IDENTIFICATION OF MOLECULAR MARKERS LINKED TO EARLY FLOWERING IN *Eucalyptus grandis*[1]

IDENTIFICAÇÃO DE MARCADORES MOLECULARES RELACIONADOS AO FLORESCIMENTO PRECOCE EM *Eucalyptus grandis*

Ana Paula Cazerta FARRO[2, 5]; Tânia Mara BORTOLOTO[3];
Shinitiro ODA[4]; Eduardo MELLO[4]; Celso Luis MARINO[3]

ABSTRACT – Flowering time is an important trait for tree breeding because it determines the speed of generation turnover and therefore the rapidity of genetic gains, and it is of particular interest in *Eucalyptus* species. In this work, we used simple sequence repeats (SSRs), random amplified polymorphic DNA (RAPD) analysis and specific markers for flowering to evaluate early flowering segregation in a full-sibling family of *Eucalyptus grandis* and to identify molecular markers associated with the control of flowering time. A cross between a normal flowering tree (wild-type) and early flowering tree resulted in 118 progeny with a 1:1 Mendelian segregation ratio for flowering time ($\chi^2 = 0.5424$, $P > 0.05$), which suggested the action of one main gene in a locus named *Early flowering in Eucalyptus grandis (PFEg)*. The SSR marker EMBRA 02 was related to the QTL PFEg, and identified this region as a candidate for trait control. These maps may be used as the basis for a study in which can be inserted new markers in an attempt to find more loci related to early flowering characteristic on eucalyptus.

Keywords: *Eucalyptus*; flowering time; linkage map; microsatellites.

RESUMO – O tempo de florescimento é uma importante característica para o melhoramento genético de árvores, pois este determina o prazo para o surgimento de uma nova geração e a rapidez para o ganho genético de uma determinada espécie, como por exemplo, *Eucalyptus grandis*. Neste estudo foram utilizados marcadores moleculares do tipo microssatélites, *Random Amplified Polymorphism* DNA – RAPD, além de marcadores específicos para avaliar a segregação do florescimento precoce em uma família de irmãos-germanos de *E. grandis*. Foi também verificado se esses marcadores estão associados ao controle do tempo de florescimento na espécie. A progênie de 118 indivíduos avaliada foi originada do cruzamento entre uma árvore de florescimento normal e outra de florescimento precoce. A segregação da característica de florescimento precoce apresentou uma razão de 1:1 ($\chi^2 = 0{,}5424$, $P > 0{,}05$), o que sugeriu a ação de um gene principal no loco denominado *PFEg* (Florescimento Precoce em *Eucalyptus grandis*). O marcador SSR EMBRA 02 foi relacionado ao QTL *PFEg* e esta região foi identificada como uma região candidata ao controle da característica. Estes mapas poderão ser utilizados como base para um novo estudo no qual podem ser inseridos novos marcadores na tentativa de localizar mais regiões relacionadas à característica de florescimento precoce em eucalipto.

Palavras-chave: *Eucalyptus*; florescimento precoce; mapa de ligação; microssatélites.

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[2]Universidade Federal do Espírito Santo – UFES, Departamento de Ciências Agrárias e Biológicas – DCAB, CEUNES, Rodovia BR 101 Norte, Km 60, Litorâneo, 29942-540 São Mateus, ES, Brasil.
[3]Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, Departamento de Genética, Distrito de Rubião Junior s/n, 18618-000 Botucatu, SP, Brasil.
[4]Companhia Suzano Papel e Celulose, Rodovia Raposo Tavares, s/n, Km 168, 18203-340 Itapetininga, SP, Brasil.
[5]Autor para correspondência: Ana Paula Cazerta Farro – farro.ana@gmail.com

1 INTRODUCTION

Brazil is one of the largest short fiber cellulose producers in the world. Careful genetic breeding programs and widespread suitable climatic conditions have contributed to this high productivity, which is primarily centered on the *Eucalyptus* genus. Early flowering is a desirable trait in breeding programs of forest trees for shortening generation intervals. Early flowering could be a very useful trait both in breeding and experimental genetics of *Eucalyptus* and has been the focus in previous studies (Domingues et al., 2006; Missiaggia et al., 2005).

