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ORIGINAL PAPER

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Towards a genus-wide reference linkage map for *Eucalyptus* based exclusively on highly informative microsatellite markers

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Abstract A novel set of 50 highly polymorphic microsatellite markers were developed and mapped on existing RAPD framework maps of *Eucalyptus grandis* and *E. urophylla*. Together with the twenty previously developed microsatellite markers, these were used to align the existing maps for the two most commercially important *Eucalyptus* species in the tropics. Sixty-three microsatellite markers were placed on the *E. grandis* map in 11 linkage groups, and 53 on the *E. urophylla* map distributed in 10 linkage groups. Approximately 66% of the microsatellite markers segregated in a fully informative fashion, allowing the establishment of colinear syntenic linkage groups between the two maps. The 50 new microsatellite markers were highly informative, with an average of 14 alleles per locus, and average expected heterozygosity between 0.82 and 0.87. Furthermore, within the subgenus *Symphyomyrtus*, to which the vast majority of commercially important *Eucalyptus* species belong, these markers display on average 90% transportability. This set of 70 mapped microsatellite markers represents a significant step toward the development of a genus-wide reference linkage map for *Eucalyptus*. These highly multiallelic and transportable markers constitute a powerful tool for QTL discovery and validation, and can be used in directed searches for QTL allele variation across *Eucalyptus* pedigrees.

Keywords Microsatellite · Simple sequence repeats (SSR) · *Eucalyptus* · Linkage map

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Introduction

Genetic mapping has become a fundamental part of science and technology development in human, animal and plant genetics. The availability of linkage maps has made possible a wide range of studies, including the identification and positional cloning of important genes (Martin et al. 1993), the implementation of marker-assisted selection (Tanksley et al. 1996), the construction of genome-wide physical maps (Kurata et al. 1997) and the understanding of genome evolution (Bonierbale et al. 1988). This wide array of scientific opportunities has motivated the development of increasingly dense and genetically informative linkage maps for a wide range of plant species (Liu 1998).

During the last decade, a number of genetic maps based on DNA markers have been constructed for some of the most widely planted forest trees. For species of the genus *Eucalyptus*, these maps have been developed based mainly on the analysis of dominant RAPD (random amplification of polymorphic DNA) and AFLP (amplified fragment length polymorphism) markers (Grattapaglia and Sederoff 1994; Verhaegen and Plomion 1996; Gaiotto et al. 1997; Marques et al. 1998), segregating in a double pseudo-testcross configuration in F1 families, or a combination of RFLP and RAPD markers segregating in various configurations in an outbred F2 (Byrne et al. 1995). These maps have provided sufficient genome coverage for the identification of QTL regions with a significant effect on the expression of important traits (Grattapaglia et al. 1995, 1996; Vaillancourt et al. 1995; Byrne et al. 1997a, 1997b;

Verhaegen et al. 1997; Marques et al. 1999; Shepherd et al. 1999). A variety of molecular marker classes and pedigree types have been used in these experiments. QTLs have been detected in F1, inbred or outbred F2 and half-sib families with or without clonal replicates.

Although several genome maps of eucalypts have been constructed, due to the low information content and transferability of dominant markers (Brondani et al. 1997) and the low throughput of RFLP marker analysis, the use of the linkage information tends to remain restricted to the mapping pedigree employed, limiting the sharing and comparison of QTL data. Furthermore, the opportunities for precise dissection of multiallelism at QTLs in outbred eucalypts are limited. Therefore, a key obstacle to progress in the identification and understanding of quantitative trait variation in *Eucalyptus* and, by consequence, in marker-assisted selection for complex traits, has been the limited polymorphic information content of the molecular marker classes used to date (Grattapaglia 1999).

Recently, existing *Eucalyptus* linkage maps of anonymous markers have been enriched by the incorporation of a number of important candidate genes involved in lignification (Gion et al. 2001; Kirst et al. 2001). Although the mapping of candidate genes is an important approach to understanding the molecular basis of quantitative variation, mapping of expressed sequences is more time-consuming, and such markers typically display limited polymorphic information content for linkage mapping. To efficiently tackle high-throughput mapping experiments and integrate existing linkage maps and associated QTL information for *Eucalyptus* pedigrees, a map based solely on microsatellite markers with alleles segregating from both parents would be most efficient. The distinct value of microsatellites arises from their multiallelic nature, codominant inheritance, abundance and wide distribution in the genome, and the ability to detect sequence variation by a simple PCR assay. Furthermore, these markers have proven to be highly transferable from genome to genome within species and, frequently, among genetically related species.

With this aim in mind, we have been developing a reference map for the genus *Eucalyptus*, based exclusively on microsatellite markers. In an earlier study (Brondani et al. 1998) we described the development and characterization of an initial set of 20 microsatellite or SSR (Simple Sequence Repeat) markers and demonstrated their extraordinary polymorphism content for genetic analysis in *Eucalyptus*. The objectives of this study were: (1) to map a set of 50 novel microsatellite marker loci on the existing RAPD framework linkage maps for *Eucalyptus grandis* and *E. urophylla*; (2) to characterize the genetic information content of these 50 marker loci, which will be used as anchor-loci for QTL discovery across eucalyptus pedigrees; and (3) to establish syntenic linkage groups, investigate locus order and rate of recombination between colinear markers in the maps of the two species.

Materials and methods

Plant material

Genetic mapping of microsatellite loci was performed on a mapping population of 92 F1 individuals derived from a cross between *E. grandis* (genotype 44), used as the female parent, and *E. urophylla* (genotype 28). Both species belong to the same subgenus *Symphyomyrtus*. This same mapping population was used earlier for the development of RAPD linkage maps (Grattapaglia and Sederoff 1994). A panel of 32 genetically unrelated adult trees, 16 *E. grandis* and 16 *E. urophylla* trees, obtained from a germplasm bank, were used for the characterization of the genetic information content of the mapped loci. Genomic DNA was extracted from young expanding leaves using the miniprep method described by Doyle and Doyle (1987), with modifications described for *Eucalyptus* by Grattapaglia and Sederoff (1994).

