

Power of Microsatellite Markers for Fingerprinting and Parentage Analysis in *Eucalyptus grandis* Breeding Populations

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We report the genetic analysis of 192 unrelated individuals of an elite breeding population of *Eucalyptus grandis* (Hill ex Maiden) with a selected set of six highly polymorphic microsatellite markers developed for species of the genus *Eucalyptus*. A full characterization of this set of six loci was carried out generating allele frequency distributions that were used to estimate parameters of genetic information content of these loci, including expected heterozygosity, polymorphism information content (PIC), power of exclusion, and probability of identity. The number of detected alleles per locus ranged from 6 to 33, with an average of 19.8 ± 9.2 . The average expected heterozygosity was 0.86 ± 0.11 and the average PIC was 0.83 ± 0.16 . Using only three loci, it was possible to discriminate all 192 individuals. The overall probability of identity considering all six EMBRAPA microsatellite markers combined was lower than 1 in 2 billion. An analysis of the sample size necessary to estimate expected heterozygosity with minimum variance indicated that at least 64 individuals have to be genotyped to characterize this parameter with adequate accuracy for most microsatellites in *Eucalyptus*. The high degree of multiallelism and the clear and simple codominant Mendelian inheritance of the set of microsatellites used provide an extremely powerful system for the unique identification of *Eucalyptus* individuals for fingerprinting purposes and parentage testing.

Deoxyribonucleic acid (DNA) polymorphisms provide a powerful tool for quantifying the existing levels of genetic variation in breeding and production populations of forest trees. Molecular markers can be used to estimate the extent of genetic divergence between individuals selected to compose such populations, resolve several issues of individual identity, including varietal protection, and investigate alleged parent-

age in open-pollinated breeding systems. Several technologies are available today to resolve such questions. In *Eucalyptus*, dominant markers such as random amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP) have been used for clonal fingerprinting of eucalypts (Keil and Griffin 1994), analyses of genetic variation in germplasm banks (Nesbitt et al. 1995) and seed orchards (Marcucci et al. 2003), and estimation of outcrossing rates in breeding populations (Gaiotto et al. 1997). Dominant markers are very limited in their ability to precisely determine parentage and frequently present problems when conclusively establishing absolute identity between two individual trees due to artifact polymorphisms. Dominant markers can readily be used to establish that two individuals are not the same, but the statement that two individuals are identical is usually only approximate and no formal statistics can be attached to this assertion.

A simple polymerase chain reaction (PCR) assay of polymorphisms at simple sequence repeats (SSRs), also known as microsatellites, is certainly the most efficient way of resolving issues of identity. SSRs are typically codominant and multiallelic, with expected heterozygosity frequently greater than 0.7, allowing precise discrimination even of closely related individuals. Due to the specificity of the PCR assay and its high information content, it also allows the determination of identity between individuals based on formal estimates derived from allele frequencies. As microsatellite marker information can be easily shared between laboratories based on primer sequences, interlaboratory comparisons of data are straightforward, improving cooperative efforts in the standardization of fingerprinting data.

Microsatellite markers have been developed for some of the main plantation forest tree species, such as *Pinus sylvestris* (Kostia et al. 1995), *Pinus radiata* (Smith and Devy 1994), *Quercus* (Dow et al. 1995), and poplar (Rahman and Rajora 2002; van der Schoot et al. 2000). Large microsatellite

collections are available for *Pinus taeda* (245 SSRs) (Williams and Auckland 2002) and more than 500 SSRs are being developed based on the first draft of the *Populus trichocarpa* genome sequence (Tuskan J, personal communication). We have recently reported the development, genetic characterization, and linkage mapping of 70 SSR loci in *Eucalyptus grandis* and *Eucalyptus urophylla* and showed their excellent potential for mapping and individual identification (Brondani et al. 1998, 2002). Rapid and reliable typing of perennial crops has been achieved with microsatellites, including grapevines (Thomas et al. 1994), apples (Guilford et al. 1997), *Citrus* (Kijas et al. 1995), pine (Devey et al. 2002), and poplar (Rahman and Rajora 2002; Rajora and Rahman 2003). Recently interest in this technology has grown even more with the possibility of using multilocus SSR profiles as part of the evidence for the development and registration of new cultivars in annual and perennial crop variety protection (Diwan and Cregan 1997; Dore et al. 2001).