The age of the plant at the first flowering in *Eucalyptus grandis* W. Hill ex. Maiden takes place at 2 to 3 years in Australia. In other species of the genus, like *Eucalyptus dunnii* Maiden, flowering occurs at 7 to 10 years (Eldridge et al., 1993).

The wide range of climatic conditions in Brazil means that eucalypt species flower at different ages. For, example, *Eucalyptus urophylla* S.T. Blake produces seeds within two years, whereas *E. grandis* flowers at the same age that it does in Australia, around three years of age and produces seeds in four years (Mora et al., 1981). However, despite this well-known flowering pattern, few *E. grandis* individuals actually flower earlier, including those in nurseries (Missiaggia et al., 2005). For example, an *E. grandis* tree that flowered at one year of age was identified in the breeding program of the Suzano Papel e Celulose Company.

The genetic control of flowering time has not been completely established, although a candidate gene is the transcription factor *ELF1*. This factor, which is associated with the meristem transition from a vegetative to reproductive state, has been isolated in *E. grandis* (Dornelas et al., 2004) and *E. globulus* Labill (named *LFY*) (Southerton et al., 1998). The *ELF1* factor can be mapped through polymorphisms in segregating populations (Thamarus et al., 2002).

In this study we examined the segregation of early flowering in a full-sib family of *E. grandis* and identified one genomic region linked to this trait.

2 MATERIALS AND METHODS

2.1 Plant Material and Early Flowering

A full-sib family of *E. grandis* (118 individuals) was obtained by controlled pollination and showed early flowering in the field. The maternal progenitor, which originated from Zimbabwe (Africa), flowered early at one and a half years of age, whereas the paternal progenitor, from the Floresta Estadual Edmundo Navarro de Andrade (Brazil), flowered at three years of age. The population was part of the Suzano Papel e Celulose Company breeding program and was planted in four lines, with 2 x 3 m of spacing between the trees, in an area of 0.072 ha in the production unit (52C068) at Fazenda Santa Eliza (lat 23º52'42'', long 47º59'00'', altitude 660 m), on July 1, 1998.

Early flowering in this population was assessed by examining all branches of each tree, approximately one and half year after seed germination (in February, 2000). The individuals were classified as early flowering when they showed some floral structures (mature or immature buttons, flowers or bracts) on their branches.

2.2 DNA Extraction, RAPD and SSR Analysis

DNA was isolated from fresh leaves using the CTAB (cetyltrimethyl-ammonium bromide) method (Grattapaglia and Sederoff, 1994). The high molecular weight DNA was checked for quality and quantity by electrophoresis in 0.8% agarose gel with λ DNA standards (Invitrogen).

Two hundred and thirty-four RAPD primers were used. The polymorphism of each primer was initially screened against the progenitors and the most polymorphic markers were then selected and screened in the entire population. RAPD polymerase chain reaction (PCR) reaction mixtures (13 μL) contained the following components and concentrations: 10 mM Tris-HCl, pH 8.0, 1.9 mM $MgCl_2$, 0.3 μM of 10-mer primer (Operon Technologies), 0.8 mM of dNTP (Invitrogen), 0.5% BSA (bovine serum albumin), 15 ng of template genomic DNA and 0.9 U of *Taq* DNA polymerase (Invitrogen). DNA amplifications were done in an MJ PTC100™ incubator (MJ Research, Watertown, MA, USA) using the following program: 3 min at 96 ºC for 1 cycle, 1 min at 92 ºC, 1 min at 35 ºC and 2 min at 72 ºC for 40 cycles, with a final cycle of 2 min at 72 ºC. The amplification products were separated on 1.5% agarose gels stained with ethidium bromide, in 1 x TBE buffer. The band profiles of each primer were scored manually on two occasions for the presence (1) or absence (0) of co-migrating fragments in all accessions. Only reproducible bands were used in the subsequent analysis. The scored fragment sizes ranged from 200 bp to 2500 bp.

