E. grandis* clone 44 and *E. urophylla* clone 28. *Arrows* indicate the segregating marker

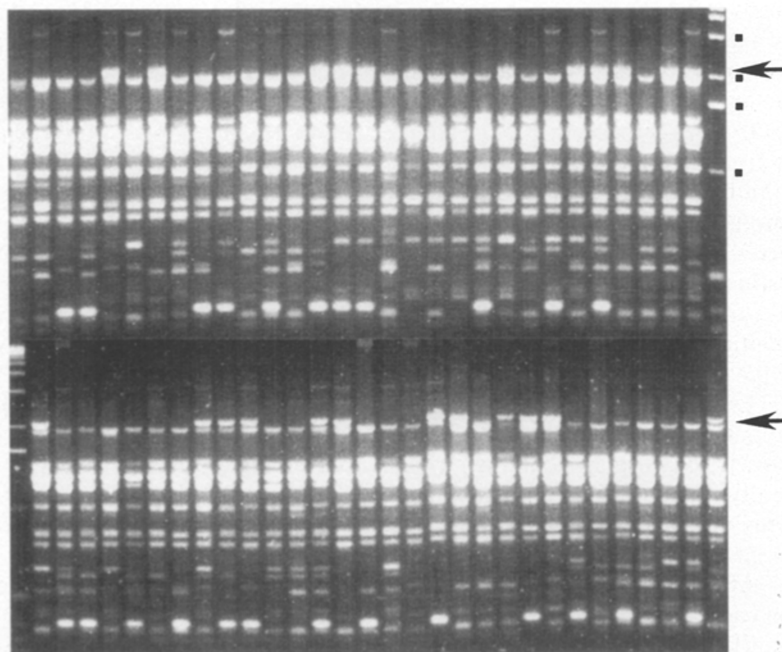

Fig. 5 Segregation of RAPD marker N13_2169 from *E. grandis* clone 44. N13_2169 was found to co-segregate in coupling with a QTL controlling the number of stump sprout cuttings (LOD 2.4, P=0.003, Tables 1 and 2). Single-locus estimates of the proportion of the phenotypic variance explained by this QTL were 10.8% (MAPMAKER-QTL) and 14% (QTLSTAT). *Last lane* in top panel and *first lane* in bottom panel are 1-kb ladder size standards. From left to right, top panel shows the RAPD profile for 30 F_1 progeny; bottom panel shows an additional 28 progeny and the two parents, *E. urophylla* clone 28 and *E. grandis* clone 44. *Arrows* indicate the segregating marker

Discussion

Linkage map construction

In this study, linkage maps of RAPD markers that segregated in the pseudo-testcross configuration were used to locate quantitative trait loci. These single-tree linkage maps had been previously constructed based on the co-segregation analysis of markers for 62 individuals. The sample size was increased for the QTL analysis reported here. Maps constructed *de novo* based on 122 individuals conserved the linear order of framework marker loci. Counting each marker out of the original order as an event (e.g., a switch in the order of two adjacent markers counts as one event), there were a total of seven order changes out of 100 markers in *E. grandis* (7%) and ten changes out of 83 markers in *E. urophylla* (12%). Therefore, on the average, only about 10% of the markers had their orders changed when increasing the sample size almost two-fold (from 62 to 122).

Keats et al. (1991) pointed out that the 1000:1 support threshold guideline for building framework linkage maps was conservative and would only prove adequate from em-

pirical studies. Our results show that even when a relatively limited sample size (*n*=62) is used to build linkage maps, the adoption of the 1000:1 support to include markers into a more likely framework order results in a robust map, whose order is essentially the same as that of an experiment using twice as many progeny. These results also indicate that a two-step approach would be adequate to optimize the extensive genotyping work necessary in QTL mapping experiments. In the first step, segregation data for a large number of markers (>250) would be gathered only for a subset (about 60) of the mapping population. Preliminary framework maps with a 1000:1 support for order would be constructed for both parents. Then, in a second step, an extended set of progeny would be genotyped only for a selected group of evenly spaced framework markers, followed by a finer search with all the markers available in potential regions of interest.

