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Localized mapping of RAPD markers in *Eucalyptus grandis*

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ABSTRACT - *Studies on the QTL expression and the adequacy of methodologies involved in the analysis of molecular markers are important to make an effective use of molecular markers in genetic forest improvement programs possible. The technique of localized mapping was used aiming at the saturation of a specific genomic region in the linkage map of an E. grandis (G1) genotype with new RAPD markers. To saturate the region of interest containing one QTL for volumetric growth, five of the 265 oligonucleotides tested for polymorphisms among the DNA clusters have seven RAPD markers mapped in the same group of the marker R4_1300, proving the efficiency of the technique in localized mapping in specific regions of genetic linkage maps. Such results are necessary to make the application of molecular markers in genetic improvement programs for Eucalyptus feasible.*

Key words: QTL, *Eucalyptus grandis*, RAPD, localized mapping.

INTRODUCTION

Molecular markers at the DNA level have allowed the construction of genetic linkage maps of forest species such as *Eucalyptus* (Grattapaglia and Sederoff 1994, Byrne et al. 1995, Bundock et al. 2000), *Populus* (Liu and Furnier 1993, Bradshaw et al. 1994) and conifers (Plomion et al. 1995, Sewell et al. 2000, Kondo et al. 2000, Li and Yeh 2001) as well as the localization of genomic regions that control economically important traits in these species (Groover et al. 1994, Benet et al. 1995, Bradshaw et al. 1995, Grattapaglia et al. 1995, Plomion 1995, Sewell et al. 2000).

Once the molecular markers that co-segregate with the desired traits are identified, high resolution mapping in the genomic regions that contain the QTLs frequently becomes necessary. When these studies aim at cloning genes or a better localization of the loci that control traits of interest, the construction of high density genetic maps becomes fundamental (Reiter et al. 1992). According to Michelmore et al. (1991) and Reiter et al. (1992), techniques of DNA cluster analysis can be used to fill little saturated regions ("gaps") in genetic maps and to localize additional markers in a strategy denominated localized mapping.

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The analysis method designated “Bulked Segregant Analysis” (BSA) (Michelmore et al. 1991) based on phenotypic data allows the fast identification of markers in specific regions of the genome by the comparison of two DNA clusters of individuals originated from a segregant progeny. In each cluster, the individuals are identical to the phenotype of interest and different to other traits. This technique has been used in the mapping of monogenic traits such as disease resistance in agricultural (Michelmore et al. 1991, Poulsen et al. 1995) and forest crops (Benet et al. 1995, Lehner et al. 1995, Junghans et al. 2003) and in QTL mapping (Wang and Paterson 1994).

In this situation the technique of “localized mapping” (Reiter et al. 1992) provides the saturation of specific genomic regions of linkage maps, from specific loci to chromosomes or entire linkage groups based on segregant genotype clusters. DNA clusters are constructed based on the genotype of molecular markers of a determined genomic region, searching for new polymorphic markers that map at this same local.

In the present study, the technique of localized mapping (Reiter et al. 1992) was used for the saturation of a specific genomic region (marker R4_1300) by the use of new molecular RAPD markers, and for the validation of the genetic linkage map of *Eucalyptus grandis* (Grattapaglia and Sederoff 1994) for the progeny of an interspecific crossing between *E. grandis* and (U3) *E. urophylla* (U3) (Campinhos 1996). Localized mapping was used with the objective of increasing the resolution degree of the genetic map in a specific region of the genome that contains one QTL for volumetric growth (Grattapaglia and Sederoff 1994).

MATERIAL AND METHODS

2.1. Plant material

A full-sib progeny of 44 individuals originated from the interspecific crossing between a *E. grandis* tree (G1), used in the study of Grattapaglia and Sederoff (1994), and a genotype (U3) of *E. urophylla*, previously used in a study on the stability of QTL expression for growth (Campinhos 1996) was used.

2.2. Genomic region studied

A QTL at a distance of approximately 12cM from the marker R4_1300, localized in linkage group 5 of the previously constructed linkage map for a *E. grandis* tree was used in this study (Grattapaglia and Sederoff 1994).