Twenty SSR primers (EMBRA) developed and mapped by Brondani et al. (1998) were randomly labeled with either of the three fluorescent dyes: 6-FAM (blue), HEX (green) and NED (yellow). SSR PCR reactions were done in a volume of 11 µL of reaction containing 10 mM Tris-HCl, pH 8.0, 5 µM of each primer, 0.125 U of *Taq* DNA polymerase, 1.8 mM $MgCl_2$, 2.5 mM dNTP and 10 ng of DNA. By using three fluorescent dyes it was possible to perform the PCR reactions in *triplex* (i.e. with three different primer pairs) systems. DNA amplifications were done in an MJ PTC100™ thermocycler (MJ Research, Watertown, MA, USA) using the following program: 5 min at 95 ºC for 1 cycle, 1 min at 95 ºC, 1 min at 56 ºC and 1 min at 72 ºC for 29 cycles, with a final cycle of 5 min at 72 ºC. The PCR products were subsequently diluted 1:10 in water and 3 µL of a mix containing loading buffer and a molecular size marker (Rox) were added. The amplification products were separated on 4.25% polyacrylamide gels in an ABI PRISM 377 DNA sequencer (run conditions: 3,000 V, 60 mA, 200 W for 2.5 h at 51 ºC). The fragments were detected and their size determined by using the programs Genescan and Genotyper (ABIPRISM).

2.3 Genotyping of Candidate Markers Related to Flowering

Since the *Eef1* QTL promotes early flowering in eucalyptus seedlings 60-90 days after seed germination (Missiaggia et al., 2005), we mapped this QTL using flanking markers detected with the following primers: EMBRA 27 - forward: ATAACCACACCAATCTGCA; reverse: TATAGCTCGAACGCTCAAC and EMBRA 164 - forward: CCTTGTTGAGCTCCTGTCT; reverse: ACTATCAGCGTCCTGCAA. The amplification reactions were done under the same conditions as for the other SSRs, except that electrophoresis was done in 6% polyacrylamide gels that were subsequently stained with silver nitrate and the fragment size then determined manually.

The *EgLFY* flowering gene of *E. grandis* (Dornelas et al., 2004), which shares homology with the *LFY* gene of *Arabidopsis*, is related to the initiation of flowering in this species. The gene fragment of *EgLFY* was amplified with the primers proposed by Dornelas et al. (2004) (forward: CTTCCTCCTCCAAGTCCAATC; reverse: TGGCGGAGCTTGGTGGGGACA). The PCR reaction mixtures (10 µL) contained the following components at the concentrations indicated: 10 mM Tris-HCl, pH 8.0, 0.5-3 mM $MgCl_2$, 0.5 µM of each primer, 1 mM of dNTP (Invitrogen), 7.5 ng of template genomic DNA and 0.5 U of *Taq* DNA polymerase (Invitrogen). The DNA amplifications were done in a temperature gradient thermocycler (Eppendorf) using the following program: 5 min at 94 ºC for 1 cycle, 30 s at 94 ºC, 1 min at 43 ºC and 1 min at 72 ºC for 35 cycles, with a final cycle of 7 min at 72 ºC. The amplification products were separated on 1% agarose gels in 1 x TBE buffer. The gels were stained with ethidium bromide, photographed and examined manually for the presence of bands.

The *SEEF* marker (SCAR – sequenced characterized amplified region) for *Eucalyptus* early flowering, developed to detect flowering in the same studied population and with 60% of efficiency to early flowering detection (Domingues et al., 2006), was used. Amplifications were performed using SEEF primers: forward-AGCGTCCTCCCTCCCGACTGAAAC and reverse-AGCGTCCTCCTAGGGCTTACGATG. PCR reaction mixtures (13 µL) contained the following components at the concentrations indicated: 10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 0.2 µM of each primer, 1 mM of dNTP (Invitrogen), 0.5% BSA, 10 ng of template genomic DNA and 1 U of *Taq* DNA polymerase (Invitrogen). DNA amplifications were done in an MJ PTC100™ thermocycler (MJ Research) using the following program: 3 min at 96 ºC for 1 cycle, 1 min at 94 ºC, 1 min at 65 ºC and 2 min at 72 ºC for 35 cycles and a final cycle of 5 min at 72 ºC. The amplification products were separated on 1% agarose gels in 1 x TBE buffer. The gels were stained with ethidium bromide, photographed and examined manually for the presence of bands.