In this study we extend the characterization of microsatellites in *Eucalyptus* and demonstrate their application for DNA fingerprinting by analyzing the multilocus profiles of 192 individuals of a *E. grandis* breeding population. Our objectives were (1) to estimate allele frequencies for these six loci in *E. grandis*; (2) to characterize these SSR loci for several parameters of genetic information content, including number of alleles, expected heterozygosity, polymorphism information content (PIC), power of exclusion, and probability of identity; (3) evaluate the minimum number of individuals necessary to estimate expected heterozygosity for microsatellite loci in *E. grandis*; and (4) verify the power of discrimination of this set of loci for individual fingerprinting in this breeding population.

Materials and Methods

Plant Material and DNA Extraction

Plant material used for DNA extraction was obtained from 192 individual trees selected in provenance/progeny trials established at Aracruz Celulose S.A. (Espírito Santo, Brazil). These trials were established with seeds collected from selected individuals growing in natural populations in Australia and one individual selected from a commercial plantation in Zimbabwe. These 192 individuals constitute one of the long-term breeding populations of *E. grandis* and are used in several breeding procedures that require correct individual identification, including controlled crosses and seed orchard establishment. Total genomic DNA was extracted from adult leaf tissue following a modified protocol described earlier (Brondani et al. 1998).

Microsatellite Genotyping

Six microsatellite loci developed earlier (Brondani et al. 1998) were selected for this study: EMBRA4, EMBRA5, EMBRA10, EMBRA11, EMBRA15, and EMBRA16. These loci were selected based on the following criteria: (1) ease of interpretation of the amplified alleles in regular silver-stained polyacrylamide gels to allow widespread use by several labs

that do not necessarily have access to automated sequencers; (2) minimum stutter of PCR products; (3) high polymorphism based on a preliminary characterization; (4) absence of null alleles as observed in parallel paternity testing studies (Ribeiro V, unpublished data); and (5) map position so as to satisfy the premise of independent segregation to allow the use of the product rule in individual identification. Markers EMBRA11 and EMBRA16 belong to the same linkage group 1, however, they are effectively independent, as their map distance is greater than 50 cM (Brondani et al. 1998). Microsatellite marker amplification and detection were performed as described earlier (Brondani et al. 1998). The amplified products were separated on 4% denaturing polyacrylamide gels stained with silver nitrate (Bassam et al. 1991) and sized by comparison to a 10 bp DNA ladder standard (GIBCO, Rockville, MD) on a computer screen. The three most frequent alleles for loci EMBRA4, EMBRA5, EMBRA10, EMBRA15, and EMBRA16 had their size confirmed by sequencing in an automated DNA sequencer (PerkinElmer/ABI Prism 377) and the information was used to assess the accuracy of allele scoring. Allele sizes were estimated using the software Seqaid II (Rhoads and Roufa 1990), taking into consideration the expected allelic series in base pairs for the locus. Multiplex loading in the same gel was carried out for up to three microsatellite loci simultaneously.

Data Analysis

The gels were scanned and scoring was carried out manually on a computer screen. Based on the estimates of allele frequencies from genotypes at the EMBRA microsatellites in this sample of 192 individuals, the following parameters of genetic information content were estimated: (1) expected heterozygosity; (2) PIC (Botstein et al. 1980); (3) probability of genetic identity (I) (Paetkau et al. 1995), which corresponds to the probability of two random individuals displaying the same genotype; and (4) paternity exclusion probability (Q) (Weir 1996), which corresponds to the power with which a locus excludes an erroneously assigned individual tree from being the parent of an offspring. The combined probability of paternity exclusion, $QC = 1 - [\prod(1 - Q_i)]$, and the combined probability of genetic identity, $IC = \prod I_i$, were also estimated for the combined battery of loci.

