SCIENTIA FORESTALIS
n. 67, p.53-63, abr. 2005

Obtainment of *Eucalyptus* spp. hybrids aided by molecular markers – SSR analysis

Obtenção de híbridos *Eucalyptus* spp. auxiliado por marcadores moleculares microssatélites

Júpiter Israel Muro-Abad
Rodrigo Barros Rocha
Cosme Damião Cruz
Elza Fernandes de Araújo

RESUMO: O estudo da diversidade genética entre genitores é uma importante etapa de qualquer programa de melhoramento onde a heterose é acentuada. Estudos preditivos da heterose vêm sendo utilizados no melhoramento genético de *Eucalyptus* visando à obtenção de melhores combinações híbridas. Neste trabalho foi realizado um estudo da diversidade genética entre 40 genitores provenientes de dois delineamentos em dialelo parcial circulante 10 x 10 entre *Eucalyptus grandis* e *E. urophylla*, no qual os cruzamentos foram estabelecidos em estudo anterior com base na diversidade genética utilizando marcadores moleculares RAPD. Foram avaliados 17 locos microssatélites que resultaram na amplificação de 75 formas alélicas com valor de heterozigose média de 26,14% (considerada baixa). Os valores de distância genética variaram de 10 a 100% e a média geral de dissimilaridade foi de 64,62% (considerada elevada). A análise de agrupamento dos 40 genitores revelou elevada diversidade e dois grupos espécie-específicos, apesar de alguns genótipos estarem fora de seu grupo espécie-específico. Comparando-se com os grupos formados pelos marcadores RAPD, os marcadores microssatélites foram mais eficientes na discriminação das espécies. Valores de correlação entre marcadores RAPD e microssatélites foram baixos e/ou negativos. Marcadores microssatélites foram eficientes na discriminação de genitores de *E. grandis* e *E. urophylla*.

PALAVRAS-CHAVE: Diversidade genética, *Eucalyptus grandis*, *E. urophylla*, Melhoramento florestal, RAPD

ABSTRACT: The study of genetic diversity among parents is an important stage of any breeding program where heterosis is exploited. The prediction of heterosis has been extensively used in *Eucalyptus* to obtain better hybrid combinations. In this study, 40 *Eucalyptus grandis* and *E. urophylla* parents (two 10 x 10 circulant partial diallel crosses have been analyzed for their genetic diversity. The crosses were produced in a previous study based on the parent's diversity using RAPD molecular markers. Seventeen microsatellites amplified 75 allelic forms, and gave 26.14% of heterozygous. The genetic distance values varied from 10 to 100%, whereas the mean genetic distance was 64.62%, which was considered high. The cluster analysis for the 40 parents showed high diversity and brought forth two groups (one for each species), although some genotypes were outside their species-specific group. In comparison with the groups formed by the RAPD markers, microsatellite markers were more efficient for discriminating species. However, the correlation values among RAPD and microsatellite markers were low and negative. Microsatellite markers were efficient to discriminate parents of *E. grandis* and *E. urophylla*.

KEYWORDS: Genetic diversity, *Eucalyptus grandis*, *E. urophylla*, Forest improvement, RAPD markers, SSR markers

INTRODUCTION

The genus *Eucalyptus* comprises the most commonly used species for the production of short fiber cellulose in Brazil, which are mostly obtained from clonal plantations (Gomes and Couto, 1986). The main advantages of clonal plantations compared to the planting of seedlings are the homogeneity within stands and the easiness of silvicultural pre- and post-harvest operations. Elite clones can be obtained from commercial plantations or test populations for improvement (Ferreira, 1992).

Theoretically, a great part of the developed *Eucalyptus* clones are of hybrid origin, spawned by natural interspecific crossing. The performance of this hybrid material is of great interest for breeding eucalypt, considering the routine use of large-scale vegetative propagation, allowing the use of the complete genetic value of an individual. In this context, breeding methods that allows the use of intraspecific and interspecific hybrid combinations should be preferred, thus optimizing heterosis in the segregant population (Muro-Abad et al., 2001). The methodology of reciprocal recurrent selection has brought forth remarkable results, since it allows combinations among populations.

E. grandis and *E. urophylla* are key species for eucalypt breeding in the cellulose sector. They own complementary characteristics for technological wood production: wood quality (*E. grandis*) and resistance to canker (*E. urophylla*), as well as the typical hybrid vigor of the genus.

The efficiency of exploiting heterosis depends, besides the methodology, on the inherent genetic variability of the breeding population. The parental choice and determination of their combining ability is crucial for the success of the breeding program (Griffing, 1956; Cruz and Regazzi, 1994).

The use of molecular marker information to assist breeding programs may accelerate the results of field tests, which are time-consuming and require a large experimental area. This kind of molecular tool has also been incorporated into other phases of breeding programs (Pigato and Lopes, 2001).