2.4 Data Analysis

Chi-square analyses, single-locus analysis and grouping of markers were done with the GQMOL 2.1 software (Cruz and Schuster, 2004) that allows linkage analysis involving markers with different segregation types. All of the markers were tested for Mendelian segregation using Chi-square analysis (χ^2) ($P > 0.05$). The settings utilized were: "backcross" to test the testcross markers (1:1 segregation) and F_2 for the intercross markers (3:1 segregation).

The maps were constructed for each parent using the double-pseudo-testcross mapping strategy (Grattapaglia and Sederoff, 1994). The marker linkage groups from the maternal and paternal progenitors were determined using an LOD threshold > 3 and a recombination fraction < 0.3. The Kosambi mapping function (Kosambi, 1944) was used to convert recombination units into genetic distances (cM).

3 RESULTS

3.1 Flowering Early Segregation

A Mendelian segregation ratio of 1:1 ($\chi^2 = 0.5424$, $P > 0.05$) was observed in the evaluation of the flowering in the field, with 63 individuals showing early flowering and 55 having no floral structures at one and a half years of age. The results suggest that early flowering is controlled by one locus with main effect. The normal time for flowering is dominant and the early flowering recessive. Brazil has one of the more advanced breeding programs of eucalypt in the world, with consistent gains in productivity over the years, but little is known about the genetic control of adaptive traits and traits related to the biology of the species.

3.2 Segregation Analysis of Molecular Markers

Fifty RAPD primers (21.4% of the 234 primers tested in the progenitors) displayed polymorphism and were used for mapping. Four hundred and forty-eight bands were obtained, 284 (63.4%) of which were suitable for mapping, with sizes ranging from 200 bp to 2000 bp and no significant χ^2. The number of RAPD bands with a 1:1 segregation varied from one to 14 (mean = 6.9) bands per RAPD primer.

Of the 20 SSR primer pairs, 18 displayed polymorphic bands with three segregation models (Table 1). The χ^2 test revealed five of the 18 markers differ from Mendelian segregation ratios ($P < 0.05$) (Table 1). Although this distorted the segregation by 27.8%, these markers were not excluded from the analysis to verify the position them on the parental maps.

Table 1. SSR primers and the respective amplified fragments used to map the maternal and paternal progenitors in this study, together with the expected segregation rates and χ^2 values for each marker.

Tabela 1. Primers SSR e seus respectivos fragmentos amplificados usados para mapear os progenitores materno e paterno deste estudo, juntamente com as segregações esperadas e valores de qui-quadrado para cada marcador.

Primer	Fragments in bp (alleles)		Segregation pattern	χ^2
	Maternal	Paternal		
EMBRA01	88/90	88/104	1:1:1:1	17.729*
EMBRA02	118/130	114/126	1:1:1:1	1.449
EMBRA03	128/130	130/136	1:1:1:1	20.69*
EMBRA04	80/104	96/104	1:1:1:1	0.235
EMBRA05	106/106	106/116	1:1	3.77
EMBRA06	130/150	132/144	1:1:1:1	0.666
EMBRA07	142/160	146/160	1:1:1:1	1.173
EMBRA08	138/168	152/166	1:1:1:1	13.05*
EMBRA09	116/120	116/120	1:2:1	22.426*
EMBRA10	138/144	138/138	1:1	0.305
EMBRA11	128/130	136/138	1:1:1:1	4.236
EMBRA12	134/152	134/152	1:2:1	1.139
EMBRA15	96/96	98/102	1:1	0.321
EMBRA16	180/204	180/204	1:2:1	9.58*
EMBRA17	128/132	130/132	1:1	1.445
EMBRA18	116/138	136/152	1:1:1:1	3.242
EMBRA19	162/162	158/160	1:1	1.309
EMBRA20	120/130	116/130	1:1:1:1	2.896

3.3 Linkage Map Assembly

The maternal progenitor map contained 174 markers that consisted of 164 RAPD (70 intercrosses + 94 testcrosses), eight SSRs, the flowering QTL *PFEg*, and the candidate SSR marker EMBRA 27. Of these markers, 101 (58.0%) were linked in 18 groups, including 12 larger groups (varying from 5-16 markers). The linkage groups varied in size from 12.66 cM to 179.13 cM (Figure 1).