Minimum Sample Size for Estimation of Expected Heterozygosity

In order to evaluate the minimum number of individuals required to adequately estimate expected heterozygosity for the microsatellite markers in *E. grandis*, the variance of the expected heterozygosity for each locus, $\text{var}(\hat{H}_{e_l})'$, was calculated considering samples of size 16, 32, 64, and 128 using the gene diversity variance formula (Weir 1996):

$$\text{Var}(\hat{H}_{e_l})' = \frac{2(1+f)}{n} \left[\sum_{i=1}^n p_{li}^3 - \left(\sum_{i=1}^n p_{li}^2 \right)^2 \right], \quad (1)$$

where f corresponds to Wright's fixation index estimated for each locus, n is the sample size, and p_{li} is the frequency of the i th allele at the l th locus in the population.

Based on the variance, estimates of the expected gene diversity, $\varepsilon(\hat{H}_{e_i})'$, the standard error, $\hat{S}_{e_i}(\hat{H}_{e_i})'$, and bias, $\hat{B}_{ias_i}(\hat{H}_{e_i})'$, were obtained:

$$\varepsilon(\hat{H}_{e_i})' = \left(1 - \frac{1+f}{2n}\right) H_{e_i} \quad (2)$$

$$\hat{S}_{e_i}(\hat{H}_{e_i})' = \sqrt{\text{Var}(\hat{H}_{e_i})} \quad (3)$$

$$\hat{B}_{ias_i}(\hat{H}_{e_i})' = \varepsilon(\hat{H}_{e_i}) - (H_{e_i}). \quad (4)$$

The coefficient of variation of the mean square error (CV_{mse}), a measure of accuracy, was calculated to graphically evaluate the optimal minimum sample size. The CV_{mse} considers the whole variation along the parametric value, taking into account the values of the standard error [equation (3)] and bias [equation (4)]:

$$CV_{mse}(I) = \frac{\sqrt{[\hat{B}_{ias_i}(\hat{H}_{e_i})]^2 + \hat{S}_{e_i}(\hat{H}_{e_i})^2}}{H_{e_i}}. \quad (5)$$

Genetic Distances Among Individuals

Multilocus genotypes for the 192 individuals were used to estimate genetic distance. Considering the heterozygous nature of the genotype data the “shared allele distance” estimator between individuals was used (Bowcock et al. 1994; Jin and Chakraborty 1993):

$$D_{SA_i} = 1 - P_{SA_i} \quad P_{SA_i} = \frac{\sum_j^r S}{2r}, \quad (6)$$

where the number of shared alleles S is summed over all loci r .

Results and Discussion

Microsatellite Markers

The six EMBRA loci amplified a total of 119 alleles, yielding a minimum of six (EMBRA11) and a maximum of 33 alleles (EMBRA16), with an average of 19.8 ± 9.2 alleles per locus (Table 1). Other similar studies involving the analysis and characterization of microsatellites in *Eucalyptus* species have also detected loci with highly variable numbers of alleles and, consequently, information content (Brondani et al. 1998; Byrne et al. 1996; Marcucci et al. 2003). Some of this variability is explained by the fact that SSRs may be preferentially located in low-copy transcribed regions of plant genomes (Morgante et al. 2002). Microsatellite EMBRA5 showed the highest expected heterozygosity ($H_e = 0.936$) and PIC ($= 0.933$) values (Table 1), reflecting the large number of alleles observed and similar allele frequency distribution in the population when compared to the other microsatellites (Figure 1). The lowest H_e and PIC were found in microsatellite EMBRA11 (0.645 and 0.524, respectively). Very similar results were obtained in a study that involved the analysis of four microsatellite loci in a population of 20 unrelated individuals of *Eucalyptus nitens*, where the expected heterozygosities ranged from 0.72 to 0.91 (Byrne et al. 1996),

Table 1. Parameters of genetic information content of six EMBRA microsatellite loci estimated from the breeding population of *E. grandis*

Microsatellite marker	Number of alleles	H_e	PIC	I	Q
EMBRA4	19	0.850	0.898	0.075	0.941
EMBRA5	26	0.936	0.933	0.008	0.875
EMBRA10	15	0.851	0.835	0.038	0.719
EMBRA11	6	0.645	0.524	0.168	0.434
EMBRA15	20	0.903	0.895	0.017	0.811
EMBRA16	33	0.930	0.910	0.038	0.864
Overall	19.8 ^a	0.862 ^a	0.833 ^a	2×10^{-9b}	0.99997 ^b

H_e , expected heterozygosity; PIC, polymorphism information content; I , probability of identity; Q , power of exclusion.