Among the most widespread molecular marker techniques (RFLP, RAPD, AFLP, and microsatellites), RAPD is the most commonly used for forest species (Williams et al., 1990; Welsh and McClelland, 1990) due to its rapidity and easiness of implementation. It allows genome-wide sampling without requiring previous knowledge of the target crop genome (Rocha et al., 2002). RAPD have been used to study eucalypt and among its many utilities, we emphasize the characterization of diversity in *E. grandis* and *E. urophylla* for diallel cross design (Muro-Abad, 2000), identification of somaclonal variation in micropropagated *Eucalyptus* spp. clones (Laia et al., 2000), analysis of the genetic diversity in commercial *Eucalyptus* spp. clones (Muro-Abad et al., 2001, Rocha et al., 2002), construction of genetic maps, and QTL detection (Grattapaglia and Sederoff, 1994; Verhaegen et al., 1997; Marques et al., 1999).

In spite of the high applicability of RAPD, the production of dominant markers and their low reproducibility are the main limitations of this methodology.

Microsatellite markers or SSR (Simple Sequence Repeat) is a newer class of markers used for distinguishing codominant characters and various allelic forms in a breeding population (Weising et al., 1998). This marker represents a region of short sequences (2 to 5) repeated in the genome, usually flanked by conserved sequences (Weising and Kahl, 1998). These sequences can be detected and amplified by PCR (Polymerase Chain Reaction) using specific primers. For the detection of microsatellite sequences, it is necessary firstly, to construct an enriched genomic library that allows one to design specific primers for each locus. Several SSR loci, as well as their oligonucleotides, have been described for *Eucalyptus*, which were proved efficient for genetic mapping (Brondani et al., 1998; Brondani et al., 2002).

The main objective of this study was the characterization of genetic diversity using SSR markers of *E. grandis* and *E. urophylla* elite parents of circulant partial diallel designs constructed using RAPD information.

MATERIAL AND METHODS

Plant material

E. grandis and *E. urophylla* were chosen as parents of a circulant partial diallel using RAPD data for grouping and crossing recommendation. These parents were collected from progeny tests coming from different regions of Maranhão, Minas Gerais and Bahia. The crosses involved 10 parents of each species with three crosses per parent, totalizing 150 crosses. The designs are as

follow: diallel I, cluster grandis 1 (cG1) x cluster urophylla 7 (cU7); diallel II (cG2 x cU6), diallel III (cG3 x cU5), diallel IV (cG6 and cU3) and diallel V (cG5 x cU1). For a more detailed description see Muro-Abad (2000).

Two of these designs were analyzed in this work using microsatellites markers (gG2 x gU6 and gG6 and gU3), resulting in a total of 40 parents analyzed.

DNA extraction

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

SSR amplification

Amplification reactions were performed according to Brondani et al. (1998) modified by Rocha et al., 2002. The amplifications were carried out in a thermal cycler (Programmable Thermal Controller – 100, MJ Research Inc.). Twenty specific primers developed by Brondani et al. (1998) were used.

Data analysis

Scoring of SSR bands was performed considering 1 for the presence and 0 for the absence of a DNA fragment. The presence or absence of a determined band (similar size) in all genotypes compared indicated similarity, whereas presence in one and absence in the other indicated dissimilarity. Data were analyzed using GENES (Cruz, 1997) software, given that when there were homozygous genotypes for one determined fragment, a new row in the binary data matrix was taken; so that two homozygous genotypes for the same locus have two similarities. The genetic distance values were calculated using the Jaccard's coefficient using the following formula:

$$Sii' = a / a+b+c, \text{ where}$$

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'.

The cluster analysis was carried out using the UPGMA method- Unweighted Pair Group Method Using Arithmetic Averages provided by STATISTICA 4.2. To aid the group characterization, the Tocher cluster method, as described by Cruz and Regazzi (1994), was also performed.

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

$$He = 1 - \sum_{j}^{i} P_{ij}^{2}$$

where P is the jth frequency of a given allele.

RESULTS

SSR analysis

Seventeen microsatellite loci were analyzed (Table 1) for the 2 diallel, consisting of 20 parents for each species (*E. grandis* and *E. urophylla*). Seventy-five allelic forms were amplified in the investigation of these loci. Allele number varied between 3 and 7 per locus, with mean of 4 allelic forms per locus. The minimum percentage heterozygosis value was 8.82% for the locus EMBRA 10, and the maximum 42.86% for the locus EMBRA 18, whereas the mean heterozygosis was 26.14%.

The analyses showed that in some microsatellite loci, such as EMBRA 8, specific allelic forms prevail in each species, and in other loci, such as EMBRA 19, the same alleles are shared by both species (Figure 1A and 1B).

Based on electrophoretic analyses of microsatellite amplification, a matrix of genetic distances was obtained using the arithmetic complement of Jaccard's coefficient. Genetic distance values varied from 10% between the genotypes G234 and G204 to 100% between G204 and G198. The general dissimilarity mean was 64.62%.