2.3. Construction of composed DNA samples

Two samples, one positive and the other denominated negative were composed of 10 different genotypes each. Each one of the samples contained 250 ng equimolar DNA of each one of the genotypes. The positive sample consisted in individuals that presented marker R4_1300, while the negative was formed by the DNA of individuals whose marker was absent.

2.4. DNA extraction and RAPD analysis

The total genomic DNA was obtained according to a procedure standardized previously by Grattapaglia and Sederoff (1994), as well as the amplification reactions of the DNA by the RAPD technique.

The RAPD markers were identified by the name of the oligonucleotide provided by the manufacturer (Operon Technologies, Alameda, CA), followed by the fragment size it amplified (in base pairs). A subjective value of the intensity of the amplified fragment is given, separated by a backslash, where 1 stands for the least intense, 3 for the most intense and 2 for the value of the intensity mean.

2.5. Cluster screening for polymorphism

The two compound DNA samples of the progeny and of the respective parents (G1 and U3) used in the study on the expression stability of growth QTLs, were evaluated using 265 oligonucleotides (OPAJ15 to OPAX20), different from those previously used in the construction of *E. grandis* maps (Grattapaglia and Sederoff 1994). Polymorphic DNA fragments that were among the two clusters and originated of the *E. grandis* parent were considered potential markers linked to marker R4_1300, in group 5 of the linkage map. All evaluated RAPD markers were of maternal origin (*E. grandis*) and should segregate in the proportion 1:1 in generation F_1, as expected in a “pseudo” crossing test (Grattapaglia and Sederoff 1994).

Screening was realized to determinate the linkage of the new markers to marker R4_1300 in two phases. In the first, the variation in the band intensity was evaluated, where potentially polymorphic markers were selected. In the second phase, each one of the selected markers was tested for four individual samples of each cluster, only searching for the present or absent markers in at least three of the four individuals. This second selection was used for the confirmation of the polymorphisms obtained in the first selection.

2.6. Linkage analysis

The RAPD markers, selected after the two screening phases were tested for a set of 44 individuals of the progeny. The recombination frequency and the mapping position in relation to R4_1300 and the other two markers

(X15_600 and Y17_1500) were estimated; both belong to group 5 of the *E. grandis* map. The markers were analyzed for presence (1) or absence (2) of the amplified fragment. Missing data were identified as (0).

The chi square test (c^2) (a=0.05) was used to test the conformity of the RAPD marker segregation with the expected proportion of 1:1, as in a crossing test. Markers with a segregation differing significantly from the expected proportion at a level of 5% were excluded from the mapping analysis. The molecular RAPD marker data were duplicated and inversely recodified, in order to allow the detection of genetic linkage of markers in repulsion phase (Grattapaglia et al. 1995a). The Mapmaker program 2.0 for Macintosh (Lander et al. 1987) was used, considering a minimum "LOD score" of 3.0 and a maximum q (recombination fraction) of 0.40, to verify which selected markers were linked to marker R4_1300 and to the markers X15_600 and Y17_1500. The distances were computed in centiMorgans (cM) using Kosambi's mapping function.

2.7. QTL analysis for the genomic region

The linkage analysis of the new and the previously mapped RAPD markers with growth traits (height at 12 and 17 months) was tested by the individual marker analysis using the t test (α=0.05).

RESULTS AND DISCUSSION

This study demonstrates that it is possible to use the methodology of localized mapping for saturation of specific regions of genetic maps in eucalyptus. Two samples of 10 individuals were used for the saturation of a genomic region of a *E. grandis* linkage map, containing one QTL for volumetric growth, with new molecular RAPD markers. According to Michelmore et al. (1991), using 10 individuals in each sample, the probability of a marker that is not linked to the marker of reference being polymorphic among the clusters is $2x10^{-6}$, in other words, this is the probability of Error type I.