QTL mapping

Similarity between regression analysis and interval mapping analysis in QTL mapping has been observed previously (e.g., Doebley and Stec 1993; Stuber et al. 1992). We compared two interval mapping methods. With the exception of three genomic regions, declared significant by one method and only close to the threshold by the other, both interval mapping methods yielded very similar results for QTL detection. The significance thresholds adopted (LOD 1.6 and P=0.01) were generally comparable. This was not surprising considering that both methods use only slightly different algorithms. Darvasi et al. (1993) estimated numerically that, at a marker spacing of 10 cM and 11 markers per chromosome, a LOD score of 1.53 – corresponding to a per-marker type-I error rate of 0.0084 – ensures a 0.05 per-chromosome type-I error. With an infinite number of markers, the LOD threshold would have to increase to 1.96. These estimates were obtained under a backcross model. In our study, marker spacings along both maps were similar, 10.6 cM for *E. grandis* and 9.5 cM for *E. urophylla*. The number of markers per chromosome averaged 14 for *E. grandis* and 15 for *E. urophylla*. Therefore, the stringency adopted to declare a QTL in this study seems satisfactory.

The power for QTL detection was limited due to the small sample size available (Fig. 3). In a true backcross, a sample size of at least 500 individuals would be necessary to achieve an average power of 0.64 for detecting a QTL with a standardized gene substitution effect of d=0.25; for d=0.5 the power was always close to 1 (Darvasi et al. 1993). In spite of the small sample size, some gain in power was probably achieved in our experiment by using clonal replicates of the F_1 individuals. Clonal replication essentially increases the heritability of the trait (Bradshaw and Foster 1992). Heritability was shown to play a crucial role in determining the magnitude of additive genetic variance at any QTL that can be detected as statistically significant (Lande and Thompson 1990). Knapp and Bridges (1990) argued that if all the additive genetic variance is accounted for by markers, an additional replication of a clone increases statistical power by an amount equivalent to adding another offspring genotype. Finally, Strauss et al. (1992) estimated that a sample size of 200 would be needed to detect half of the additive genetic variance at α=0.01 when the within-family trait heritability is 0.5 and five effective QTLs control the trait.

No estimates of heritability are available for the traits investigated. However, we were able to estimate an upper limit to broad-sense heritability by calculating repeatability. In our experimental conditions repeatabilities were in the order of 0.4 to 0.6. Assuming that by clonal replication we increased our sample size from about 100 to a "virtual" sample size of 200 (Bradshaw and Foster 1992), and using our estimates of repeatability as an upper limit of heritability, our experimental results agree with the theoretical arguments discussed above. Standardized gene substitution effects for the QTLs detected in our study (Δ_1 Tables 2 and 4) were typically between 0.5 and 0.7 phenotypic standard deviations, and the smallest effect detected was 0.45. Our mapping experiment was efficient to detect only major-effect QTLs. However, a significant proportion of the genetic variation could be accounted by the QTLs mapped. For FWS, repeatability 0.59, ten QTLs were detected accounting for 89.0% of the genetic variation; for %Root, repeatability 0.52, the four QTLs detected account for an estimated 67.1%; while for #Cutt, repeatability 0.42, six QTLs account for 62.7%. At this point we could only speculate on the proportion of the genetic variation that is due to additive effects.

LOD 1.0 support intervals for QTL positions were typically around 30–50 cM. Discrepancies in the most likely QTL location within the marker interval were observed between the two interval mapping methods, although in 75% of the cases the most likely position was either in the same or in an adjacent interval. From simulation studies, Darvasi et al. (1993) concluded that the confidence intervals for QTL map location can be rather broad, in some cases essentially covering the whole chromosome, and relatively independent of marker density. For standardized gene-substitution effects equal to 0.5, a sample size of 1000 individuals would be required to reach an 11-cM confidence interval. Taking these simulations into account, and given the size of our experiment, we would not expect to be able to precisely locate QTLs beyond the level of assigning them to linkage groups. The QTL positions reported herein are therefore tentative. While the issue of precise QTL location would be crucial for map-based cloning efforts, it should not represent a significant obstacle for marker-assisted breeding. The extreme markers bracketing the 1.0 LOD support interval could be used for ensuring successful selection for the favorable QTL allele.

Pseudo-testcross QTL mapping

In the "pseudo-testcross" mapping strategy we exploited the high levels of heterozygosity of outbred individuals and the efficiency of the RAPD assay to uncover large num-

bers of genetic markers in an informative mating configuration (Grattapaglia and Sederoff 1994). In the present study we have extended the combined use of the "pseudo-testcross" mapping strategy and RAPD markers to map the first QTLs for species of *Eucalyptus*. To our knowledge this is also the first such analysis of QTL mapping using RAPD markers in trees, and the first attempt to understand the genetic architecture of commercially important traits related to vegetative propagation by rooted cuttings and micropropagation.