The hierarchic cluster analysis UPGMA was carried out considering the 40 parents and each diallel separately. The general cluster analysis (Figure 3) produced two large groups, using 67.5% genetic distance as threshold value. Each group is predominantly formed by one species only. The first group consists of in majority *E. urophylla* genotypes, including no more than four *E. grandis* genotypes (G224, G204, G234, and G198). The second group consists mainly of *E. grandis* genotypes, including genotype U425.

Table 1
List of the microsatellite loci amplified, number of alleles per locus, genotypes in heterozygosis, total genotypes analyzed per locus, and percent heterozygosis per locus and general mean.
(Relação dos locos microssatélites amplificados, número de alelos por locos, genótipos em heterozigose, total de genótipos analisados por loco, heterozigose percentual por loco e média geral)

Oligonucleotide	Nr of Alleles	Genotypes in heterozygosis	Percentage of Heterozygosis
EMBRA 1	5	8	21.05
EMBRA 2	5	15	37.50
EMBRA 3	4	5	13.51
EMBRA 4	5	12	31.58
EMBRA 5	7	17	42.50
EMBRA 6	4	12	35.29
EMBRA 7	4	5	25.00
EMBRA 8	4	7	17.50
EMBRA 9	3	3	10.00
EMBRA 10	4	3	8.82
EMBRA 11	3	10	26.32
EMBRA 13	4	11	28.95
EMBRA 14	5	8	22.86
EMBRA 15	3	3	21.43
EMBRA 17	6	11	35.48
EMBRA 18	5	12	42.86
EMBRA 19	4	7	20.00
Total	**75**	**149**	
Mean	**5**	**9.93**	
Percentage of heterozygosis among clones			**26,14**

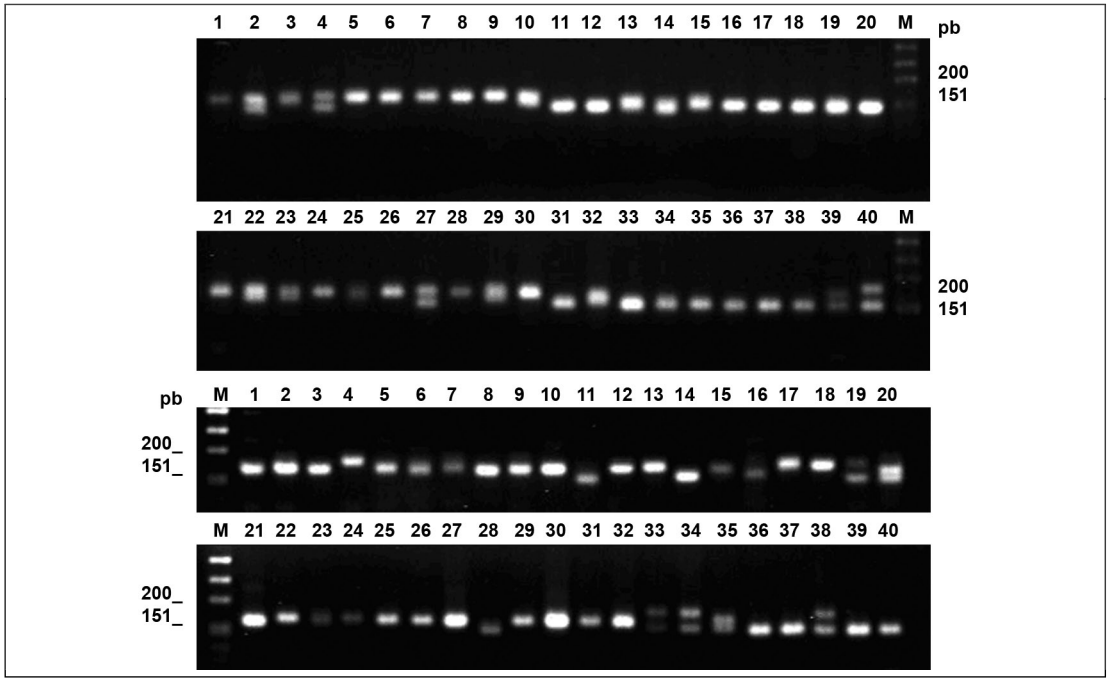

Figure 1
Agarose gel electrophoresis (2,7%) of the amplification of microsatellite loci EMBRA8 (A) and EMBRA19 (B) for 40 parents of *E. grandis* (1 – 10 and 11 – 20) and E. urophylla (11 – 20 and 31 - 40).
(Eletroforese em gel de agarose 2,7% da amplificação dos locos microssatélites EMBRA8 (A) e EMBRA19 (B) para 40 genitores de *E. grandis* (1 – 10 e 11 – 20) e E. urophylla (11 – 20 e 31 a 40))

