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Fingerprint and genetic diversity analysis of *Eucalyptus* spp. genotypes using RAPD and SSR markers

"Fingerprint" e análise da diversidade genética em genótipos de *Eucalyptus* utilizando marcadores RAPD e microssatélites

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RESUMO: Marcadores moleculares apresentam grande potencial para o controle de qualidade em programas de melhoramento. Os objetivos deste trabalho foram: caracterizar um conjunto de marcas moleculares exclusivas para cada um dos genótipos analisados, determinar o valor de heterozigose entre os indivíduos para recomendação de futuros cruzamentos e avaliar a manutenção da variabilidade genética ao longo do programa de melhoramento. Quinze genótipos superiores de grande potencial para a propagação clonal foram analisados com dois tipos de marcadores: RAPD e microssatélite. A análise dos resultados consistiu da descrição do padrão de bandas, cálculo de índices de similaridade e de diversidade genética. Foram analisados 210 fragmentos de DNA gerados pela amplificação com 29 oligonucleotídeos RAPD e foram identificadas 80 formas alélicas na amplificação, utilizando 20 oligonucleotídeos específicos para regiões microssatélites. Os dendrogramas calculados para cada tipo de marcador são ligeiramente diferentes. O índice de coincidência foi calculado para os valores superiores e inferiores dos dendrogramas, respectivamente 69% e 72%. A utilização conjunta destas duas técnicas de marcadores permite aliar a alta cobertura do genoma proporcionada pela técnica de RAPD com a alta repetibilidade das bandas microssatélites, possibilitando a obtenção de um conjunto de marcas único e altamente informativo para os genótipos analisados.

PALAVRAS-CHAVE: Programas de melhoramento, Marcadores moleculares, RAPD, SSR, *Eucalyptus*, Proteção de cultivares

ABSTRACT: Molecular markers have great potential for use in quality control in breeding programs. It were used RAPD and SSR markers for obtaining an exclusive fingerprint for the 15 genotypes studied, for recommending crosses based on hybrid vigor and for genetic diversity analysis during breeding programs. Fifteen *Eucalyptus* hybrids with high potential for clonal propagation were analyzed. Band patterns are described and indices of similarity and genetic diversity were calculated for each marker type. For the SSR markers the percentual of heterozigotes were also estimated. Two hundred ten DNA fragments were generated by the RAPD amplifications using 29 random primers; and 80 allelic forms were identified through amplification using 20 primers specific for microssatelite regions. Dendrograms calculated for these two types of markers were slightly different. Coincidence indices for the superior and

inferior values were 69% and 72% respectively. Combined use of these two marker techniques takes advantage of the thorough genome coverage provided by RAPD markers and the high reproducibility of SSR bands, and has enable us to obtain exclusive and highly informative data set for the genotypes analyzed.

KEYWORDS: Breeding programs, Molecular markers, RAPD, SSR, *Eucalyptus*, Cultivar protection

INTRODUCTION

Eucalyptus (Myrtaceae) species are commonly employed in Brazilian reforestation programs and constitute the majority of the world's planted hardwood forests (Eldridge et al., 1994). To attend the growing demand for wood, Brazilian planted forests increased by more than 3 millions ha between 1985 and 1994 (Abracave, 1996) and genetic breeding programs are being carried out to obtain raw material of better quality. Considerable gains have been achieved by reforesting large areas with selected clonal plants (Muro Abad, 2000).

In breeding programs there is a great concern in the choice of the genotypes that will be used as genitors. Beyond the individual performance, the genetic divergence among the genitors must be considered, for the success of the breeding programs do not be early committed (Cruz, 1990). Studies on genetic divergence have been of great importance in breeding programs involving hybridizations, because they supply an indicative that the genitors, when breed, can improve the hybrid vigor in the population. (Cruz, 1990; Muro Abad, 2000; Baril et al., 1997).

Among the possible uses of molecular markers for quality control in genetic breeding programs, we include the recommendation of crosses based on the genetic divergence of genitors (Tsaftaris, 1995; Lanza et al., 1997; Dias et al., 2001), confirmation of individual identities in the field, legitimacy of crosses and cultivars developed (Keil e Griffin, 1994; Vaillancourt et al., 1998; Laia et al., 2000).