^a Average of the estimated value across all loci.

^b Combined probability of identity and exclusion, considering all markers.

showing that *Eucalyptus* microsatellites are usually very informative.

Probability of Identity and Power of Exclusion

The probability of identity (I) expresses the likelihood of finding two individuals with the same genotype for a certain loci in the population. This probability was very low in most cases, ranging from 0.17 (EMBRA11) to less than 0.01 (EMBRA5). Considering that every locus segregates independently, assuming therefore that they are in linkage equilibrium, the chance of finding identical genotypes in the population when the six EMBRA microsatellites are included in the analysis corresponds to the product of probabilities of the identity of every locus. The combined estimate was 2×10^{-9} , meaning that the chance of finding two individuals with the same genotype in the population is almost null. This power of discrimination was confirmed when we analyzed the whole group of individuals with the microsatellite that showed the highest H_e and PIC values (EMBRA5) and detected that 43% of them had a unique genotype. When a second microsatellite was included in the analysis (EMBRA16), this proportion increased to 95%. Finally, with a third microsatellite (EMBRA15), all individuals were discriminated. Similar results were obtained with a breeding population of *Eucalyptus dunnii*, when four microsatellite markers could discriminate nearly all 46 individual trees selected to compose a seed orchard (Marcucci et al. 2003). The power of exclusion (Q) indicates the probability of excluding a nonparent from paternity or maternity. As expected, the power of exclusion was high for all the microsatellites analyzed. It ranged from 0.434 (EMBRA11) to 0.941 (EMBRA4), with an average of 0.774. The combined power of exclusion, which is the exclusion probability considering all six EMBRA loci, was greater than 99.99%, indicating that these loci are appropriate to determine parentage in eucalypts beyond any reasonable doubt.

Accuracy of Estimates of Heterozygosity

EMBRA microsatellites with large numbers of alleles in low frequencies showed a small reduction in the coefficient of