The SSR marker EMBRA 02 and the *PFEg* were linked in the maternal map (LG-08) at a distance of 35.09 cM with a LOD of 3.0 (Figure 1). Thamarus et al. (2002) found a linkage between EMBRA 02 and two flowering genes: AGE1 – *Agamous homolog* (Harcourt et al., 1995), at 89 cM, and ELF1 – *Leafy homolog* (Southerton et al., 1998) at 36 cM.

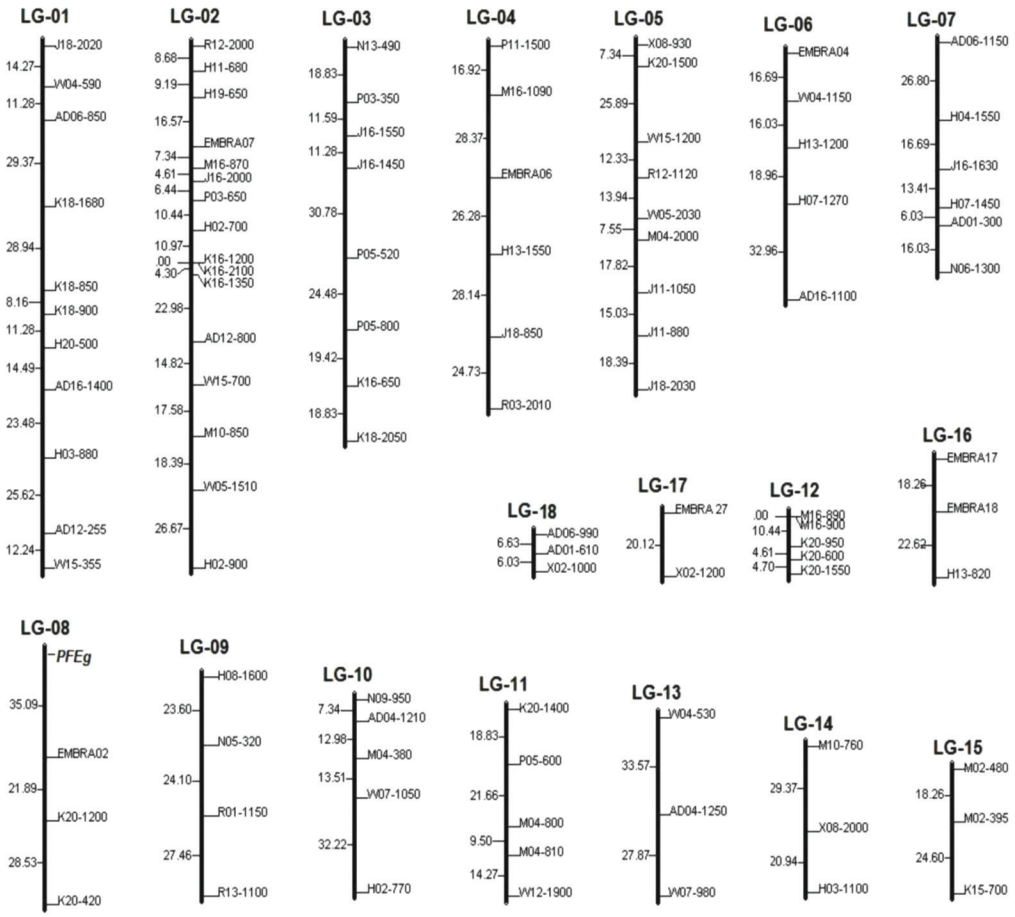

Figure 1. Maternal molecular linkage map for *Eucalyptus grandis*. The markers are shown on the right and genetic distances (centimorgans – cM) on the left of each linkage group (LG). Genetic distances were calculated with the Kosambi mapping function (Kosambi, 1944). In LG-08 it was found the PFEg locus linked to the EMBRA02 marker at a distance of 35.09 cM.