According to Brazilian legislation, the protection of genetic material depends on the utilization of easily identifiable phenotypic traits that are stable over several generations. Considering the *Eucalyptus* long life cycle, it would take many years to prove misappropriation of a cultivar. In these cases the use of molecular markers would solve most of the difficulties (Becher et al., 2000) as they can be used for the early identification of a cultivar and when dealing with transformed plants, they allow an accurate identification of alterations in the genetic material as a result of genomic manipulation.

The RAPD technique uses arbitrary sequence primers for random amplification of genomic loci (Willians et al., 1990), resulting in unique band patterns for each genotype. It is a low cost methodology that can be easily implemented and allows simultaneous analyses of a great number of genotypes (Ferreira e Grattapaglia, 1995). The low informative content per loci and some reproducibility problems are the main limitations of the RAPD technique.

More consistent markers can be obtained by the study of genomic regions containing simple repeated sequences. The amplification of the microsatellite regions allows the identification of highly polymorphic, repetitive markers able to precisely distinguish even closely related individuals (Brondani et al., 1998). The use of primers complementary to the conserved DNA sequences that flank microsatellites permits the individual amplification of these highly polymorphic regions. The high polymorphism of these

segments is due to the presence of different numbers of simple repeated sequences. Microsatellites revolutionized mammalian genetics in the 1980's (Hamada et al., 1982) and they have recently been applied within the *Eucalyptus* genus (Brondani et al., 1998; Kirst, 1999).

The objectives of this study were to characterize a set of molecular markers able to identify the studied genotypes, to determine genetic divergence for recommending future crosses and to evaluate the genetic variability retained during the course of a breeding program.

MATERIAL AND METHODS

Plant material

Fifteen *E. grandis* and *E. urophylla* hybrids identified as C1 to C15 were analyzed. They were selected in commercial planting of *E.grandis* with characteristics of natural hybrids in altitude from 800 to 1000 meters with precipitation of 1300-1500mm/year and water deficit from 100 to 150 mm/year, latitude of 18° S and longitude of 42° with annual medium temperature from 20° to 21° C. The genotype C6 was selected in commercial planting of *E.grandis* with characteristics of natural hybrids in altitude of 300 meters, with precipitation of 1000-1200mm/ano and water deficit from 200 to 250 mm/year, latitude of 19°S and longitude of 42°W, with temperature annual average from 22 to 23°C. Healthy, mature leaves were collected from the selected individuals, packed on ice and transported to the Laboratório de Genética Molecular e de Microrganismos - BIOAGRO, where they were stored at – 20° C for posterior total DNA extraction.

DNA extraction

DNA was extracted using the method described by Doyle and Doyle (1990), with the following modifications: 1% insoluble PVP and 0.4% β-mercaptoethanol were used in the extraction buffer.

RAPD amplification

Amplification reactions were performed according to Willians et al. (1990). A total of 29 random primers (Operon Technologies Inc., Alameda, CA) were used in this analysis. The amplified products were analyzed by electrophoresis in 1.5% agarose gel and stained with ethidium bromide. The bands were visualized on an ultraviolet transilluminator, using Eagle Eye ™ video system (Stratagene).

SSR amplification

Amplification reactions were performed according to Brondani et al. (1998) with modifications in the annealing temperature and in the time of the cycles used for amplification. The amplifications were carried out in a thermal cycler (Programmable Thermal Controller – 100, MJ Research Inc.) programmed for an initial step at 94°C for two minutes and five initial cycles of 94°C for 1 minute, 60°C for 1 minute, with a 1°C per cycle reduction in annealing temperature from 60°C to 56°C and 72°C for one min. These initial cycles were followed by 38 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, followed by a final extension step at 72°C for 7 min. Twenty specific primers developed by Brondani et al. (1998) were used.

Data analysis

Scoring of RAPD and SSR bands was done considering presence (1) or absence (0) of a determined DNA fragment for different samples. The presence of a determined band (similar size) in all genotypes compared indicates similarity, while presence in one and absence in the other indicates dissimilarity. The data were analyzed by the statistical program GENES (Cruz, 1997). The genetic distance values were calculated using Nei Li's coefficient (Nei e Li, 1979), according to the formula:

$$S_{ii'} = 2a / a+b+c,$$

Where:

a = bands or the same allelic form present in genotypes i and i',

b = bands or the same allelic form present in genotype i,

c = bands or the same allelic form present in genotype i'.