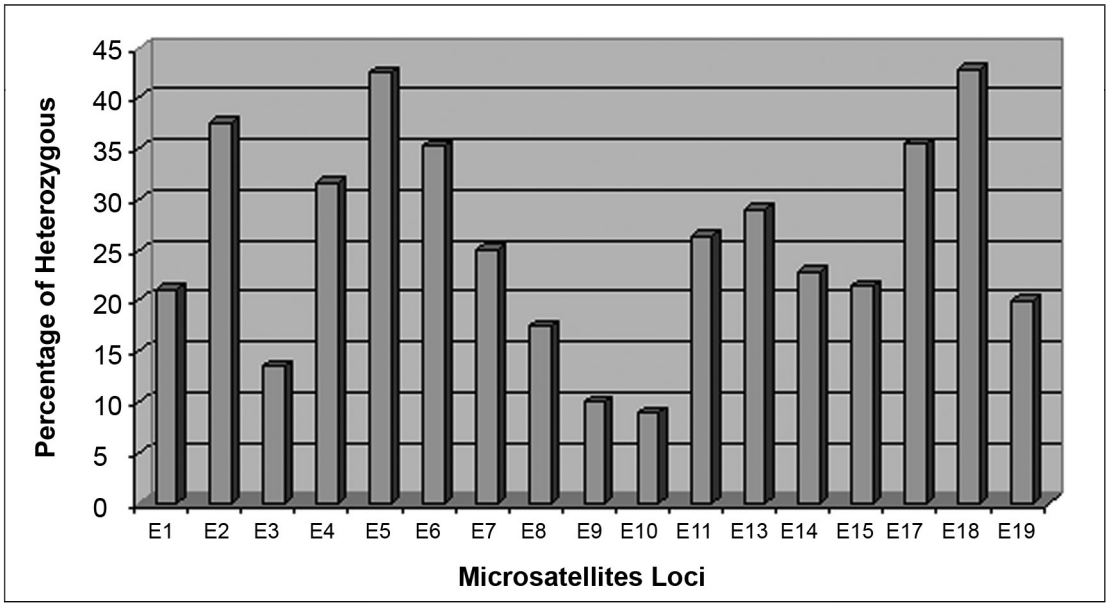

Figure 2
Percent distribution of heterozygosis presented in the 17 microsatellite loci EMBRA for 40 parents of *E. grandis* and *E. urophylla*.
(Distribuição do percentual de heterozigose apresentado para os 17 locos microssatélites EMBRA, para 40 genitores de *E. grandis* e *E. urophylla*)

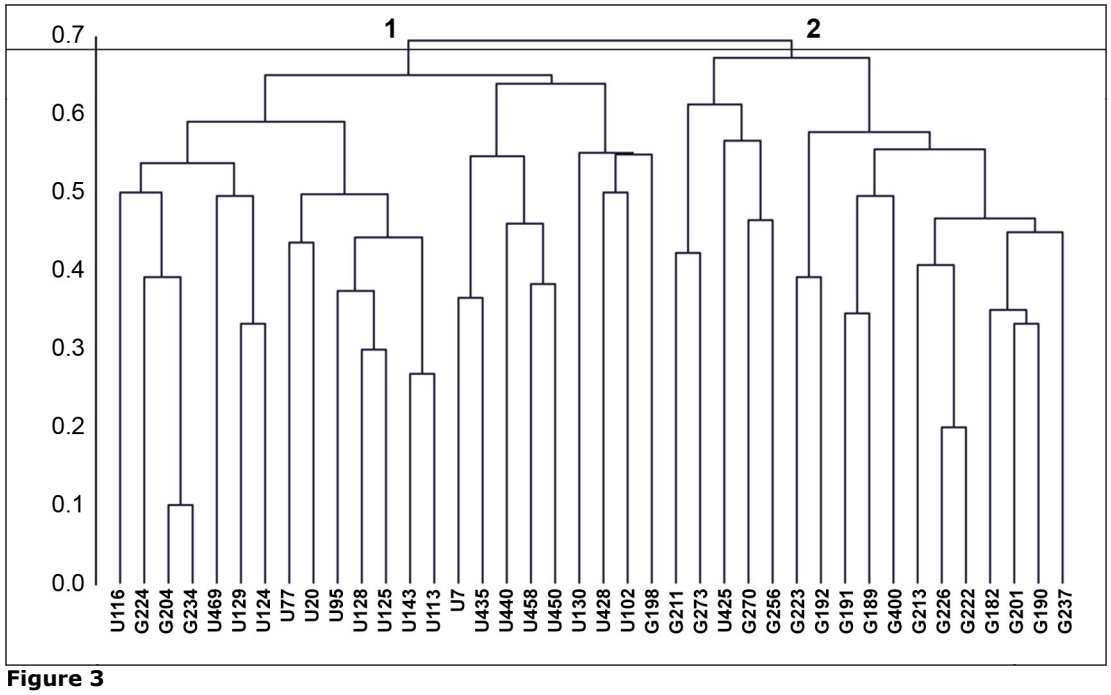

Figure 3
Dendrogram for the hierarchical cluster using UPGMA, based on the arithmetical complement of Jaccard's index for microsatellite markers of 40 parents of *E. grandis* and *E. urophylla*.
(Dendrograma da análise de agrupamento hierárquica pelo método UPGMA, com base no complemento aritmético do índice de Jaccard para marcadores microssatélites; de 40 genitores de *E. grandis* e *E. urophylla*)