The pseudo-testcross strategy is based on the selection of single-dose markers present in one parent and absent in the other. As a result, the genetic linkage maps are individual-specific, and no RAPD markers are in common between the two maps. This same concept extends to QTL mapping. The QTLs mapped in this study are individual-specific. We cannot establish homologies of linkage groups or homologies of QTLs in the two maps at this time. Such homologies will await the localization of common RAPD marker loci on both maps. Conservation of RAPD markers and their linkage relationships in maps of different individuals will depend on the presence of the same RAPD marker loci and their allelic state. In a previous study in *E. grandis*, we have determined that approximately 33% of mapped RAPD markers were conserved across individuals of widely distinct origins, and approximately half of those also segregated (Grattapaglia and Sederoff 1994). Furthermore, the conservation of favorable marker/QTL associations across individuals will depend essentially on the extent of linkage disequilibrium between marker and trait loci in the population (see below).

QTL mapping in crop plants has usually relied on the availability of inbred lines that frequently were chosen to differ specifically with respect to QTLs affecting the traits of interest. When these inbred lines are crossed to produce segregating F_2 or BC families, a large amount of linkage disequilibrium is generated and quantitative trait associations with alternative marker genotypes can be readily measured. In outbred populations, such as those of domestic animals and most forest trees, QTL mapping strategies involving inbred pedigrees are generally not applicable due to a significant genetic load and time constraints. For most traits of interest, populations are generally polymorphic at both the QTL and marker loci, and the degree of linkage disequilibrium that can be generated by crossing populations is limited. As pointed out by Soller (1991), in such populations, mapping can be based on the disequilibrium necessarily found within individual families within a single population. The pseudo-testcross QTL mapping approach explores precisely this source of disequilibrium by virtue of the specific coupling relationships between marker alleles and QTL alleles in the parents of the family. In this study we relied on the existing linkage disequilibrium within an interspecific full-sib family to identify genetic factors controlling traits related to vegetative propagation in *Eucalyptus*. Maximum-likelihood methods for QTL mapping in full-sib families have been presented, targeting specifically those cases where several unrelated families with few individuals are available (Knott and Haley 1992). As pointed out by those authors, where family sizes are large enough it may be possible to use least squares-based methods to find marker-QTL linkages within single pedigrees without the need to accumulate evidence on individual markers across pedigrees. With increasing numbers of full sibs the linkage phase can be accurately determined and the power of QTL detection increases substantially. Furthermore, additional genotype data from grandparents obtained from three-generation pedigrees provides information only on the phase of marker linkage in the parents. It has, however, little or no impact on the increase in mean test statistic (QTL detection power) unless only small families are available (Knott and Haley 1992). In *Eucalyptus*, as well as in most forest tree species, large full-sib families are available or can be readily produced. Three-generation pedigrees are typically rare.

QTL mapping in the pseudo-testcross configuration relies on within-family linkage disequilibrium to establish marker/trait associations. Separate QTL analyses are carried out for each parent of the cross under the conventional backcross model. For this reason, in the pseudo-testcross QTL analysis, dominant RAPD markers provide essentially the same amount of information as co-dominant RFLPs with the obvious advantage of speed in marker data gathering. Evidently, as in a true testcross, no intralocus interactions such as dominance can be estimated in the pseudo-testcross. However, when compared to the conventional backcross model for QTL mapping, the pseudo-testcross differs in two main aspects. First, because of the undomesticated nature of the species for which this strategy is attractive, no prior genetic information is available about the parental genotypes and no planned "construction" of QTL genotypes is possible. Therefore, the only QTLs that can potentially be detected are those that are heterozygous in the parents and where the differential effect between the alternative QTL alleles is relatively large. Knott and Haley (1992) pointed out that in outbreeding populations under selection, even in the best situation, i.e., when a QTL has only two alleles, on average 50% of parents do not produce segregating gametes at this locus.

Secondly, because of the likely heterozygosity at any QTL locus in both parents, the quantitative value of alternative marker genotypes is measured as the effect of one allelic substitution averaged over the potentially two alternative alleles inherited from the other parent. If the genotype of one parent at the QTL locus is Q_1Q_2, and for the other parent Q_3Q_4, the QTL analysis essentially tests the difference between the average trait value of $(Q_1Q_3+Q_1Q_4)$ versus $(Q_2Q_3+Q_2Q_4)$ in the first parent and $(Q_3Q_1+Q_3Q_2)$ versus $(Q_4Q_1+Q_4Q_2)$ in the second parent. Also, the specific intralocus interactions that might take place and affect the final phenotype cannot be taken into account in the analysis. Therefore, more genetic "noise" is present in the system since the effect of the QTL allele substitution is measured against a genetically heterogeneous background both at the locus as well as at the rest of the genome. We cannot predict to what extent this "noise" should introduce a certain level of bias in the estimates of the magnitude of

QTL effect and so adversely affect the power of QTL detection. The differences between the multipoint estimates of total phenotypic variation and the arithmetic sum of the estimates for each individual QTL might partly be a result of such bias due to non-additive sources of genetic variation.