Cluster analysis was carried out using the UPGMA method– Unweighted Pair Group Method Using Arithmetic Averages provided by STATISTICA version 4.2. To aid the characterization of the groups the Tocher cluster method, as described by Cruz e Regazzi (1994), was also performed.

Genetic diversity was calculated accord to Nei (1978) as:

$$\hat{H}e = 1 - \sum_{j}^{i} Pij^2$$

where P is the jth frequency of a given allele.

RESULTS

RAPD analysis

Two hundred ten DNA fragments generated by the RAPD amplifications were analyzed. Of this total, 134 fragments were polymorphic (63.8%) and 76 monomorphic (36.2%). An average of seven DNA fragments were amplified per primer, with a range of three to 13 DNA fragments per primer.

The average genetic distance value was 16.2%, with the smallest value (5.7%) found between individuals C7 and C8 and the largest value (26%) between individuals C2 and C6. The genetic diversity value found for the 15 individuals was 0.77.

To estimate of the efficacy of the chosen hierarchical method the correlation value among the matrix calculated by the Nei Li coefficient and the matrix calculated using the dendrogram data was considered (cofenetic correlation). The cofenetic correlation value calculated was 82.6%. The dendrogram shows a clear separation among groups formed, which was confirmed by the Tocher cluster analysis method (Table 1). In order to evaluate the reliability of the clusters formed the binary dataset was also submitted to bootstrapping. The data were reconstructed 1,000 times by resampling the bands with replacement. In that way, the number of times, expressed as a percentage, that a cluster is repeated can be considered to the validity of the clusters. The bootstrap values greater than 50% are show in the dendrogram (Figure 1).

To minimize the poor reproducibility of the RAPD bands amplifications reactions were repeated and only the DNA fragments inside an interval of defined size (493 to 2027 bp) were used to determine the individual genetic fingerprints.

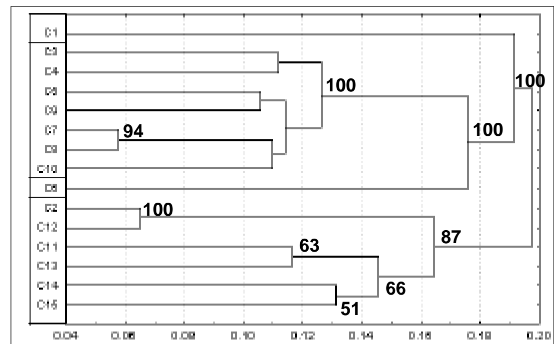

Figure 1
Cluster analysis showing the averages of the genetic distances among 15 *Eucalyptus* genotypes based on the RAPD data. Bootstrap values greater than 50% are shown on the corresponding node for each cluster. The genotypes grouped according to the Tocher method are separated each other.
(Dendrograma mostrando a média das distâncias genéticas entre 15 genótipos de *Eucalyptus* baseado nos dados da técnica de RAPD. Valores de "bootstrap" maiores que 50% estão mostrados nas bases dos ramos. Os grupos confirmados pelo método de Tocher estão separados um dos outros)

Table 1
Identification of groups identified by the Tocher optimization method of the RAPD data.
(Grupos identificados pela técnica de agrupamento de Tocher calculados utilizando os dados do marcador RAPD)

Groups	Genotypes
< 1 >	7 8 5 9 10 3 4
< 2 >	2 12 11 14 13 15
< 3 >	6
< 4 >	1

SSR analysis

For analysis of the SSR regions 20 primers projected by Brondani et al. (1998) were used. Of these only 16 primers amplified fragments which could be separated in 2.5% agarose gel (Figure 2). An average of five allelic forms were found per primer with a variation of three to seven microsatellite loci per primer. Low percentages of heterozygous loci per primer were found, on average: about 46% of the loci analyzed were heterozygous.