Figura 1. Mapa de ligação molecular maternal para *Eucalyptus grandis*. Os marcadores estão apresentados à direita e as distâncias genéticas (centimorgans – cM) à esquerda de cada grupo de ligação (LG). As distâncias genéticas foram calculadas com a função de mapeamento de Kosambi (Kosambi, 1944). No LG-08 é encontrado o loco PFEg ligado ao marcador EMBRA02 a uma distância de 35,09 cM.

The paternal progenitor map contained 191 markers that consisted of 178 RAPD (70 intercrosses + 108 testcrosses) and 13 SSRs. Of these markers, 101 (52.9%) were linked in 17 groups formed by 13 larger groups (4-13 markers) (Figure 2). The size of these groups varied from 21.26 cM to 191.23 cM.

Some linkage groups may represent the same chromosome. To examine this possibility, the location of homologous groups was assessed using intercross markers (3:1 segregation) and SSR. Seven groups in the maternal map were paired with 10 groups in the paternal map (Figure 3). Overall, the maps showed 20 common markers that allowed seven groups in the maternal map to be paired with one or more groups in the paternal map (Figure 3).

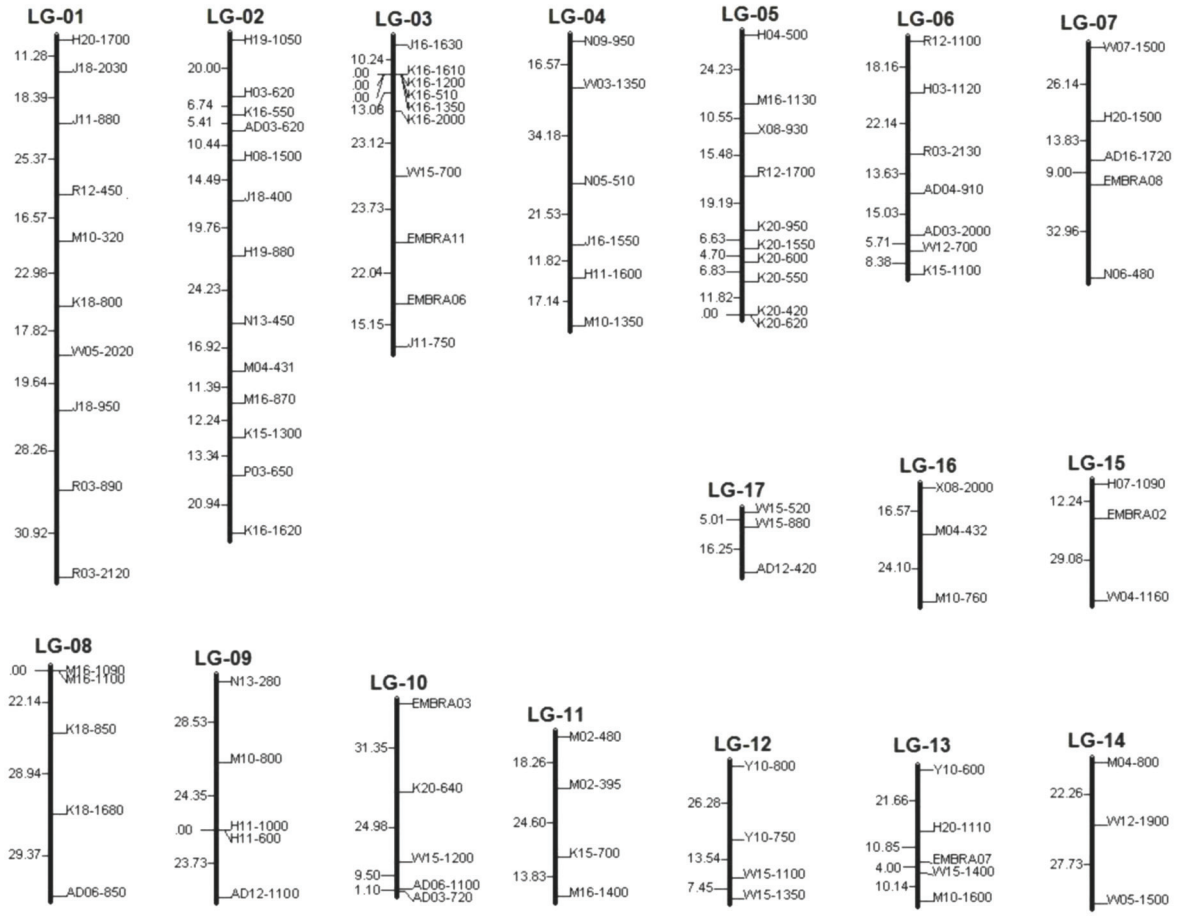

Figure 2. Paternal molecular linkage map for *Eucalyptus grandis*. The markers are shown on the right and genetic distances (centimorgans – cM) on the left of each linkage group (LG). Genetic distances were calculated with the Kosambi mapping function (Kosambi, 1944).

Figura 2. Mapa de ligação molecular paternal para *Eucalyptus grandis*. Os marcadores estão apresentados à direita e as distâncias genéticas (centimorgans – cM) à esquerda de cada grupo de ligação (LG). As distâncias genéticas foram calculadas com a função de mapeamento de Kosambi (Kosambi, 1944).

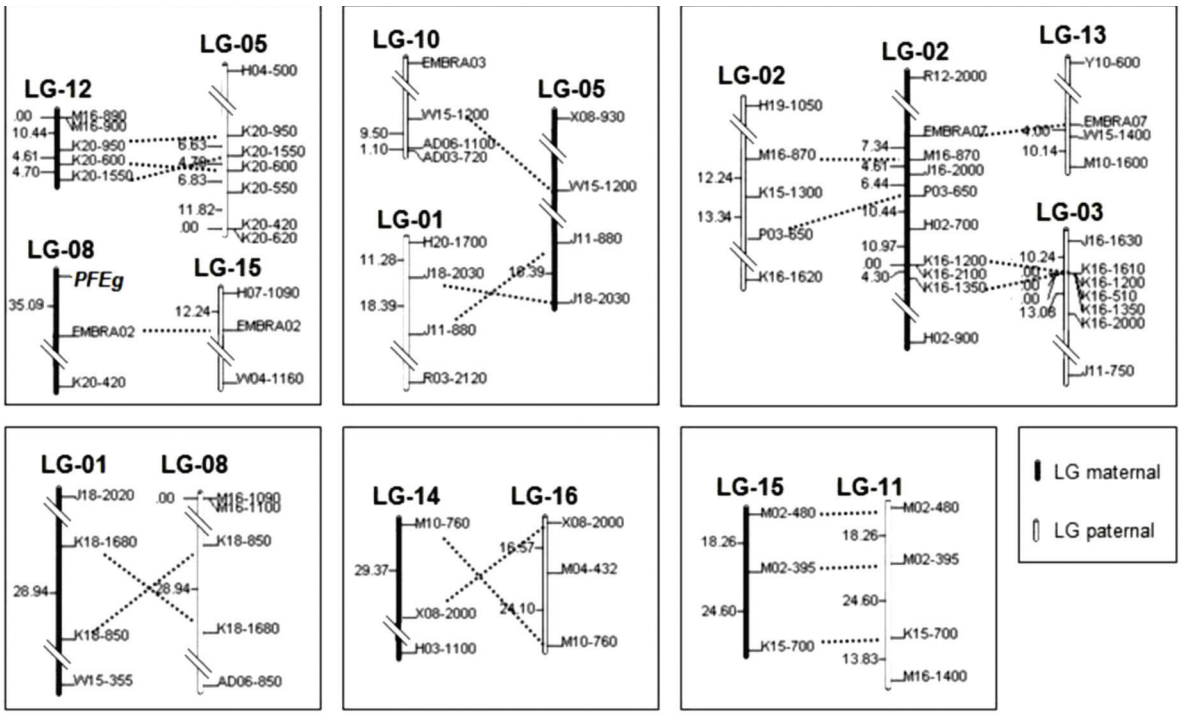

Figure 3. Comparison of the maternal and paternal progenitor linkage groups based on molecular markers common.

Figura 3. Comparação entre os grupos de ligação dos progenitores paterno e materno baseada em marcadores moleculares em comum.