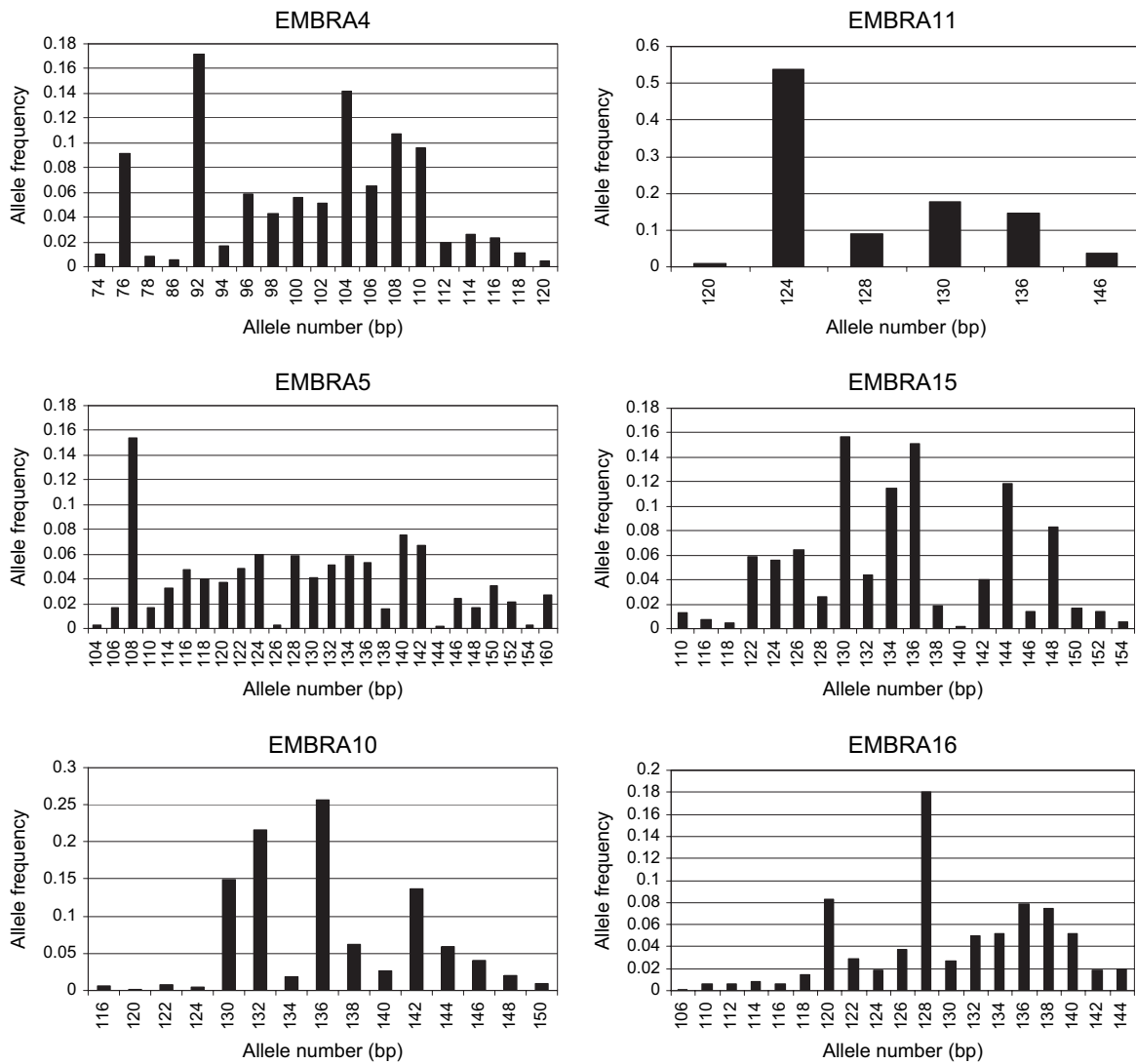


Figure 1. Allele frequency distributions of six EMBRA microsatellite loci in the breeding population of *E. grandis*.

variation of the mean-squared error when samples of 64 and 128 individuals were considered, indicating that this size should be adequate for accurate estimations of H_e . Most of the microsatellites analyzed in this study were included in this situation, with the exception of EMBRA11. To obtain the same accuracy that was obtained with the other loci, more than 128 individuals would have to be analyzed with this microsatellite (Figure 2). These results refer only to the microsatellites that were analyzed in this study and for the population of individuals that were typed. However, considering that microsatellites have a similar pattern of allelic distribution in different species (Valdes et al. 1993), we can infer that several studies involving their characterization may be incurring significant errors, indicating incorrect H_e values when these were obtained with small samples. A sample size of 64 was considered satisfactory when we analyzed the bias of the H_e of all microsatellites (Figure 2). It is important to notice that the bias of H_e always had negative values. This behavior was related to the increasing number of

rare alleles that were observed when the sample size was extended, contributing to the detection of higher H_e values, and was particularly interesting because it showed that H_e was always underestimated in relation to the population parameter.

Genetic Distances Among Individuals

Based on the information obtained at the six microsatellite loci, shared allele distance coefficients were estimated for all the pairwise comparisons of the 192 trees (data not shown). The average distance between individuals was high (0.857) and ranged from 0.333 to 1. Of the 18,336 pairwise distances, there were 3 between 0 and 0.4, 24 between 0.4 and 0.5, and 458 between 0.5 and 0.6, that is, 97% of the pairwise distances between individuals were estimated to be greater than 0.6.