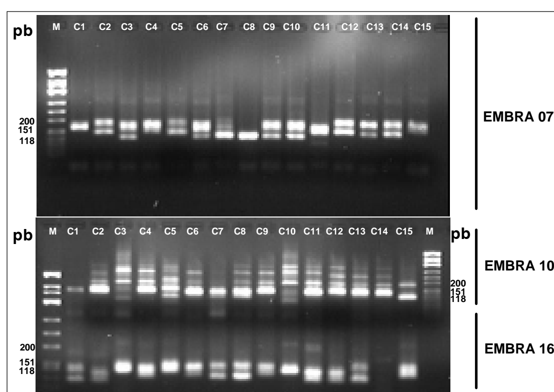

Figure 2
Agarose gel (2.5%) electrophoresis of amplified microsatellite DNA fragments of 15 *Eucalyptus* genotypes using the primers EMBRA 07, EMBRA 10 and EMBRA 16. The standard marker is ∅X phage DNA cleaved with the enzyme Hind I.
(Eletroforese em gel de agarose (2,5%) de fragmentos de DNA amplificados de 15 genótipos de *Eucalyptus* utilizando os oligonucleotídeos específicos para regiões microssatélites EMBRA 07, EMBRA 10 e EMBRA 16. O padrão de tamanho utilizado é o DNA do fago ∅X clivado com a enzima Hind I)

The average genetic distance among the individuals was 61% with low standard deviations. The smallest genetic distance value was found between genotypes C7 and C8 (12.5%), and the largest genetic distance value (93%) between C7 and C11.

The dendrogram was slightly different from that generated using the RAPD data. Agreement between values in the two matrices was estimated from the coincidence coefficient, which represents the correlation between the highest and lowest genetic distance values, starting from the average value of each matrix (Cruz e Regazzi, 1994). The coincidence coefficient for the highest values was 73% and 69% to the inferior values. The cofenetic correlation calculated for the SSR data set was 81.3%.

The SSR binary dataset was also submitted to the bootstrapping and the cluster method of Tocher used to aid the characterization of the clusters (Figure 3 and Table 2). The genetic diversity value was calculated to be 0.98.

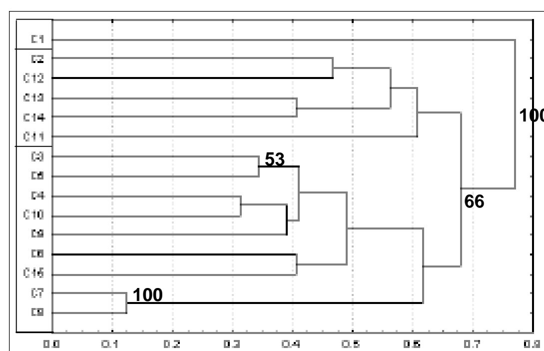

Figure 3
Cluster analysis showing the averages of the genetic distances among 15 *Eucalyptus* genotypes based on the SSR data. Bootstrap values greater than 50% are shown on the corresponding node for each cluster. The genotypes grouped according to the Tocher method are separated each other.
(Dendrograma mostrando a média das distâncias genéticas entre 15 genótipos de *Eucalyptus* baseado nos dados da técnica de Microssatélites. Valores de "bootstrap" maiores que 50% estão mostrados nas bases dos ramos. Os grupos confirmados pelo método de Tocher estão separados um dos outros)

Table 2
Identification of groups formed by the Tocher optimization method.
(Grupos identificados pela técnica de agrupamento de Tocher calculados utilizando os dados do marcador microssátelite)

Groups	Genotypes
< 1 >	7 8 15 6 10 3 9 4 5
< 2 >	2 12 11 13 14
< 3 >	1

DISCUSSION

Reproducibility of the amplified bands pattern is a prerequisite for characterization of exclusive molecular markers for each single genotype. The partial reproduction of bands generated by RAPD is one of the major limitations to the use of this technique for molecular characterization. Standardization, particularly of concentration and quality of amplified DNA, may contribute to a greater similarity in results among different laboratories. Repetition of a sample subset using the same DNA showed few or no variation among amplification patterns.

In comparison to the band patterns produced by RAPD, the bands generated by amplification of the microsatellite regions are fully reproducible among laboratories, have high discriminatory power, and the study of only a few loci allows the precise discrimination among individuals. Poor separation of amplified fragments in agarose gels and production of non-specific fragments were the main limitations of this method (Figure 2).

Performing a preliminary electrophoresis at low voltages proved essential for adequate separation of DNA fragments, and the use of amplification cycles with initially high annealing temperatures reduced the amplification of non-specific DNA fragments. The use of polyacrylamide denaturing gel and Taq DNA Polymerase of high quality would certainly improve the accuracy of the technique. However, these procedures become rather cumbersome and costly if many genotypes must be analyzed. The allelic pattern produced by the 16 primers analyzed allows the precisely discrimination of the genotypes from any other one.