Van Eck et al. (1994) compared qualitative and quantitative analysis of a known locus controlling tuber shape in a cross between heterozygous potato clones. In their pseudo-tescross QTL analysis (analysis on each parent separately) the effect of a QTL allele in one parent might go unnoticed due to the masking effect of a stronger allele at the locus, contributed by the other heterozygous parent. To alleviate this problem they recommend using markers that detect different polymorphisms in both parents, such as fully classified co-dominant RFLPs segregating 1:1:1:1. However, their results also show that the pseudo-testcross analysis correctly detected the *a priori* known QTL. The highest significance peak (lowest *P* value) was found exactly at the marker more closely linked to the QTL in the maternal parent, corroborating the previously determined position by the qualitative analysis. For QTL mapping they used analysis of variance and a small sample size (n=50), both conditions prone to limited QTL detection power, particulary when an allele of weaker effect is under scrutiny. Had they used a larger progeny size and interval mapping methods, the missed effect might have been detected. However, analyzing large progenies with RFLP markers becomes a very time-consuming task. Furthermore, only a small subset of RFLP markers will be fully classified in any particular cross. The speed and ease of RAPD markers in the pseudo-testcross configuration allows undertaking a much larger genotype analysis both in terms of sample size and the number of markers compensating for the lower information content per marker locus. Furthermore, as pointed out by Williams et al. (1990), pairs of dominant RAPD markers closely linked in repulsion are essentially as informative as co-dominant markers. Highly polymorphic microsatellite markers would evidently be very powerful in this respect.

A potential problem that may arise in the pseudo-testcross QTL analysis is when spurious linkages occur between markers in both maps due to sampling variation in the genotypic data. If one of the markers involved in such linkages happens to be linked to a QTL in one of the parents, it may potentially lead to falsely declaring a QTL on the map of the other parent. In our study we specifically tested for this possibility, by analyzing jointly the marker data sets of both parents. At a relaxed LOD threshold (LOD 3.0) six spurious linkages were detected. Only one case, a spurious linkage (LOD 3.1) between marker X15_1600 (*E. urophylla* , group 4) and markers I4_1360 and X15_1050 (*E. grandis*, group 1), could have led to a potentially erroneous QTL detection. The marker in *E. urophylla* is linked to the strongest QTL detected for %Root. A LOD 1.3 for %Root was estimated for the marker bracket in *E. grandis*. Although it did not reach the significance threshold adopted (LOD 1.6), regions such as these could accidentally be interpreted as a QTL. Typically, such spurious linkages can be identified easily by the low LOD score and by the fact that they involve only one or two localized markers in the group, while true linkages always involve several, if not all, markers in the group. To further verify the spurious nature of these marker linkages, or potential spurious QTL detection, the markers involved from both maps can be removed and the linkage and QTL analyses performed again.

Marker-assisted selection for vegetative propagation in *Eucalyptus*

In spite of the low power for QTL detection stemming from the heterogeneous nature of the QTL mapping design and the limited sample size, we were able to map a number of QTLs controlling significant proportions of the phenotypic and genetic variation in vegetative propagation traits. QTLs controlling somatic embryogenesis (Armstrong et al. 1992) and in vitro androgenesis (Cowen et al. 1992) have been identified in maize. Recently, RAPD markers linked to two known genes that control somatic embryogenesis in alfalfa have also been mapped (Yu and Pauls 1993). The traits investigated in these studies were found to be under the control of a few genomic regions with large effects. For example, a set of five markers (three of them linked) explained 82% of the phenotypic variance for the percentage of immature embryos forming embryogenic callus (Armstrong et al. 1992). Our results also indicate that a few loci, or loci clusters, control most of the variation in micropropagation response in *Eucalyptus*, as ten regions were identified controlling an estimated 89.0% of the genetic variance.

