



## Genetic divergence in *Eucalyptus* spp. clones by molecular RAPD markers

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**ABSTRACT** - The genetic divergence in 20 *Eucalyptus* spp. clones was evaluated by multivariate techniques based on 167 RAPD markers, of which 155 were polymorphic and 12 monomorphic. The measures of genetic distances were obtained by the arithmetic complement of the coefficients of Jaccard and of Sorenso-Nei and Li and evaluated by the hierarchical methods of Single Linkage clustering and Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Independent of the dissimilarity coefficient, the greatest divergence was found between clones 7 and 17 and the smallest between the clones 11 and 14. Clone clustering was little influenced by the applied procedure so that, adopting the same percentage of divergence, the UPGMA identified two groups less for the coefficient of Sorenso-Nei and Li. The clones evidenced considerable genetic divergence, which is partly associated to the origin of the study material. The clusters formed by the UPGMA clustering algorithm associated to the arithmetic complement of Jaccard were most consistent.

**Key-words:** Dissimilarity, clustering analysis, eucalypt, RAPD.

### INTRODUCTION

The importance of commercial forests that supply enough wood to satisfy the increasing demand of the national and international sales market is generally acknowledged, besides representing a viable alternative for the protection of environments in a critical stage of degradation (Pogianni 1988). This alternative becomes more viable when species of the genus *Eucalyptus* are used, given the great adaptability and high yields under the conditions in Brazil.

However, the intense silviculture in Brazil does

not fully feed the wood demand, neither in quality nor in yield (Mora and Garcia 2000), in spite of the high competitiveness in the forestry sector, owing to the favorable climate conditions and the technological level presently used in Brazilian silviculture. The wood supply or production can be increased by an expansion of the cultivation area and/or yield increase. One of the ways of raising the yields of our commercial forests is through genetic improvement, by the identification of promising genotypes on commercial or experimental plantations, of the recombination of selected genotypes and

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controlled crosses, for example, based on the knowledge on the genetic divergence in superior genotypes.

An effective way of detecting genetic variability in different accessions is by means of molecular markers, as for example the RAPD (*Random Amplified Polymorphic DNA*). The RAPD technique is based on the amplification of the DNA fragment; an arbitrary primer sequence is hybridized in opposite direction of the target, by means of denaturation, annealing and extension cycles by the enzyme Taq polymerase (Ferreira and Grattapaglia 1998).

The study of genetic variability through molecular RAPD markers provides high gain at low costs, shortly after the genetic divergence is detected, resulting in considerable time-savings for forest species. It further allows the indication of parents for the development of specific hybrids, to maintain the genetic divergence in the following improved generations, without an expressive narrowing of the genetic base in future cycles of the improvement programs (Caixeta et al. 2003).

The objective of this study was to estimate the genetic divergence in 20 commercial *Eucalyptus* spp. clones, based on RAPD markers, in support of improvement and hybridization studies with a view to the maintenance of the genetic variability.

## MATERIAL AND METHODS

### PCR with non-specific primers (DNA amplification)

Leaf samples of plantlets of 20 *Eucalyptus* spp. clones were subjected to molecular analyses (Table 1), multiplied through minicuttings at a mean age of 85 days, through the RAPD technique that uses non-specific primers, as described by Williams et al. (1990). The primers used in this study were obtained from a collection of the University of British Columbia – Nucleic Acid – Protein Service Unit (Canada).

This study was conducted at a laboratory of the Universidade Estadual Paulista (FCAV/UNESP), Campus de Jaboticabal, state of São Paulo.

### DNA extraction from plant tissue

Young leaves (generally with the fourth leaf totally unrolled) were collected from each access, identified and taken to the local of extraction of total DNA, according to the extraction methodology described by

Lodhi et al. (1994) with modifications, according to the following procedures: 0.1 g fresh plant tissue was ground in liquid nitrogen with a porcelain mortar and pestle, 1 mL extraction buffer (20 mM EDTA; 100 mM Tris-HCl and the pH adjusted to 8.0 with HCl. Then, 1.4 M NaCl and 2% (v/v) CTAB) and 0.2%  $\beta$ -mercaptoethanol was added, the mixture shaken and transferred to a 2 mL microcentrifuge tube.