In the initial evaluation of the 265 oligonucleotides, 47 (17.74%) were selected for containing a total of 77 apparently polymorphic markers among the clusters (Figure 1). The mean of 1.64 markers per oligonucleotide was mainly owing to a few oligonucleotides that amplified three to four polymorphic fragments. Most of the oligonucleotides (60.4%) presented only one potentially polymorphic marker. The smallest fragment evaluated in this phase had approximately 400 base pairs (pb) and the greatest 3.000 bp. The mean size was 1.124 ± 516 bp. In this first phase of selection, the variation in the band intensity in both samples was sufficient that the marker were considered potentially polymorphic and, consequently, selected. This little rigorous criterion resulted in the selection of false positives, avoiding the loss of the molecular markers actually linked to marker R4_1300.

Only 9.1% of the markers selected in the first and 26.9% in the second screening phase were effectively linked, considering the cluster parameters LOD = 3.0 and θ = 0.40. The selection of polymorphisms based on the difference of band intensity in the first screening, and the marker selection segregating up to 3:1 in both clusters of four individuals of the second screening were little strict in the selection of polymorphic markers, showing that the low rigor used at the two screening levels resulted in the selection of RAPD markers that belonged to other linkage groups of the map. The less rigorous analysis at both screening levels may seem inefficient at first because of the large number of selected markers, while in fact it furnishes a great enrichment in markers linked to the target locus. The second phase of fragment screening to be used in the linkage analysis with marker R4_1300 was therefore carried out with a relatively high number of markers selected in the first phase.

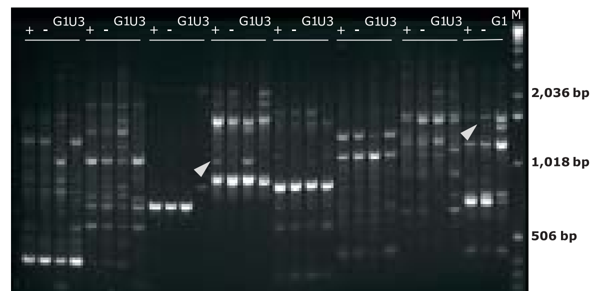

Figure 1. Amplification pattern obtained by means of the RAPD reaction using the compound samples as DNA template, "+" (positive sample for marker R4_1300) and "-" (negative sample for marker R4_1300), and the parents "G1" (female) and "U3" (male). M = Marker of fragment size of DNA. bp=base pairs. The arrows indicate two of the polymorphisms selected amongst the samples

Curiously, in spite of the proportion of 16.25% of selected oligonucleotides initiators, Bundock et al. (2000) obtained a mean of 5 markers per oligonucleotide similar to the present study, apparently segregating in the proportion of 1: 1 in a progeny derived from a crossing of *E. globulus* of different origins. Possibly, the smaller proportion of markers found in the localized mapping (1.67) occurs since the focus is on a specific region, even when

considering the crossing among *E. grandis* and *E. urophylla*.

In the second screening phase, 26 markers were selected among the 77 found in the first screening phase, based on 21 oligonucleotides. At the most, those markers that presented a potentially recombinant individual in each one of the clusters were considered, that is, a presence:absence relation of, at the most, 3:1 or 1:3. In this phase, the approximate fragment size varied from 600 to 1.700 bp with a mean of 1.058 ± 312 bp.

In relation to the mean size and standard error (in base pairs) of the fragments selected in the first for the second level of selection, it was verified that the mean size remained practically the same (from 1.124 to 1.058). The standard error was however reduced by more than 200 bp (516 to 312), suggesting that the majority of the molecular RAPD markers, over 1.500 bp and below 500 bp, is less repeatable in successive analyses. Obviously, markers beyond these limits are selected when they are of easy interpretation and reproductions throughout the evaluations. Li and Yeh (2001) for example obtained 328 RAPD markers that were used for the construction of a *P. contorta* subsp. *latifolia* linkage map that varied from 260 to 3080 base pairs.