QTLs of major effect were also identified controlling stump sprouting and adventitious rooting response. However, while a major-effect QTL for rooting was identified, all the QTLs mapped for stump sprouting were of relatively smaller and equal effect. Given the interspecific nature of our pedigree, comparisions of the genetic architecture of the two traits might not be entirely legitimate. Furthermore, unlike the controlled conditions achieved for in vitro propagation assessments, seasonal effects have long been known to have a strong influence both on sprouting and rooting responses in *Eucalyptus* (Fazio 1964; Blake 1972; Cremer 1973). It will be interesting to compare these results with those of experimental runs carried out at different times or locations. In spite of all this, the majority of the explained variation for %Root could be accounted for by the three genomic regions inherited from the *E. urophylla* parent. These included a strong QTL controlling over 20% of the phenotypic variation, which in our experimental conditions corresponds to over 40% of the genetic variance. We also found that the *E. grandis* parent was responsible for most of the inherited variation in the ability to form shoots both in vitro and by stump sprouting. These results agree with the prior information available on the general behavior at the species level regarding these traits. *E. grandis* is the most widely and intensely bred and planted species due to its rapid growth and extensive adapt-

ability. *E. grandis* is considered to sprout and micropropagate well (Hartney 1980) and is frequently employed in hybridization programs to improve such traits in species such as *E. nitens* and *E. deglupta* (Zobel 1993). On the other hand, hybridization to *E. urophylla* typically improves the ability of *E. grandis* to form roots on cuttings.

High levels of intra- and inter-specific variation in vegetative propagation response have been observed in *Eucalyptus*. It is fairly common that superior families or individuals in respect of volume production are identified, but success with rooted cuttings from them is prohibitively low for production purposes (Campinhos and Ikemori 1980; Van Wyk 1985). Typically, large numbers of superior families and individuals have to be generated so that high selection intensities can be applied to obtain productive genotypes that can also be vegetatively propagated. The manipulation of environmental factors known to affect the rootability of cuttings has been undertaken with variable success (reviewed by Hartney 1980). However, to move vegetative propagation technology to a production level, clone-specific requirements become impractical, and a rather robust protocol is necessary. To achieve this goal, genetic manipulation of vegetative propagation response through interspecific hybridization has been the method of choice. Molecular-marker-assisted breeding for vegetative propagation traits would be a highly desirable way to track the inheritance and segregation of important genomic regions on an individual basis. This should substantially accelerate the introgression of these traits into breeding populations, as well as facilitate the indirect pre-screening of individual clones, so reducing the number of individuals that need to undergo the time-consuming assessments of sprouting and rooting response.

The pseudo-testcross QTL mapping strategy involves the construction of genetic linkage maps of molecular markers and the identification of QTLs for individual genotypes. This approach is particularly attractive because it mitigates the obstacle of linkage equilibrium faced by marker-assisted breeding in outbred species such as forest trees. As many have pointed out, with linkage equilibrium, marker-trait associations established in one cross would not hold in a second pedigree, because marker and QTL alleles would be randomly associated at the population level (Soller 1978; Beckmann and Soller 1983; Lande and Thompson 1990; Strauss et al. 1992). In the present study we have shown that the within-family linkage disequilibrium can be used to identify genomic regions controlling quantitatively inherited traits related to vegetative propagation in *Eucalyptus*. With this approach one can contemplate performing QTL analysis in any available full-sib family generated from undomesticated and highly heterozygous organisms such as forest trees. Another relevant aspect of our work is that QTL mapping seems feasible on two-generation pedigrees of the kind commonly available in breeding programs. This is particularly important because it opens the possibility of using already existing families in retrospective QTL analyses, and so allowing one to gather the necessary quantitative data in an acceptable time.

Although the pseudo-testcross QTL mapping information is generated on an individual basis, the progressive accumulation of individual linkage maps with subsets of common markers among them will make obvious the relationships of QTLs in different maps. This will eventually lead to the identification of general regions associated with trait expression. However, even though such regions might be found, we expect that a multiplicity of QTLs controlling economically important traits exist at the population level. This should be particularly true for genetically heterogeneous species such as forest trees. For a similar phenotypic expression, different QTLs can act in different ways in different individuals, depending on the inherent genetic background and the kinds of selective pressures that the individuals have been subject to. Even in highly domesticated crops, such as maize and tomato, some experimental evidence exists on the issue of inconsistency of QTL expression across populations suggesting heterogeneity of QTLs (Tanksley and Hewitt 1988; Beavis et al. 1991). In the single-tree QTL approach adopted in the present study, marker/trait associations are established at the individual level, and therefore substantial linkage disequilibria are expected to be maintained. Close linkages established between markers and QTLs could be followed for several subsequent generations of selection and recombination. To optimize QTL mapping in each individual tree, larger progeny sizes than the ones used in this study would be required in the initial detection step to improve the power of detection. In subsequent generations, however, the number of markers genotyped could be substantially reduced as only those particular marker segments containing the QTLs of interest would be tracked. Progeny sizes could then vary depending on the number of genomic regions targeted at selection to increase the probability of recovering genotypes with the correct QTL allele profiles.

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