A separate cluster analysis using the UPGMA methodology was performed for each diallel. The first diallel between groups gG2 x gU6 (Figure 4B) gave two groups with threshold value of 68%. The first group comprises mainly *E. grandis* and the included *E. urophylla* genotype U425a. The second group contains only *E. urophylla* genotypes.

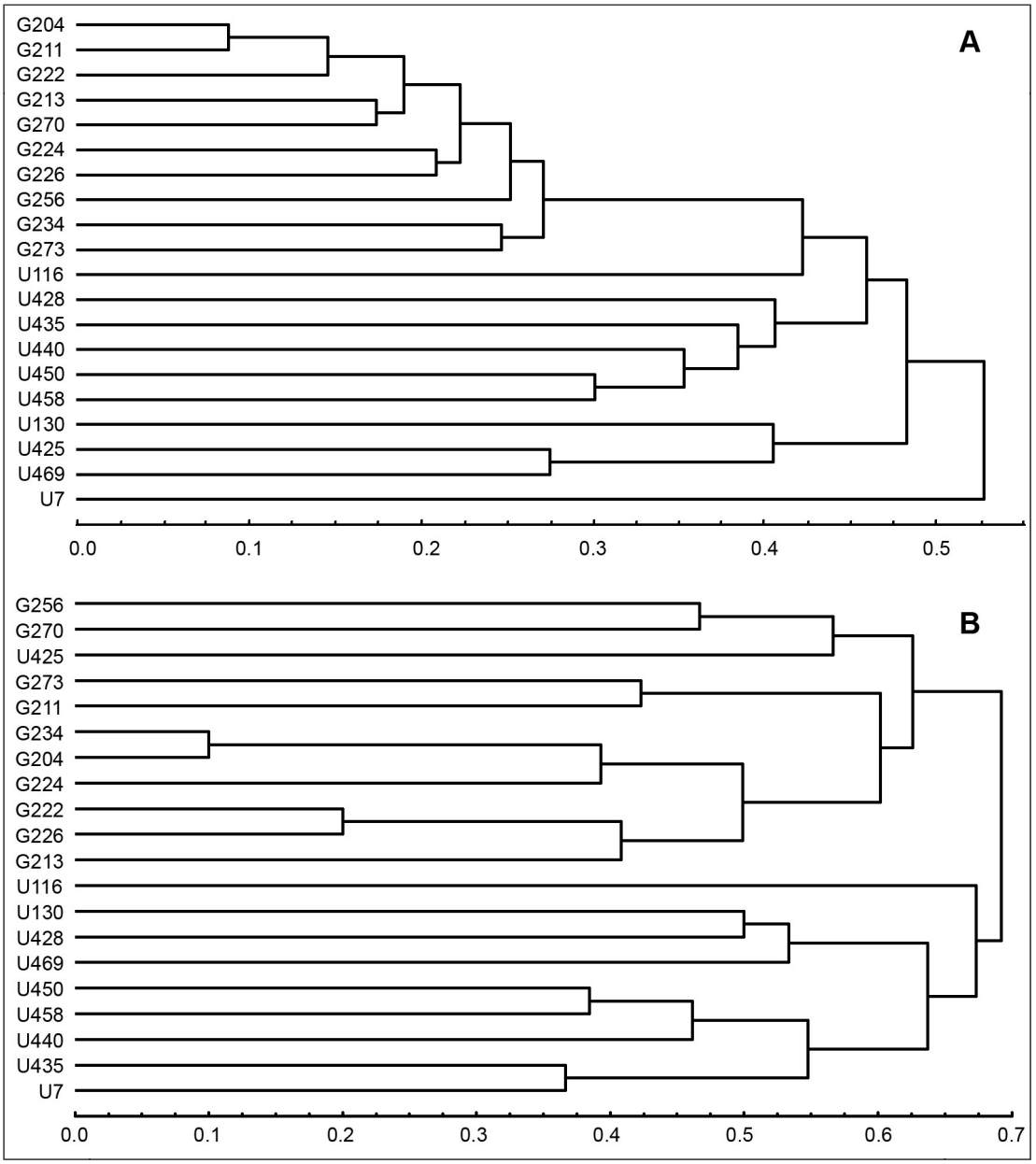

Figure 4
Hierarchical cluster analysis of genotypes derived from the circulant partial diallel gG2 x gU6 using UPGMA, based on data obtained with RAPD (**A**) and SSR (**B**).
(Análise de agrupamento hierárquico pelo método UPGMA de genótipos pertencentes ao dialelo parcial circulante gG2 x gU6, com base em dados obtidos por marcadores RAPD (**A**) e SSR (**B**))