3. 4 Candidate Molecular Markers

The primer EMBRA 164 did not provide ideal amplification of the progenitors and F1 offspring. However, the primer EMBRA 27 yielded amplification products of 130-134 bp and was not linked with the flowering gene expressed in our crosses; linkage was observed only with the RAPD marker X02-1200 at a distance of 20.12 cM (LG-17 – Figure 1). These results indicate that early flowering observed in the present segregated population is not related to the *Eef1* locus (Missiaggia et al., 2005).

Amplifications done with the primers described by Dornelas et al. (2004) revealed unspecific fragments (280 – 350 bp) and the data were not used in the final map.

The SEEF marker (Domingues et al., 2006) showed recombination ratio of 40% with the *PFEg*. The SEEF was not mapped because in the map design was considered the minimal distance of 30 cM and LOD score 3.0. This marker is cis-linked in the paternal progenitor with normal flowering (Domingues et al., 2006).

4 DISCUSSION

4.1 Field Analysis of Flowering Phenotype

The early flowering trait has a segregation ratio of 1:1, this can be explained by the cross between a recessive progenitor (maternal) and a heterozygous progenitor (paternal) for a locus with main effect called *PFEg*. Missiaggia et al. (2005) found a 1:1 segregation ratio in a family with flowering in 60-90 days. The marker SRR EMBRA 27, linked to the *Eef1* locus (Missiaggia et al., 2005), was not shown to be linked to the *PFEg* locus identified in this study. This indicates the existence of multiple genomic regions involved in the time for the initiation of flowering in *Eucalyptus*.

The initiation of flowering depends on the balanced expression of a complex network of more than 80 genes (Levy and Dean, 1998) that are regulated by endogenous and environmental factors (Araki, 2001; Dornelas and Rodriguez, 2005; Mouradov et al., 2002). Flowering time may be controlled by polygenes (Buckler et al., 2009), although the 1:1 segregation ratio for early flowering seen in our study indicated that the phenotype was controlled by one locus with main effect.

4.2 Segregation Analysis of Molecular Markers

Segregation distortions are found in genetic mapping studies of different species (Nachit et al., 2001; Ramsay et al., 2000; Risterucci et al., 2000). The segregation distortion seen here (27.8%) was lower than that reported for SSR markers in wheat (> 30%) (Nachit et al., 2001). Possible explanations for such distortion include chromosomal rearrangements (Tanksley, 1993), gametic or zygotic selection (Jacobs et al. 1995), and the presence of lethal genes (Blanco et al., 1998).

4.3 Flowering

Domingues et al. (2006) described the SEEF marker, which was demonstrated to be efficient in detecting 60% of tree with early flowering. This indicates a distance of 40 cM between the marker SEEF and the *PFEg*.

Linkage was detected between the SSR marker EMBRA 02 and the QTL *PFEg* for early flowering, with a LOD score = 3.0 (Figure 1 – LG-08). Thamarus et al. (2002) used molecular markers such as SSR and RFLP with EST probes to build a linkage map for *E. globulus*. As shown here, linkage was observed between genes for flower, such as *Leafy homolog* (*ELF1*) (Southerton et al., 1998) and *Agamous homolog* (*AGE1*) (Harcourt et al., 1995), and the SSR marker EMBRA 02. Thamarus et al. (2002) reported that *ELF1* was located 39.0 cM from the SSR marker EMBRA 02. The linkage of the flowering genes AGE and LFY and the locus *PFEg* with the EMBRA 02 marker emphasizes the connection of this linkage group to flowering in *Eucalyptus*. In *Arabidopsis thaliana* (L.) Heynh., the LFY gene is located on chromosome 5 that contains a high concentration of genes related to flowering.

The SSR EMBRA 02 marker region is a candidate for controlling flowering time, and the use of this marker as a starting point for fine mapping this region may be a useful strategy. However, since this marker is still far from the marker for early flowering, other candidate markers closer to EMBRA 02 in different linkage maps may be useful in identifying the region that controls flowering time. Another point that could allow identification of many candidate genes for early flowering, as well as candidate marker, is the genome assembly for *E. grandis* that is available at www.phytozome.net/eucalyptus.php. Identification of new markers in the genome sequence will permit testing of hypotheses regarding the involvement of those candidate genes in the early flowering phenotype in the segregating progeny.

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