Of the 26 markers selected for *E. grandis*, the proportion band absence:presence, or vice-versa in the individuals of the two clusters was 3:1 for eight markers; 3:1 in one and 4:0 in the other for nine markers; and 4:0 in both clusters for nine markers. Of the analyzed markers, six showed distortion from the expected Mendelian segregation 1:1 (α=0.05) (Table 1). The linkage analysis with Mapmaker (LOD = 3.0 and θ = 0.40) demonstrated that seven of the 20 markers were mapped in the same linkage group of the markers R4_1300, X15_600 and Y17_1500 (Figure 2). Among the seven mapped RAPD markers, six presented a proportion 4:0 or 0:4 in the two clusters of each marker in the second screening phase.

A linkage analyses of two points showed that of the seven markers mapped in the same marker R4_1300 linkage group, three formed a more distant group. The nearest marker (AR20_600) lies 24.5 cM from R4_1300 and the most distant ones, AL13_750/740 (apparently a codominant locus) 28.2 cM. In the opposite sense and also distant lies marker AP3_1400, localized at 19.1 cM. Only three of the markers lie close to R4_1300. The nearest ones were AO15_600 and AR14_1550 mapped 4.6 cM from R4_1300 and 0 cM from marker X15_600. The greatest distance between marker R4_1300 and a selected marker was 28.2 cM for the codominant locus, defined by the markers AL13_750 and AL13_740.

The linkage analysis of the new markers mapped with the trait height of 12 and 17-month-old plants was not significant at a level of 5% of probability (Table 2). Thus, the previously mapped and the new markers identified in this experiment of localized mapping were not linked to QTL for plant growth. These results suggest that the markers linked to QTL identified by Grattapaglia and Sederoff (1994) are not valid for the progeny in our study where, even with the increased saturation of the region with other markers the QTL could not be localized.

However, the linkage analysis and the relative ordination of the selected markers showed that of the seven studied markers, four were mapped at a larger distance and three more closely to marker referential (R4_1300). The sensitivity limit of the segregant clusters analysis for the detection of molecular markers linked to the referential locus comprises a recombination window of approximately 25 cM (Michelmore et al. 1991). Consequently, the greatest observed distance of approximately 28 cM is close to the theoretically expected distance.

In the QTL analysis, the QTL for volumetric growth in the genomic region saturated with the new molecular markers was not detected, suggesting that it is not possible to extrapolate the information of the QTL identified in the study of Grattapaglia and Sederoff (1994). There are at least two possible reasons for this result. The first is the existence of a QTL x genotypic composition interaction, that is, the expression of this QTL depends on other genes of secondary effects or even of other QTLs, whose presence in the analyzed individuals was deficient. In the same family used in this study (G1xU1), the QTL for growth in group 5 was not detected in a stability study (Campinhos 1996). The second is the effect of sampling since only 44 of the 88 individuals of the progeny were used in stability study of QTLs for growth (Campinhos 1996). With the reduction of the sample to the half, the t values for the tested markers were reduced to approximately a third. This shows that the sample size used (n = 44) had been insufficient for the QTL mapping, even if it had been expressed in this family.

Verhagen et al. (1997) verified that some regions of the eucalyptus genome have an effect on more than one trait. In this study, the actuation of one QTL for different traits and different QTLs for the same trait at different ages was reported, showing a significant effect of the interaction QTL versus age.

Regarding the expression of QTLs in the early and late growth cycles Sewell et al. (2000) argued that QTLs associated with wood traits could be influenced by changes of the microenvironment within the cell due to the seasonality of the activity of the same. This possibility was verified by the analysis of the QTLs associated with

Table 1. Analysis of the segregation of the RAPD markers, localized by means of localized mapping. i = band intensity: high (1), mean (2) and low (3); 1:1 = relation presence:absence of the evaluated markers; χ^2 = chi-square test for agreement with the 1:1 segregation hypothesis