Microsatellite marker analysis

The 50 novel microsatellite markers mapped and characterized in this study were obtained according to the procedures described earlier by Brondani et al. (1998). Amplification reactions for microsatellite markers were carried out in 96-well V-bottom microplates, in a total volume of 13 µl containing the following constituents: 0.3 µM of each primer, 1 U of *Taq* DNA polymerase, 0.2 mM of each dNTP, 10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1.3 µl of DMSO (50%), 7.5 ng of template DNA. Thermal cycling conditions for the PCR were as follows: 96°C for 2 min, then 29 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min; and a final elongation step at 72°C for 7 min, using an MJ Research PT-100 thermal cycler. Initial screening for polymorphism between the two parents was carried out in 3.5% agarose gels containing 0.1 µg/ml ethidium bromide in 1×TBE buffer (89 mM TRIS-borate, 2 mM EDTA pH 8.3). All markers were then screened and ultimately mapped in 4% denaturing polyacrylamide gels containing 7 M urea and 1×TBE buffer, and visualized by silver staining (Bassam et al. 1991), to ensure detection and facilitate interpretation of genetic segregation, given the potentially minimal size differences between the parental alleles.

Linkage mapping analysis

The microsatellite markers were identified by the same acronym (EMBRA, *Eucalyptus* microsatellite from Brazil) adopted earlier, followed by a sequentially assigned number. The 50 new markers were integrated into a framework map composed of RAPD and SSR markers at a likelihood support for locus order of 1000:1. Multipoint linkage analysis was carried out using MapMaker (Lander et al. 1987) version 2.0 for Mac. Likelihood for locus order was determined with the "Ripple" command. Two separate data sets were used, one for each parent, as the RAPD framework maps were constructed under a pseudo-testcross design. The microsatellite marker data showed segregation either from one of the parents only (1:1 ratio), from both parents with two alleles in common (1:2:1 ratio) or from both parents when at least three different alleles were present in a fully informative configuration (1:1:1:1 ratio). The microsatellite could therefore be mapped in one or both parents. When the same microsatellite marker locus was mapped on both maps, it allowed the prompt alignment of linkage groups of the two species. To allow for the detection of linkage of microsatellite to RAPD markers in any linkage phase, the data set was duplicated and recoded. A minimal LOD score of 3.0 and a maximum recombination fraction (θ) of 0.40 were used for the initial grouping of markers. The Kosambi mapping function was used to transform values for the recombination fraction to estimate map distances. For each pair of linked markers, a Z test was performed to test for significant differences between recombination fractions in *E. grandis* (female) and *E. urophylla* (male).

Analysis of SSR loci for genetic information content

The genetic information content of the 50 new markers was estimated by genotyping a panel of 32 genetically unrelated trees (16 of *E. grandis* and 16 of *E. urophylla*). The allele sizes were determined by comparison to a 10-bp ladder size standard (BRL). All 50 loci were characterized by determining the number of alleles at individual loci, the observed heterozygosity (H_{obs}) and expected heterozygosity (H_{exp}), defined as

$$H = 1 - \Sigma(\rho_1)^2, \quad (1)$$

Here p_i is the frequency of the i^{th} allele in the population sample studied. Average expected heterozygosity was also estimated, defined by

$$H_{AV} = \Sigma H_n / n, \quad (2)$$

Here n is the total number of loci. Separate analyses were carried out for the two *Eucalyptus* species and estimates were also obtained for the two species combined.

Results and discussion

Linkage analysis

All 50 markers reported herein were successfully mapped onto the framework maps, giving a total of 70 polymorphic microsatellite markers distributed throughout the *Eucalyptus* genome (Table 1). All loci displayed Mendelian inheritance and segregated accordingly in the mapping population. Of the 50 loci analyzed here, 44 (88%) were heterozygous and segregated in the female parent (*E. grandis*), 36 (72%) were heterozygous and segregated in the male parent (*E. urophylla*) and 30 (60%) segregated in both parents in a fully informative fashion with three or four different alleles (Fig. 1). At a LOD score ≥3 ($\theta = 0.40$), 47 (94%) markers could be placed onto the RAPD framework maps for the two parents. The exceptions were the loci EMBRA9, EMBRA36 and EMBRA44, with a LOD score ≥2, which were placed in the most likely interval in the linkage map (Fig. 2). The integration of these 50 microsatellite markers into the previous map (Brondani et al. 1998) generated a map with a total of 53 SSR markers placed on the *E. urophylla* map, and 63 placed on the *E. grandis* map; 46 of these markers (66%) could be mapped in both parents.

Seven loci were only mapped on the *E. urophylla* map (EMBRA63, EMBRA66, EMBRA64, EMBRA46, EMBRA7, EMBRA62, EMBRA38) and 17 only on the *E. grandis* map (EMBRA43 EMBRA41, EMBRA24, EMBRA51, EMBRA8, EMBRA31, EMBRA50, EMBRA32, EMBRA28, EMBRA20, EMBRA48, EMBRA59, EMBRA2, EMBRA39, EMBRA29, EMBRA26 and EMBRA25). Two markers (EMBRA32 and EMBRA50) co-segregated in the mapping population. However, these markers differ in their internal repetitive sequences (Table 1), and display different polymorphic information contents (see Table 2), suggesting that they represent different but very closely linked loci. No significant clustering was observed in this sample of 70 mapped microsatellite markers, which include both AG and AC repetitive motifs. This distribution indicates that extensive coverage of the *Eucalyptus* genome should be readily achieved based exclusively on this class of hypervariable markers, in the same way as with anonymous RAPD and AFLP markers (Fig. 2).