In conclusion, this study demonstrates the power of discrimination of microsatellite markers in *E. grandis* and

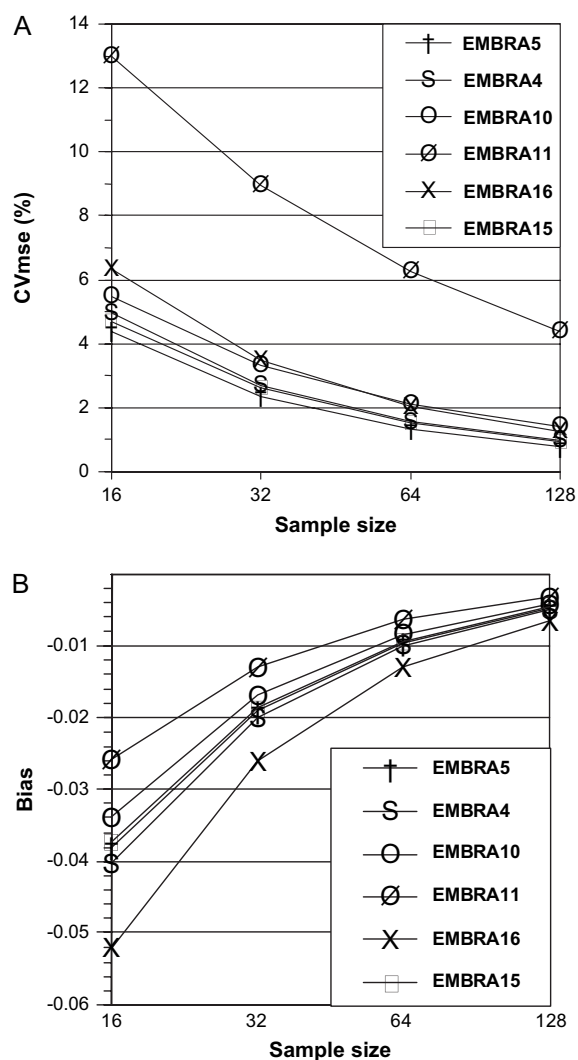


Figure 2. (A) Reduction of the coefficient of variation of the mean-squared error of the expected heterozygosity with increasing sample size for six EMBRA microsatellites, based on Weir's gene diversity variance formula (Weir 1996). (B) Reduction of the bias of the expected heterozygosity, with increasing sample size, for six EMBRA microsatellites. Bias was estimated based on Weir's gene diversity formula (Weir 1996).

their high potential for future use in issues concerning plant variety protection, as well as their application in the assessment of the existing genetic variation, genetic distance, and determination of parentage among individuals of a breeding population. Genetic analysis with microsatellites should be of interest to eucalypt tree breeders as complementary descriptors for varietal protection, particularly of vegetatively propagated elite clones. Interest in the use of microsatellites for this purpose has become even greater after acceptance by the USDA Plant Variety Protection Office of SSR allelic profiles as evidence of the uniqueness of new cultivars (Diwan and Cregan 1997). Microsatellites have been shown to be almost twice as informative as dominant markers (RAPD and AFLP) and

much more informative than RFLPs in soybean (Powell et al. 1996), and approximately six times more informative than RAPD and nine times more informative than allozyme in poplar (Rajora and Rahman 2003), being the ideal marker for discriminating individuals and for parentage determinations. Information on the genetic distance among individuals in a breeding population is an important tool for forest tree breeders performing guided crosses, which are more likely to produce more widely segregating progenies and consequently increase the probability of obtaining higher-value transgressive individuals to be propagated as elite clones.

The microsatellites used in this study could be confidently scored using a simple and accessible silver-stained detection technology, allowing widespread use by several laboratories. We are now optimizing these same loci and others in larger multiplex systems based on fluorescent detection. Although less accessible in general, fluorescent detection improves scoring of dinucleotide repeats, as demonstrated for *Eucalyptus sieberi* (Glaubitz et al. 2001). We expect that these systems will add a significant power of resolution for distinctness, uniqueness, and stability (DUS) tests in the varietal protection of eucalypt clones, especially when closely related individuals are under scrutiny. A database of multilocus genetic profiles of elite clones could be immediately used to implement genetic identity tests by electronic comparison of multilocus profiles between questioned and reference samples in quality control procedures in the context of breeding programs and varietal protection.

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