Marker	i	1:1	χ^2	Marker	i	1:1	χ^2
R4_1300[1]	2	23:21	0.0909	AN10_800	1	24:20	0.3636
X15_600[1]	3	21:23	0.0909	AO8_1410	3	18:26	1.4545
Y17_1500[1]	2	20:24	0.3636	AO8_1400	3	26:18	1.4545
AJ15_1100	3	28:16	3.2727	AO14_950	2	28:16	3.2727
AJ20_1100	1	26:18	1.4545	AO15_600	3	23:21	0.0909
AK5_1200	2	22:22	0	AP3_1700	2	20:24	0.3636
AK19_1500	2	14:30	5.8182*	AP3_1300	2	23:21	0.0909
AL4_950	1	30:14	5.8182*	AQ14_1100	2	36:8	17.8181*
AL13_750	3	22:21	0.0233	AQ14_900	2	36:8	17.8181*
AL13_740	3	21:22	0.0233	AQ16_1300	1	15:29	4.4545*
AM2_650	1	20:24	0.3636	AR14_1550	1	21:23	0.0909
AM8_800	1	15:29	4.4545*	AR20_600	1	23:21	0.0909
AM17_750	3	24:20	0.3636	AS2_1050	3	25:19	0.8182
AN5_900	3	24:20	0.3636	AS6)1400	2	27:17	2.2727
AN5_1000	2	22:22	0				

*distorted marker of the 1:1 segregation ($P < 0.05$)
[1]previously mapped markers

the specific weight of *Pinus taeda* wood, where most of the QTLs were specific for early or late cycles. This complexity can hamper the detection of QTLs associated to wood traits, which is an alternative hypothesis for the non- detection of the QTL for volumetric growth in the genomic region saturated with the new markers for *Eucalyptus*. Besides, within a same site the temporal expression of QTLs of a single individual could be significantly influenced by genotype x environment interactions and/or by the plant development stage (Sewell et al. 2000).

Using the same principle of localized mapping, other studies evidenced the potential of the technique in the saturation of specific genome regions. Giovannoni et al. (1991) used DNA clusters constructed based on molecular genotypes for the identification of DNA markers near the two genomic intervals, containing regulator abscission genes of the pedicel and of fruit ripening in tomato. Using this technique, Reiter et al. (1992) realized the localized mapping of 23 additional RAPD markers in chromosome 1 of *Arabidopsis thaliana*. Churchill et al. (1993) demonstrated the applicability of the method, by means

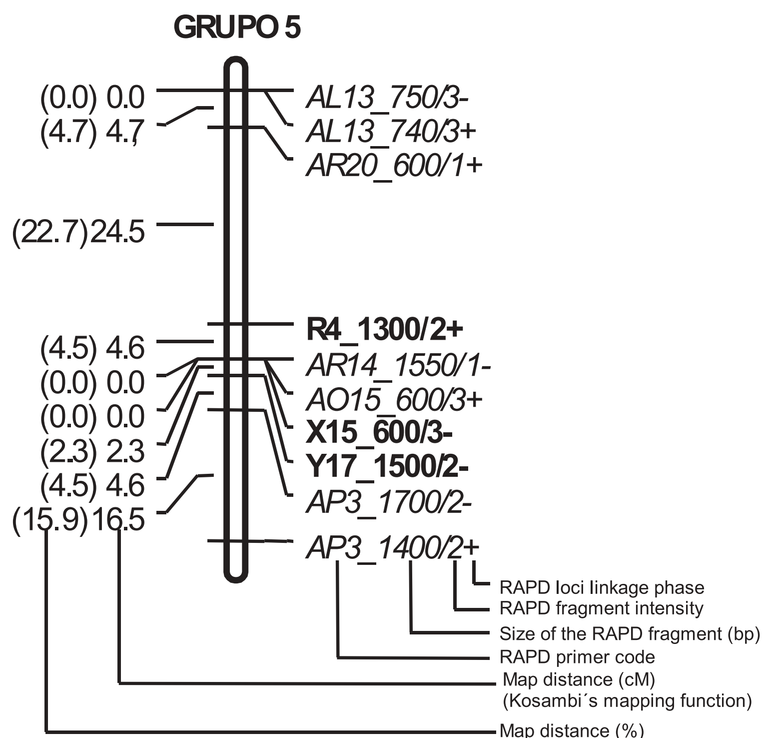

Figure 2. Genetic linkage group previously constructed by Grattapaglia and Sederoff (1994) corresponding to group 5 of the *E. grandis* linkage map, showing the previously mapped markers (bold) and the new molecular markers linked to the preceding (italic). RAPD markers are identified by the oligonucleotide code (Operon Technologies), fragment size in base pairs, fragment amplification intensity and linkage phase (+ or -)