The analysis of the second diallel, between groups gG6 x gU3 (Figure 5B), clearly shows two groups as well, with genetic distance of 60% as threshold value. The first group contains *E. grandis* genotypes, whereas the second mostly *E. urophylla* genotypes, with the insertion of G198. Three groups are formed with a threshold value of 58%, one for each species, and a third with only the genotypes G198 and U102.

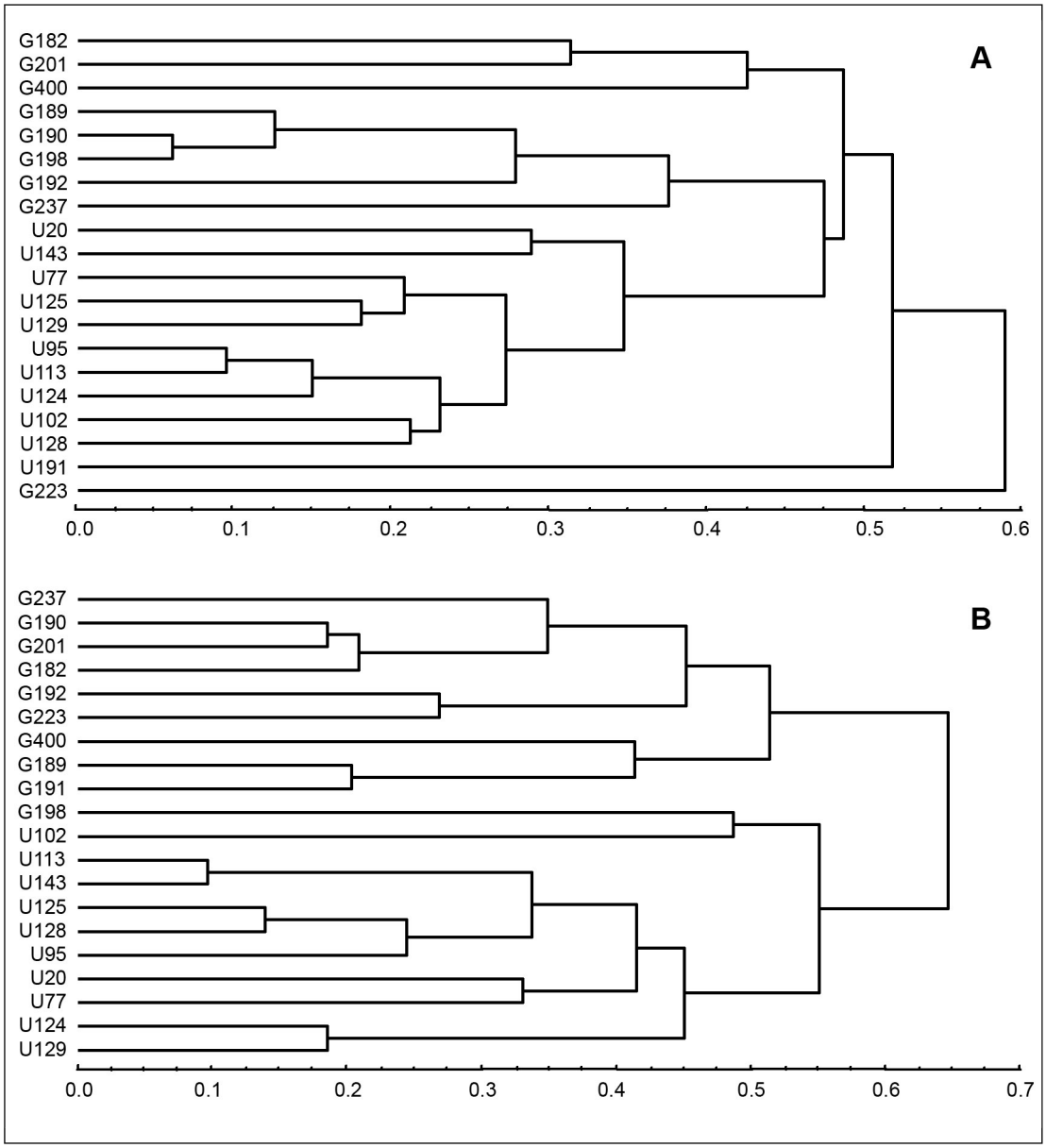

Figure 5
Hierarchical cluster analysis of genotypes derived from the circulant partial diallel gG6 x gU3 using UPGMA, based on data obtained with RAPD (A) and SSR (B).
(Análise de agrupamento hierárquico pelo método UPGMA de genótipos pertencentes ao dialelo parcial circulante gG6 x gU3, com base em dados obtidos por marcadores RAPD (A) e SSR (B))

The Tocher method grouped the 40 genotypes into 13 clusters, considering the value (θ) of 50% (θ - the maximum among the minimum distance values used in the Tocher method to decide for the inclusion of an individual into a group). The clusters formed do not necessarily comprise one single species, as shown in Table 2. Group 2 is the largest with 9 genotypes, whereas groups 12 and 13 hold only 1 genotype.