Identification of the most heterogeneous groups can be used to guide the recommendation of crosses between clonal genitors seeking the maintenance of the genetic diversity and the hybrid vigor in the population. In this case, crossbreeding between genetically different individuals is recommended, rather than that involving individuals belonging to related genetic groups.

The two dendrograms based on data from the two molecular techniques are slightly different, but, taken together, they allow the identification of the most divergent individuals. Significant correlations were found between the two dendrograms (Figures 1 and 3).

The main differences between the two dendrograms are the different groups confirmed by the Tocher technique and the change in grouping of the least genetically divergent pairs C6 - C15 and C7 - C8.

The cofenetic correlation values calculated for the RAPD and SSR markers are high and show that the UPGMA cluster method is indicated to cluster the genotypes.

High genetic diversity values were found among the analyzed genotypes. Breeding programs with *Eucalyptus* trees are still quite recent in contrast to those for typical agricultural species. The few selected populations still retain much of the inherent genetic variability of the original species. The molecular analysis results will allow the monitoring of the genetic variability during the breeding program.

The percentage of heterozygous microsatellite loci was analyzed as a second estimate of genetic variability. The percentage of polymorphic loci found (P=46%) was lower than that observed by Brondani (1998) and Kirst (1999) respectively P=86% and P=57%. In his work, Kirst (1999), discuss about the effect of the number of genotypes in the estimative of the genetic diversity in *Eucalyptus* spp. He estimated the percentage of polimorphic loci with 16, 32, 64, 128 genotypes and observed standard deviations as high as 10% in some genetic diversity estimatives considering 16 genotypes. Brondani et al. (1999) also observed an imbalance in the expected proportion of heterozygous loci considering the allelic

frequencies founded in their study. According to Brondani et al. (1999) the conservation of heterozygous microsatellite loci is generally lower than expected due to self-pollination within *Eucalyptus*.

Besides these two factors, the analyses of few genotypes and the natural percentage of self-pollination, the electrophoresis in agarose gel may have contributed to the low proportion of polymorphic loci founded, once this technique do not allow the identification of allelic differences due few nucleotides. The analysis of additional genotypes and the use of more refined techniques, like the electrophoresis in polyacrylamide denaturing gel, are necessary to increase the precision of studies of genetic diversity using SSR markers.

Developed genetic material is protected in several countries. In Brazil, the cultivar protection law (Law n° 9.456, April 25, 1997) establishes criteria for cultivar protection. The protection depends on describers based on phenotypic features that must be stable across many generations. Although molecular markers show high polymorphism, stability, and repeatability, they are still not used as describers. The set of markers obtained allows the accurate identification of the studied genotypes from any other one. Adoption of molecular markers for crop description, as already done in several countries, will help prevent illegal appropriation of genetic resources.

CONCLUSIONS

✓ The utilization of RAPD and SSR markers was satisfactory for the molecular fingerprint and clustering of the analyzed genotypes. The combined use of these two markers allows to ally the high genomic coverage provided by RAPD markers with the high reproducibility of the SSR fragments enabling the obtainment of a highly informative set of markers for the analyzed genotypes.

✓ These two molecular techniques produced a set of molecular markers that allows the accurate identification of each genotype studied from any other. However the high reproducibility of the SSR markers makes them the best choice for molecular characterizations.

✓ Although the RAPD bands are comparatively less informative than that produced by the SSR technique the use of the SSR bands for genetic diversity studies depends on the analysis of more genotypes and on the use of more refined electrophoresis techniques.

✓ At least three more divergent groups can be identified in the cluster analysis; the group formed only by the genotype C1, a second group formed by the genotypes C3, C4, C5, C7, C8, C9, C10, and a third group formed by the genotypes C2, C11, C12, C13, C14. Although bootstrap values have been smaller than 50% to the terminals branches of dendrogram more divergent associations confirmed by both molecular techniques can be identified inside these three groups.

✓ The SSR amplification initially using cycles with high annealing temperatures that decrease every cycle, reduces the amplification of non-specific fragments; while the accomplishment of a preliminary electrophoresis at low voltages allows the separation of the fragments.

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