Table 2. Analysis of growth QTL in the saturated region. T Test and probability P of rejection of the hypothesis of association among the markers mapped in group 5 of the linkage map of *E. grandis* and the trait height with 12 and 17 months

	height (17 months)		**height** (12 months)	
Marker	**t**	**P**	**t**	**P**
R4_1300[1]	-0. 531	0. 5982	-0. 518	0. 6073
X15_600[1]	0. 576	0. 5679	0. 481	0. 6328
Y17_1500[1]	0. 327	0. 7455	0. 219	0. 8277
AL13_740	-0. 536	0. 5945	-1. 052	0. 2991
AL13_750	0. 536	0. 5945	1. 052	0. 2991
AO15_600	-0. 576	0. 5679	-0. 481	0. 6328
AP3_1700	-0. 446	0. 6580	-0. 557	0. 5802
AP3_1300	0. 685	0. 4972	0. 401	0. 6903
AR14_1550	0. 576	0. 5679	0. 481	0. 6328
AR20_600	-0. 674	0. 5038	-1. 086	0. 2838

[1]previously mapped markers

of the high resolution mapping of a region of chromosome 5 of tomato containing a regulator gene of fruit ripening. In a study of localized mapping in peach (*Prunus persica*) and using the BSA technique, Chaparro et al. (1994) localized 15 RAPD markers linked to the Gr ("red leaf") loci and the isoenzyme Mdh-1 in linkage group 5 of the constructed map.

The use of different markers is an alternative for the increase of the resolution such as for the construction of maps. Particularly, Bundock et al. (2000) verified the usefulness of the RAPD markers in association with microsatellites for the construction of *E. globulus* maps. This approach to the use of different markers was also proposed by Kondo et al. (2000) who mapped a resistance gene to pine needle gall midge based on RAPD markers, understanding the need for more closely linked markers; localized mapping could equally contribute to the saturation of the linkage map close to the gene of interest.

After the construction of genetic linkage maps moderately saturated with molecular markers and the localization of regions of interest in the map, the technique of localized mapping in the search for the saturation of

these locals is possible, facilitating the marker-assisted selection or the realization of map-based gene cloning. Although it was not possible to validate the QTL for growth (Grattapaglia and Sederoff 1994), the determination of more markers near the region of the marker referential R4_1300 was therefore possible, demonstrating the potential of the technique of localized mapping in the saturation of specific genomic regions of a *Eucalyptus* linkage map. Even in the absence of markers linked to plant growth QTLs we observed an increased saturation of the map of linkage group 5 (Figure 2), which can be used for *E. grandis*. Alternatives for the detection of the studied QTL would therefore be reevaluations of the QTL for this species as well as for the progeny used in the present study, of the sample size of the progeny and even of the marker type.

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Mapeamento localizado de marcadores RAPD em *Eucalyptus grandis*

RESUMO - *Estudos a respeito da expressão de QTLs e da adequação de metodologias envolvidas na análise de marcadores moleculares são importantes para se viabilizar a utilização efetiva de marcadores moleculares em programas de melhoramento genético florestal. Objetivando-se saturar com novos marcadores RAPD uma região genômica específica no mapa de ligação de um genótipo de* E. grandis *(G1), utilizou-se a técnica de mapeamento localizado. Na saturação da região de interesse, contendo um QTL para crescimento volumétrico, dos 265 oligonucleotídeos testados para polimorfismos entre os agrupamentos de DNA, cinco possuíam sete marcadores RAPD que mapearam no mesmo grupo do marcador referencial R4_1300, comprovando-se assim a eficiência da técnica no mapeamento localizado em regiões específicas de mapas de ligação genética. Resultados como este são necessários para a viabilização da aplicação de marcadores moleculares em programas de melhoramento genético de* Eucalyptus.

Palavras-chave: QTL, *Eucalyptus grandis*, RAPD, mapeamento localizado.

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