To compare the distance values obtained with RAPD and SSR, the Pearson correlation and coincidence coefficients between the obtained dissimilarity values were estimated with both marker types. For these analyses, the distance values were calculated taking the 40 genotypes together and each diallel was considered separately (Tables 3, 4). For the distance values between the 40 genotypes, the correlation among the dissimilarities produced with RAPD and SSR was – 0.188, with coincidence coefficients for the highest values of 7.9% and the lowest of 30.7%. The highest correlation values were obtained when the diallel crosses were considered separately. For diallel design 1 (gG2 x gU6) the correlation between RAPD and SSR was 0.4 with coincidence coefficients for the highest values of 38.59% and lowest of 54.38%. For diallel design 2 (gG6 x gU3) the correlation between RAPD and SSR was 0.267 with a coincidence coefficient for the highest values of 33.3% and the lowest of 52.6%.

Table 2
Tocher cluster analysis of the 40 parents *E. grandis* and *E. urophylla*.
(Análise de agrupamento por Tocher, de 40 genitores de *E. grandis* e *E. urophylla*)

Group	Genotypes								
< 1 >	G234	G204	G226	G222	G224	G201			
< 2 >	U113	U143	U458	U128	U125	U95	U20	U129	U124
< 3 >	G190	G182	G237	G192	G223				
< 4 >	G189	G191	G400						
< 5 >	U435	U7	U440						
< 6 >	G273	G211							
< 7 >	G198	U77							
< 8 >	G256	U450							
< 9 >	U102	U428							
< 10 >	G213	U130							
< 11 >	G270	U425							
< 12 >	U469								
< 13 >	U116								

Table 3
Correlation between the dissimilarity values obtained with RAPD and SSR, considering all genotypes or only those from the diallel designs 1 and 2.
(Correlação entre os valores de dissimilaridade obtidos por marcadores RAPD e SSR, considerando todos os genótipos ou apenas aqueles que fazem parte dos dialelos 1 e 2)

Variables	Var(X)	Var(Y)	Cov(X.Y)	N. of data	Correlation	Probability
RAPD x SSR	0.0231	0.0235	-0.0044	380	-0.188*	0.0319
G2xU6 RAPD x G2xU6 SSR	0.0096	0.0197	0.0055	190	0.397*	0.0
G6xU3 RAPD x G6xU3 SSR	0.0155	0.0122	0.0037	190	0.2674*	0 .027

* Significant at 5% of probability. for the test t.

Table 4
Analysis of the lowest and highest coincidence coefficients between the dissimilarity values obtained with RAPD and SSR, considering all genotypes or only those from the diallel designs 1 and 2.
(Análise de coincidência inferior e superior entre os valores de dissimilaridade obtidos por marcadores RAPD e SSR, considerando todos os genótipos ou apenas aqueles que fazem parte dos dialelos 1 e 2)

40 Genotypes			
Size of the sample: 114 (Superiors Values)			
RELATION	**N. of data**	**Coincidence (No)**	**Coincidence (%)**
RAPD x SSR	380	9	7.894%
Size of the sample: 114 (Inferiors Values)			
RAPD x SSR Dialelles 1 and 2	380	35	30.701%
Size of the sample: 57 (Superiores)			
RELATION	**N.of data**	**Coincidence(No)**	**Coincidence(%)**
G2xU6 RAPD x G2xU6 SSR	190	22	38.60%
G6xU3 RAPD x G6xU3 SSR	190	19	33.33%
Size of the sample: 57 (Inferiors Values)			
G2xU6 RAPD x G2xU6 SSR	190	31	54.38%
G6xU3 RAPD x G6xU3 SSR	190	30	52.63%

DISCUSSION

Rocha et al., 2002) analyzed 15 hybrid clones of *Eucalyptus grandis* and *E. urophylla* with 16 SSR loci, (14 of these were used in this study) and estimated the percentage of heterozygous in 46% and the number of allelic forms varying from 3 to 7. All of the 17 SSR loci evaluated showed genotypes in heterozygosis. However, maximum and mean numbers of heterozygous per locus (42.86% and 26.14%, respectively) were low, but the number of alleles is consistent with the above-mentioned study. Nevertheless, Brondani et al. (1998) observed mean heterozygous values of 57% in 15 designed microsatellite loci out of 20, and an allele number between 9 and 26, with a mean of 16.3 alleles per locus for a set of 32 *E. urophylla* and *E. grandis* trees from a germplasm bank.