Comparative mapping

Microsatellite marker loci were placed on 11 linkage groups for *E. grandis* (corresponding to the haploid chromosome number of *Eucalyptus*, n = 11) and in 10 linkage groups for *E. urophylla*, allowing the establishment of 10 syntenic homologous linkage groups between the two species (Fig 2). In *E. urophylla*, the addition of microsatellite markers to the RAPD framework map did not change the number of linkage groups. In *E. grandis*, however, a merger of groups 7 and 14 at LOD score ≥3.5 was observed. The addition of two microsatellite markers at the tip of group 7 allowed the linkage between the two groups, resulting in the reduction of the number of linkage groups from 12 to the expected final number of 11.

Marker loci mapped on both parental maps allowed the recognition of homologous linkage groups between *E. grandis* and *E. urophylla* (Fig. 2). At a likelihood support of 1000:1, the locus order was colinear in the two species for 39 (97.5%) out of the 40 loci that could be compared between the two parental maps. The exception was marker locus EMBRA27 on group 2, where the change of position needed to obtain the same order in both maps was at least 6.0^{20} times less likely. Alterations in order conservation among the parental maps have been described in other tree species (Maliepaard et al. 1998; Devey et al. 1999) and are probably due to sampling errors rather than having any biological basis. Chromosome rearrangements can occur, but are more frequent when the evolutionary distance between the genotypes is larger, favoring the occurrence of these events (Menancio-Hautea et al. 1993). The observed differences are most probably due to the relatively limited size of the mapping population, i.e. the number of meioses sampled and used to estimate recombination frequency.

The linkage group numbering adopted followed the numbers established earlier for *E. grandis* (Grattapaglia et al. 1995), the most appropriate choice as a reference species. The total number of SSR markers per linkage group varied from 2 (group 3) to 8 (group 8) for *E. grandis*, and from 2 (groups 3 and 11) to 7 (groups 1, 2, 8 and 10) for *E. urophylla*. The average distance between two markers was 10 cM for the *E. grandis* map and 9 cM for the map of *E. urophylla*. With the inclusion of microsatellite markers on the RAPD framework map, the total map length, originally estimated at 1620 for *E. grandis* and 1156 cM for *E. urophylla* (Grattapaglia and Sederoff 1994), was increased to 2088 and 1804 cM, respectively, i.e. increases of 22% and 36%, respectively. A similar increase (41%) in total map distance was observed in barley and rice after the integration of

AFLP markers into a previously constructed map (Becker et al. 1995; Maheswaran et al. 1997). On the other hand, a decrease of 38.6% in map length was observed by Paglia et al. (1998) in a conifer genetic map, when compared to the first genetic map constructed for the same species. These variations in the estimate of genome size are most likely to be due to the size of the mapping population and the stringency level adopted for assigning locus order. In this study, a stringent threshold for ordering was adopted – almost all markers were ordered with LOD ≥3 in the two maps. The three markers that could not be ordered at a LOD support ≥3 were excluded from the framework and were assigned to the most likely interval. These markers mapped to distal regions of the linkage groups and contributed enormously to increasing the extension of the linkage map.

We observed that despite the fact that 97.5% of the markers were colinear in the two species maps, differences in the estimates of recombination frequency were observed for some locus pairs. Although for 38 out of the 49 locus pairs the recombination estimated was numerically higher in *E. grandis*, only in one instance was this difference significant at the 0.01 level. The averages for two-point recombination frequency in *E. grandis* and *E. urophylla* were, respectively, 0.19 ± 0.08 and 0.14 ± 0.07 and not significantly different. This result suggests that no significant difference should be expected in the average rate of meiotic recombination between the same pairs of microsatellite markers in the male and female parents, although the possibility of localized differences in some specific genomic regions exists.

Genetic information content of microsatellite marker loci

All seventy microsatellite marker loci developed are fully transferable and genetically informative between the two species. Indeed, over 95% of these markers are fully transferable to a number of other species, including other two major species of the genus, *E. globulus* and *E. tereticornis*, that belong to different sections of the subgenus *Symphyomyrtus* (Kirst et al. 1997). The 50 new markers reported herein are in complete agreement with the first 20 markers reported previously (Brondani et al. 1998) as to the size range, mean number of alleles found per locus and polymorphic information content for the two *Eucalyptus* species under study. For the 50 microsatellite markers, we identified a total of 717 alleles in 32 individuals. The two species shared, on average, 45% of the alleles observed and alleles restricted to one species were seen at almost all loci (Fig. 3). The number of alleles detected ranged from 5 to 16 for *E. urophylla* and from 4 to 17 for *E. grandis*. Combining the data for both species, the number of alleles per locus ranged from 7 (at the least variable loci) to 22 (at the most variable ones), with an average of 14.3 alleles per locus. These variations in allele number are similar to the range observed for other species. In forest trees, a similar range was observed in, for example, *E. nitens* (5–16 in 20 individuals, Byrne et al. 1996), *Populus tremuloides* (5–11 in 36 individuals, Dayanandan et al. 1998) and *Shorea curtissi* (2–20 in 40 individuals, Ujino et al. 1998). A similar mean number of alleles at a given locus was found for both species, 10.3 for *E. grandis* and 10.5 for *E. urophylla*. The alleles varied in size between 75 to 350 bp and all of them occurred at frequencies below 0.3 in the population sample analyzed.

For the new set of 50 loci, the average observed heterozygosities were the same for the two species, separately and combined (Table 2). The average expected heterozygosity for all loci was higher than the observed values, in the range 0.85. In *E. grandis* and in *E. urophylla*, 72% and 78% of all loci, respectively, had expected heterozygosity values above 0.8. Combined estimates for the two species revealed that 96% of the estimates of heterozygosity were above 0.8. For the four marker loci based on AC repeats (EMBRA21, EMBRA22, EMBRA56 and EMBRA 70), the average number of alleles per locus (13) and the average observed and expected heterozygosity values (0.55 ± 0.17 and 0.88 ± 0.03) did not differ significantly from the values observed for AG repeats, indicating that, despite the low frequency of AC repeats in the plant genome (Morgante and Olivieri 1993), the levels of polymorphism are similar for both types of repetitive motif in *Eucalyptus*. No positive relationship was seen in *Eucalyptus* between the number of tandem repeats in AG and AC motifs, and the level of polymorphism for this set of 50 loci evaluated in a sample of 32 individuals. The highest level of polymorphism in *Eucalyptus* was observed at the locus EMBRA42 (22 alleles), a perfect dinucleotide repeat based on 15 AG repeats, and the lowest level of polymorphism (7 alleles) was detected for EMBRA62 with 18 AG repeats. Similar results – where longer repeats did not necessarily show the highest level of polymorphism – have been reported for *Pinus* (Echt et al. 1996) and *Populus* (Dayanandan et al 1998). Repetitive motif structure also did not seem to be an indicator of polymorphism. For the 8 imperfect and 9 compound sequences characterized (classified according to Weber 1990), the genetic information content was high and not significantly different from that found in perfect repeats.

The assessment of microsatellite heterozygosities was done using a panel of 32 *Eucalyptus* trees. An average 48% increase in the number of alleles was observed for six characterized microsatellite markers when the number of *E. grandis* individuals sampled was increased from 16 to 192 (Kirst 1998). However, despite this result, the estimates of expected heterozygosity did not change significantly, as most of the new alleles detected were present at low frequencies. These estimates of genetic polymorphism, although based on relatively small sample sizes, confirm the remarkable potential of microsatellite markers for distinguishing between individuals, and for parentage studies, genetic diversity

Table 1 Details of the 50 *Eucalyptus* microsatellite loci reported in this study

SSR locus	Repeat structure	Primer pairs (5′→3′)	Annealing temperature (°C)	Product size (bp)[a]
EMBRA21	$(AC)_{16}TT(AC)_{12}$	ACAAGGGAAACTTGATCG and GGACCGAACATAGCAAG	56	92
EMBRA22	$(AG)_{17}(AC)_{14}$	GCACATGCACACACGGTTG and AAGGCCAGTGGTCGTGAGTC	56	126
EMBRA23	$(AG)_{16}$	GGTTGTTTCATCTTTTCCATG and AGCGAAGGCAATGTGTTT	56	118
EMBRA24	$(GAA)_5C(GA)_6$	CGCTATAATTCTATGCCG and TGAGAGAGATATTCGCGT	56	162
EMBRA25	$(AG)_{27}TAG...(AG)_4$	TCATCAGGTTCGCTCGTT and AGTTGAGTGAACACGCGG	56	315
EMBRA26	$(AG)_{19}$	CCCACAACAAAAGGAAAG and AGAGGTGTTCGATTCAATTC	56	120
EMBRA27	$(AG)_{24}$	ATAACCACACCAATCTGCA and TATAGCTCGAACGCTCAAC	56	135
EMBRA28	$(AG)_{25}$	CAAGACATGCATTTCGTAGT and ACTCTTGATGTGACGAGACA	56	178
EMBRA29	$(AG)_{18}AGA...(AG)_{18}$	CTTCGCTCACATCAGTCTC and CAATCGAGTCAATAACATTCA	56	204
EMBRA30	$(AG)_{22}$	TTAGTTGAATCCAACCATTG and TATATAAGGTGCAAATAATAAACG	58	138
EMBRA31	$(AG)_6TTTCCC(AG)_{19}$	AATTGCCCGAGTCAAAATAC and GGAACAATGTGGTTTGGG	58	148
EMBRA32	$(AG)_{22}$	ATCAGCCTCCACGTTCTA and GGAGAAGGAAGGTGTATCAA	56	65
EMBRA33	$(AG)_{19}$	CAATTTGCATGTCCAGTTTG and GCAGAAGTTGATTGAAAGCA	58	122
EMBRA34	$(AG)_{21}$	TCAAAACCCTCTCTCTCAT and AATAAACATTTTCTTTGAACAGA	56	104
EMBRA35	$(AG)_{24}(TCC)_9$	ATTTTTGAGAGAAAAATCTTATCC and GTGGTGTGAACGAACGAT	56	205
EMBRA36	$(AG)_{29}$	TTATCGTCAATTCTTGCTTG and AATTCAGCTCAAGATTTGGT	56	155
EMBRA37	$(AG)_{16}$	CACCTCTCCAAACTACACAA and CTCCTCTCTCTTCACCATTC	56	124
EMBRA38	$(AG)_{11}(TA)_4$	GGTTCTCTAGTGAAAATGTCG and ATACATCCATCAAAGCACAA	56	126
EMBRA39	$(AG)_9(CA)_{11}$	GCATTCGTACTCATTTTCAA and GCATCGAGAGTGGATTAGTT	54	146
EMBRA40	$(AG)_{19}(CA)_4$	AAAGTATCTCCACGCTTCAT and TCCCAATCATGATCTTCAG	56	141
EMBRA41	$(AG)_{13}$	ATGATTTTGTGCGTGGAC and TCAGGTGAAAGGATGGAG	56	198
EMBRA42	$(AG)_{15}$	GAGTAAAAATTGGTTTTGAGTG and CCCTCTTTTCATTTTGTCTT	56	127
EMBRA43	$(AG)_{14}$	TCCAGGTTCATATTCACATC and CATCTCAAGTTCCTCCCT	56	145
EMBRA44	$(AG)_{16}$	GGGGTTTGTTCTGCTTAG and CCAAAGAGTTCAGCTGTG	50	208
EMBRA45	$(AG)_{18}$	GTCATTTGCACACAGTTTTC and AGTTCATAGAATGCAGAAAATG	56	102
EMBRA46	$(AG)_{20}$	GAAGTCATCATCTGTAGATTGC and ACCCATTATTCTTTGTGAGC	56	168
EMBRA47	$(AG)_{24}$	AGAACCCTCTATAAAACCCC and GGGCTAGACATGATGGAG	56	106
EMBRA48	$(AG)_{26}$	ATTTGATTCCTTCCCCAA and ATTGTAATGGAGCCTCTCAC	54	123
EMBRA49	$(AG)_{20}$	ATTATTGGTTCATATTGAAAACC and AGATAGAGATTGAGTGAGACCC	54	132
EMBRA50	$(AG)_{10}AA(AG)_{17}(AGG)_6$	TGGATGCTGTTCTCATCCT and GGGTTTCTTTGTGAAACGA	58	123
EMBRA51	$(AG)_{20}$	GATGCATTCCTTTTTTTCC and CATTCTCTTGCATCTGGAC	58	115
EMBRA52	$(AG)_2T(AG)_{26}$	TAATCAGCATTAGCGAAAGA and CGTATATGTTCAGAGTCAATCC	58	103
EMBRA53	$(AG)_{17}GT(AG)_5$	ATTAGCTTTTCTGTAACCCG and GAATGGACAAGCTCTGATG	60	130

Table 1 (Contd.)