The number of allelic forms is clearly a function of the population analyzed (Young et al., 2000). Although there are two species in this study, the possibility of natural hybridization between them allows the gene flow among these and other close populations. In contrast to germoplasm bank accesses or natural populations, clonal materials usually represent a final stage of a breeding cycle or program. Therefore, the low degree of heterozygosis in elite clones may suggest a concentration of genes derived from the few families used in the breeding programs. Besides, electrophoresis in agarose gel may also have contributed to the lower proportion of polymorphic loci founded, since this technique does not allow the identification of allelic differences due to the production of few nucleotides by some SSR loci (Rocha et al., 2002).

In spite of the low variation of heterozygosity among the loci studied, a great difference in their capacity to show clonal genetic variability was observed (Figure 2). Loci such as EMBRA5 and EMBRA18 are highly informative because of their number of allelic forms and the percentage of heterozygosity observed.

The cluster analysis with the 40 genotypes revealed variability among the genotypes used for the crosses. The separation into two groups by species proved to be efficient, misplacing only 5 genotypes. Brondani et al. (1998) and Brondani et al. (2002) stated that these SSR loci could be extrapolated to another *Eucalyptus* species belonging to a different section of the same subgenus Symphyomyrtus. The natural possibility of intersection of allelic forms among the *Eucalyptus* species in seed orchards and breeding populations in the field may be a cause of genetic similarity among genotypes of *E. grandis* and *E. urophylla* placed in different groups.

These genotypes are part of a breeding program in which circulant partial diallel designs were carried out using RAPD data to perform interspecific crossings (Muro-Abad, 2000). RAPD revealed

great genetic variability among genotypes making cluster characterization difficult. SSR were more efficient to discriminate among the species than RAPD (data not show).

When analyzed separately, SSR appeared very similar to RAPD. Two large well-distinguished groups were formed in each diallel. The first group diallel 1 (gG2 x gU6) contained predominantly *E. grandis* genotypes, with the insertion of genotype U425. The separation into two groups is in agreement with results obtained by Muro-Abad (2000) using RAPD. However, some differences can be seen. Genotype position is not the same, and one group is not more homogenous than the other, as shown with RAPD. Similar results were found in diallel 2 (gG6 x gU2); also occurring separation by species.

Differences among clusters obtained with different markers are quite common and have been reported by D'Ávila et al. (1998), who used RAMP (Random Amplified Microsatellite Polymorphic) and the parentage coefficient (COP) to study genetic diversity in Hordeum vulgare spp. In this case, there was no concordance among the clusters obtained with RAMP and COP. With the same set of SSR and RAPD, Rocha et al. (2002) verified some differences between clusters obtained with the two marker types in eucalypt clones.

In our study, the correlation values (considering the 40 genotypes and each diallel) were very low, although significant (Table 3). The coincidence values for the lowest dissimilarity means (Table 4) were also not very high. Significant percentages were only observed for the coincidence coefficient of the lowest values among the dissimilarity means for both diallel (54.4% diallel 1 and 52.6% diallel 2). Similar results were presented by Rocha et al. (2002). The greatest similarity among the lowest dissimilarity values suggests that the less divergent genotypes with RAPD are also less divergent with SSR.

CONCLUSIONS

• The analysis of the parents *E. grandis* and *E. urophylla* with SSR was satisfactory and allowed the discrimination of the two species;

• When using microsatellite markers, the parental groups used for crossing in a circulant partial diallel are divergent;

• In spite of the low correlation between the dissimilarity means using RAPD and SSR, the clustering of both markers separates the two parental groups efficiently.

AUTHORS AND ACKNOWLEDGEMENTS

JÚPITER ISRAEL MURO-ABAD é Doutorado no curso de Genética e Melhoramento do Departamento de Microbiologia / BIOAGRO da UFV – Universidade Federal de Viçosa – Viçosa, MG – 36571-000.

RODRIGO BARROS ROCHA é Mestrando do curso de pós-graduação em Genética e Melhoramento do Departamento de Microbiologia / BIOAGRO da UFV – Universidade Federal de Viçosa – Viçosa, MG – 36571-000 – E-mail: rbrgenetica@yahoo.com.br

COSME DAMIÃO CRUZ é Professor Titular do Departamento de Microbiologia / BIOAGRO da UFV – Universidade Federal de Viçosa – Viçosa, MG – 36571-000 – E-mail: cdcruz@ufv.br

ELZA FERNANDES DE ARAÚJO é Professora Titular do Departamento de Microbiologia / BIOAGRO da UFV – Universidade Federal de Viçosa – Viçosa, MG – 36571-000 – E-mail: ezfa@ufv.br

Os autores agradecem ao Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq, pela concessão de bolsas.

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