SSR locus	Repeat structure	Primer pairs (5′→3′)	Annealing temperature (°C)	Product size (bp)[a]
EMBRA54	$(AG)_{17}$	TGTATGAGGTACATCCGG and AAAGTTATCAGCGAGAGTTC	50	144
EMBRA55	$(AG)_{26}$	ATATTGACCCCTTCAAAGA and TGTCATCACCAATAATTGTT	50	178
EMBRA56	$(AG)_{23}(CA)_8$	TCATTGACATGCTGACTGT and ACTAACAGTTGAAAAGGTAAAGC	58	149
EMBRA57	$(AG)_{28}$	CCTTCTCTCTCTTGGAATAC and ATAGCCAGTGAAAGTGAGG	56	115
EMBRA58	$(AG)_{20}$	CACCAACTGGTACTATGAGGAT and TTGGCTTACGGTAGAACACT	56	143
EMBRA59	$(GA)_6GG(GA)_3AA(GA)_9$	GTTGTGCATGGGCCTCTTG and CGACGGCCAGTGAATTGTAA	52	106
EMBRA60	$(AG)_{23}$	AACAGCAGTTGCTACACCAC and GAGCGAAAAGGAGAACACC	60	114
EMBRA61	$(AG)_{22}$	GGCATAACGAGTTGTTCT and AGAGTATAATCAGCGCCT	58	186
EMBRA62	$(AG)_{18}$	TAGGACCTACAGGACCAT and GAACCCACAGTTATTTCC	55	139
EMBRA63	$(AG)_{21}$	CATCTGGAGATCGAGGAA and GAGAGAAGGATCATGCCA	58	201
EMBRA64	$(AG)_{21}$	GCTTCACTTTCAGAACACTC and AGCTCCCTTCACAAGGTA	56	270
EMBRA65	$(AG)_{18}$	GACATCTCCTCCTCAAGC and CGATATGCTACGTCTTCC	58	173
EMBRA66	$(AG)_{18}A(AG)_4$	ACTTCTTAGGCTACAGCA and GAAGGATCACGAGACATA	56	155
EMBRA67	$(AG)_{17}$	GGAAGAATCTAAGCGTCA and GAAGAAGATGAATGTAGGTG	58	218
EMBRA68	$(AG)_{20}$	GATGACTTCTCCTTCCGT and TGGCTTACGGTAGAACAC	58	108
EMBRA69	$(AG)_5TT(AG)_{19}$	ACCTTGTGATGGATGAAGC and CCCGACAAGGATGAGAAA	56	121
EMBRA70	$(CATA)_9CAT(AC)_{17}$	GTCACTGTGTTCAAGAACGTA and TTGTTGCTGATACCAATCC	56	169

[a]Product sizes are based on sequencing data

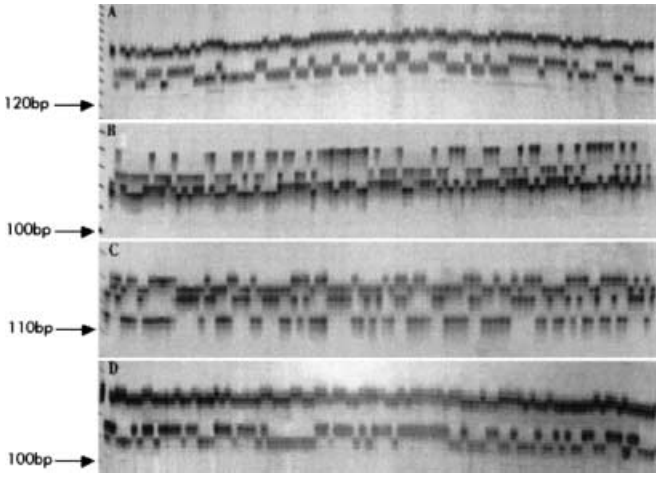
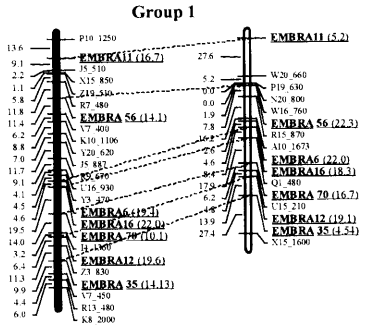


Fig. 1A–D Inheritance and segregation of fully informative SSR loci in *Eucalyptus*. Resolution and detection, by denaturing polyacrylamide gel electrophoresis and silver staining, of the SSR loci EMBRA21 (**A**), EMBRA30 (**B**), EMBRA37 (**C**) and EMBRA69 (**D**). The leftmost lane shows the 10-bp Ladder size standard (BRL), with sizes of fragments indicated in bp; lanes 2 and 3 show the data for the two parents (*E. grandis* and *E. urophylla*); the remaining lanes show the results for 94 F1 progeny individuals

assessment, certification of controlled crosses, genome mapping and marker-assisted selection (Grattapaglia 1999). Given an average expected heterozygosity value of around 0.82 for these 70 markers in the two species, and the fact that only about half of the alleles are shared, we can arrive at a reasonable estimate of the proportion of fully informative mating configurations expected in any mapping pedigree. An average of 68% (0.82×0.82) of the microsatellites described in this study are expected to segregate in a fully informative configuration in any segregating population. This estimate is very close to the proportion observed (66%) in this particular mapping population. Earlier we estimated this proportion at 80% based on the initial set of 20 markers (Brondani et al. 1998); this was most probably due to some selection for more polymorphic loci in the initial development. We expect, in fact, that 60–70% is a more realistic estimate, but this is still approximately three times higher than estimates obtained with RFLPs (Byrne et al. 1995). Furthermore, in some ongoing mapping experiments we have observed that when the parents of the pedigree are interspecific hybrids themselves, one can easily count on finding 70–80% fully informative configurations (A. Missiaggia, personal communication).


Group 1
EMBRA11 (16.7)
EMBRA 56 (14.1)
EMBRA6 (19.3)
EMBRA16 (22.0)
EMBRA 70 (10.1)
EMBRA12 (19.6)
EMBRA 35 (14.13)
EMBRA11 (5.2)
EMBRA 56 (22.3)
EMBRA6 (22.0)
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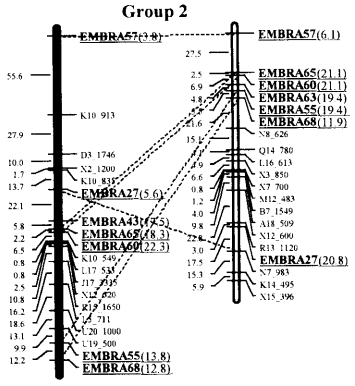
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EMBRA62(18.3)
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EMBRA55(13.8)
EMBRA68(12.8)
EMBRA57(6.1)
EMBRA65(21.1)
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EMBRA63(19.4)
EMBRA55(19.4)
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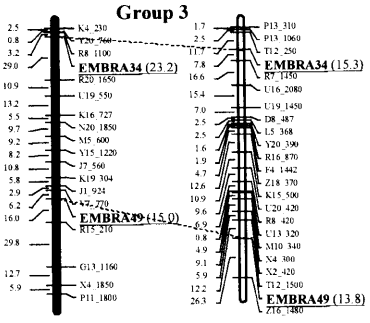
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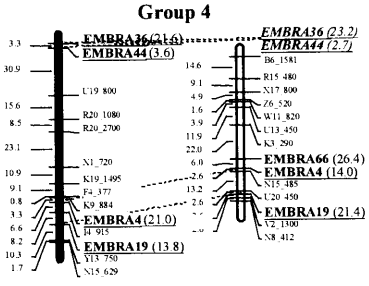
EMBRA49 (13.8)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EMBRA19 (13.8)
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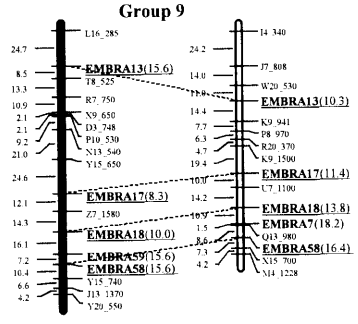
EMBRA19 (21.4)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EMBRA13(15.6)
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EMBRA59(15.6)
EMBRA58(15.6)
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EMBRA17(11.4)
EMBRA18(13.8)
EMBRA7(18.2)
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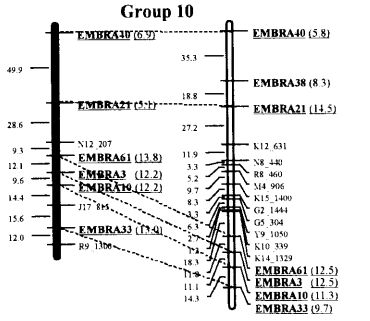
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EMBRA19 (12.2)
EMBRA33 (13.4)
EMBRA40 (5.8)
EMBRA38 (8.3)
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EMBRA10 (11.3)
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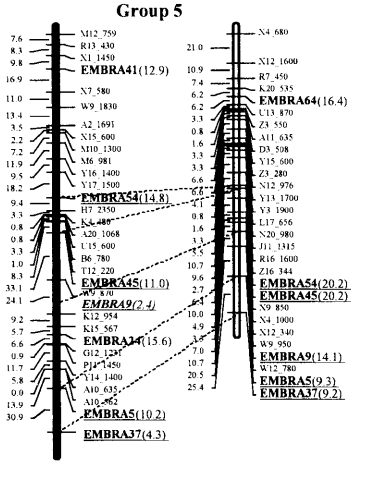
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EMBRA5(10.2)
EMBRA37(4.3)
EMBRA64(16.4)
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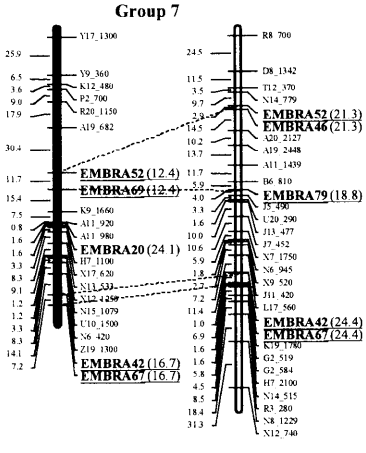
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EMBRA42 (16.7)
EMBRA67 (16.7)
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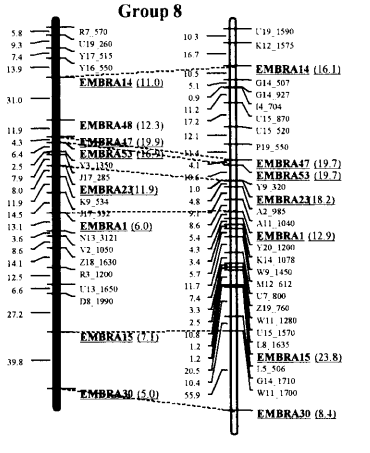
EMBRA67 (24.4)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EMBRA53 (16.0)
EMBRA23(11.9)
EMBRA1 (6.0)
EMBRA15 (7.1)
EMBRA30 (5.0)
EMBRA14 (16.1)
EMBRA47 (19.7)
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EMBRA1 (12.9)
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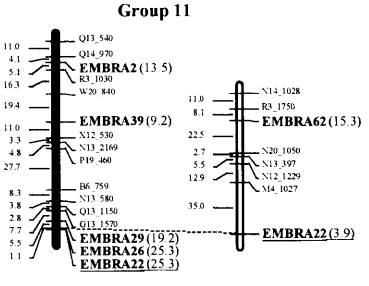
EMBRA30 (8.4)


Group 11
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EMBRA26 (25.3)
EMBRA22 (25.3)
EMBRA62 (15.3)
EMBRA22 (3.9)

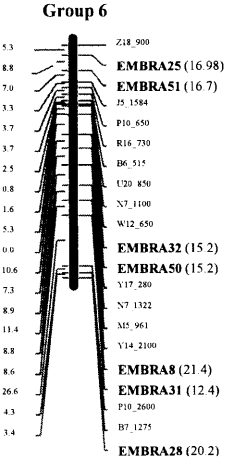
Group 6
EMBRA25 (16.98)
EMBRA51 (16.7)
EMBRA32 (15.2)
EMBRA50 (15.2)
EMBRA8 (21.4)
EMBRA31 (12.4)
EMBRA28 (20.2)

Fig. 2 Map positions of SSRs on the RAPD linkage map of *Eucalyptus*. Sixty-six microsatellite loci mapped with a LOD ≥3 were integrated into the framework map; the remaining three (indicated in *italics*), with LOD scores ≥2, were placed in the most probable interval. The alignment of 10 linkage groups was based on 46 common SSR markers (*underlined*). The *filled bars* show the *E grandis* linkage groups, the *open bars* the *E urophylla* linkage groups. LOD scores are given in *parentheses* beside the locus designations locus

A reference linkage map for the genus *Eucalyptus*

The morphological similarity and genetic compatibility in sexual crosses among *Eucalyptus* species, particularly in the same subgenus, suggest a very high rate of conservation of DNA sequences flanking microsatellite regions among species. This has been observed earlier on a smaller scale for four SSR loci by Byrne et al. (1996) between *E. nitens* and three other species, including *E. grandis*, and in a more extensive sampling both of markers and species by Kirst et al. (1997). In this latter report, the rate of transferability was above 95% within a section and still over 80% between phylogenetically more distant sections within the subgenus *Symphyomyrtus*, dropping to 25 to 59% among species from different subgenera. These results indicate that the prospects for transferring microsatellite mapping

Table 2 Linkage group assignment on the reference map, allele size ranges, allele numbers and estimates of genetic information content with observed and expected heterozygosities for 50 SSR loci in *E. urophylla* and *E. grandis*

SSR locus	Linkage group	Allele size range (bp)	No. of alleles *E. urophylla*	No. of alleles *E. grandis*	No. of alleles shared	Total No. of alleles	$H_{obs.}$ *E. urophylla*	$H_{exp.}$ *E. urophylla*	$H_{obs.}$ *E. grandis*	$H_{exp.}$ *E. grandis*	$H_{obs.}$ Combined	$H_{exp.}$ Combined
EMBRA21	10	128–170	11	11	7	15	0.68	0.84	0.73	0.86	0.70	0.89
EMBRA22	11	180–245	6	10	4	12	0.25	0.86	0.44	0.64	0.34	0.83
EMBRA23	10	118–145	8	12	5	15	0.81	0.88	0.93	0.81	0.87	0.89
EMBRA24	5	125–170	14	11	7	18	0.56	0.85	0.5	0.9	0.53	0.9
EMBRA25	6	260–350	8	9	7	9	0.81	0.84	0.66	0.82	0.74	0.84
EMBRA26	11	112–200	9	8	3	14	0.07	0.83	0.21	0.86	0.14	0.9
EMBRA27	2	100–170	13	17	9	21	0.75	0.93	0.65	0.89	0.68	0.93
EMBRA28	6	180–300	9	12	7	14	0.93	0.9	0.68	0.86	0.81	0.9
EMBRA29	11	220–310	9	9	6	12	0.81	0.83	0.75	0.85	0.78	0.89
EMBRA30	8	100–160	13	15	10	18	0.81	0.91	0.75	0.90	0.78	0.92
EMBRA31	6	135–180	12	7	7	12	0.68	0.8	0.5	0.78	0.59	0.86
EMBRA32	6	85–160	8	10	5	13	0.56	0.78	0.62	0.82	0.59	0.83
EMBRA33	10	120–180	7	10	5	12	0.43	0.75	0.87	0.76	0.65	0.82
EMBRA34	3	100–160	10	11	5	16	0.38	0.82	0.44	0.86	0.40	0.89
EMBRA35	1	110–160	9	7	5	11	0.43	0.71	0.56	0.83	0.5	0.85
EMBRA36	4	130–180	13	9	4	17	0.81	0.86	0.81	0.88	0.81	0.91
EMBRA37	5	115–165	11	14	7	18	0.75	0.91	0.75	0.83	0.75	0.88
EMBRA38	10	100–155	11	9	6	14	0.62	0.78	0.75	0.83	0.69	0.86
EMBRA39	11	120–150	5	7	4	8	0.18	0.23	0.62	0.72	0.40	0.69
EMBRA40	10	120–180	10	8	6	12	0.75	0.81	0.75	0.85	0.75	0.86
EMBRA41	5	190–220	7	8	7	8	0.87	0.82	0.66	0.69	0.75	0.8
EMBRA42	7	115–170	13	15	6	22	0.93	0.9	0.87	0.9	0.9	0.93
EMBRA43	2	95–150	10	8	6	12	0.66	0.81	0.26	0.82	0.43	0.86
EMBRA44	4	205–225	6	7	4	9	0.62	0.8	0.6	0.78	0.61	0.86
EMBRA45	5	115–160	13	9	6	16	0.78	0.84	0.62	0.9	0.7	0.91
EMBRA46	7	90–130	13	8	7	14	0.87	0.89	0.60	0.70	0.74	0.88
EMBRA47	8	110–165	13	10	6	17	0.56	0.77	0.62	0.88	0.59	0.88
EMBRA48	8	110–180	16	13	9	20	0.81	0.89	0.87	0.91	0.84	0.92
EMBRA49	3	125–195	13	10	7	16	0.81	0.84	0.81	0.93	0.81	0.91
EMBRA50	6	110–170	12	6	4	14	0.56	0.86	0.18	0.72	0.37	0.86
EMBRA51	6	95–200	12	14	7	19	0.66	0.89	0.86	0.90	0.76	0.93
EMBRA52	7	110–150	11	14	9	16	0.75	0.85	0.66	0.89	0.70	0.89
EMBRA53	8	125–195	14	9	8	16	0.66	0.77	0.8	0.88	0.73	0.86
EMBRA54	5	165–210	9	4	3	10	0.44	0.54	0.47	0.7	0.45	0.62
EMBRA55	2	190–290	5	13	5	13	0	0.73	0.62	0.86	0.43	0.87
EMBRA56	1	130–190	8	11	7	14	0.4	0.87	0.58	0.88	0.48	0.91
EMBRA57	2	95–150	11	14	8	17	0.46	0.87	0.46	0.9	0.46	0.91
EMBRA58	9	140–245	11	9	6	14	0.85	0.85	0.25	0.75	0.53	0.82
EMBRA59	9	90–130	10	6	6	10	0.62	0.77	0.68	0.79	0.65	0.84
EMBRA60	2	75–120	13	12	10	15	0.81	0.90	0.66	0.89	0.74	0.91
EMBRA61	10	170–210	10	10	8	12	0.56	0.85	0.6	0.79	0.58	0.87
EMBRA62	11	130–160	6	6	5	7	0.10	0.78	0	0.68	0.04	0.81
EMBRA63	2	175–230	11	10	7	14	0.81	0.81	0.75	0.83	0.78	0.86
EMBRA64	5	250–320	11	7	7	11	0.93	0.87	0.87	0.79	0.90	0.86
EMBRA65	2	140–260	11	12	5	18	0.46	0.86	0.35	0.89	0.41	0.89
EMBRA66	4	140–260	15	16	11	20	0.75	0.87	0.56	0.89	0.65	0.93
EMBRA67	7	115–190	11	15	9	17	0.28	0.82	0.75	0.91	0.53	0.91
EMBRA68	2	100–180	10	11	6	15	0.81	0.84	0.81	0.88	0.81	0.90
EMBRA69	7	100–140	13	12	9	16	0.43	0.88	0.75	0.89	0.59	0.91
EMBRA70	1	135–190	11	11	6	14	0.68	0.84	0.75	0.86	0.71	0.89
Mean ± SE			10.5 ± 2.59	10.3 ± 2.93	6.46 ± 1.82	14.34 ± 3.4	0.62 ± 0.23	0.82 ± 0.10	0.62 ± 0.20	0.83 ± 0.07	0.62 ± 0.18	0.87 ± 0.05

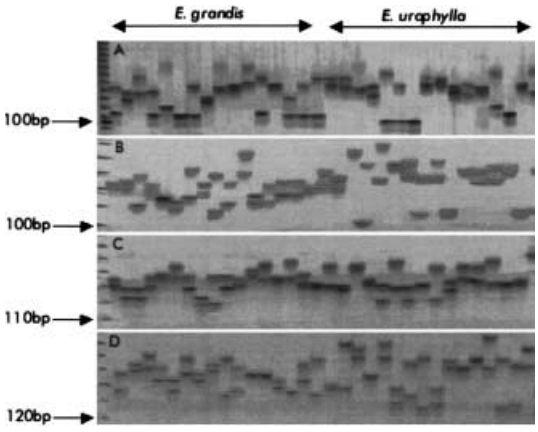

Fig. 3A–D Allelic polymorphism at SSR loci. Allelic variation at the loci EMBRA30 (**A**), EMBRA45 (**B**), EMBRA23 (**C**) and EMBRA36 (**D**) revealed on a silver-stained polyacrylamide gel loaded with samples from 16 individuals of *E. grandis* and 16 individuals of *E. urophylla*. The leftmost lane contains the 10-bp Ladder size standard (BRL), with the sizes of fragments indicated in bp

information across species in *Eucalyptus* are excellent, particularly so within a subgenus but also at a lower rate among distant subgenera. As the commercially relevant intra-and interspecific breeding efforts are carried out mainly between species of the same subgenus (*Symphyomyrtus*) we can confidently state that the mapping information reported in this work will be relevant and useful to the vast majority of molecular breeders in the world.

In conclusion, this report demonstrates the colinear arrangement of microsatellite markers along the linkage maps of two *Eucalyptus* species, and sets the stage for the final construction of a genus-wide reference map that will be useful for the great majority of commercially important species of eucalypts. Such a map will allow widespread use of these markers in several areas of *Eucalyptus* genetics, facilitating information exchange and comparison among laboratories, particularly in candidate gene mapping, QTL mapping and marker-assisted selection procedures. With the complete coverage of the *Eucalyptus* genome with microsatellites of high genetic information content, every family segregating for the trait of interest should provide marker-QTL linkage information. This will allow the comparison and integration of QTL mapping data from totally independent experiments, thus advancing QTL validation across genetic backgrounds, which is one of the crucial aspects needed for the implementation of molecular breeding in the genus. The validation of QTL positions across pedigrees will then allow a focused search for new allelic variation at known QTLs within and among species, thus expanding the opportunities for marker-assisted introgression and selection in *Eucalyptus* hybrid breeding. Finally, a dense set of mapped microsatellite markers will be of fundamental importance for anchoring EST-rich physically mapped contigs, in order to accelerate functional genomics and ultimately elucidate the mechanisms of gene expression and regulation underlying quantitative trait variation.

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