To each tube, 10 mg of PVP (polyvinyl-polypyrrolidone) (Sigma, P6755) was added, the tubes tilted gently several times to mix the material, incubated in water bath at 60 °C for 25 min and let cool down to room temperature.

Afterwards, 1 mL chloroform-isoamyl alcohol (24:1) was added, the tubes slightly shaken and then centrifuged at 10.621g for 15 min at room temperature. The supernatant was transferred to a new tube and 500  $\mu$ L NaCl 5 M and 1.000  $\mu$ L of cold ethanol (-20 °C) 95% was added. The solution was stored at -80 °C for 20 min for total precipitation of the DNA, centrifuged at 4.460g for 5 min and then centrifuged at 10.621g for another 5 min at 4 °C to form the precipitate. The supernatant was discarded and the precipitate rinsed in cold (4 °C) 75% ethanol for 1 min and thereafter centrifuged at 10.621 g and 4 °C for 5 min.

After disposing of the ethanol, the DNA precipitate was dried and resuspended with 50 mL TE buffer (Tris 10 mM, EDTA 1 mM, pH 8.0), treated with 10 mL RNase at a concentration of 10 mg/mL and incubated at 37 °C for 30 min. The DNA samples were stored at -20 °C. Based on this solution the concentration was estimated by spectrophotometry and the samples diluted to a concentration of 20 ng/mL which constituted the study solution.

### RAPD analysis

The amplification reactions were conducted in a volume of 20 mL, containing PCR buffer [200 mM Tris-HCl (pH 8.4) and 500 mM KCl]; 1.5 mM MgCl<sub>2</sub>; 10 mM dNTPs; 5 ng primers; 2.5 U of *taq* DNA polymerase; 100 ng DNA template, and water (Milli Q.) to complete a volume of 20 mL. All amplification reactions were performed in sterile PCR tubes (200  $\mu$ L). Corresponding to each reaction, a negative control was carried out with water Milli-Q instead of plant material. These reactions were performed on a thermal cycler (PTC-100 with Hot Bonnet, MJ Research, Inc., Watertown, MA) which was

**Table 1.** Relation and information of the genotypes used in this study. The origin refers to the parents and provenance to the site of selection

Clone	Identification	Origin	Provenance
1	<i>Eucalyptus grandis</i> x <i>E. urophylla</i>	Coffs Harbour (Australia) x Timor (Indonésia)	Itirapina – SP
2	<i>E. grandis</i> x <i>E. urophylla</i>	Coffs Harbour (Australia) x Timor (Indonesia)	Itirapina – SP
3	<i>E. grandis</i> x <i>E. urophylla</i>	unknown	Champion – SP
4	<i>E. grandis</i> x <i>E. urophylla</i>	Zimbabwe x Camaquã	Aracruz – ES
5	<i>E. grandis</i> x <i>E. urophylla</i>	Zimbabwe x Camaquã	Aracruz – ES
6	<i>E. grandis</i> x <i>E. urophylla</i>	Zimbabwe x Camaquã	Aracruz – ES
7	<i>E. grandis</i>	unknown	Champion – SP
8	<i>E. urophylla</i>	Timor	Jacaré – SP
9	<i>E. grandis</i> x <i>E. urophylla</i>	Zimbabwe x Camaquã	Aracruz - ES
10	<i>E. grandis</i> x <i>E. urophylla</i>	Zimbabwe x Camaquã	Aracruz – ES
11	<i>E. grandis</i> x <i>E. urophylla</i>	unknown	Champion – SP
12	<i>E. grandis</i> x <i>E. urophylla</i>	Coffs Harbour (Australia) x Timor (Indonesia)	Itirapina – SP
13	<i>E. grandis</i> x <i>E. urophylla</i>	Coffs Harbour (Australia) x Timor (Indonesia)	Itirapina – SP
14	<i>E. grandis</i> x <i>E. urophylla</i>	Zimbabwe x Camaquã	Aracruz – ES
15	<i>E. grandis</i> x <i>E. urophylla</i>	Zimbabwe x Camaquã	Aracruz – ES
16	<i>E. grandis</i> x <i>E. urophylla</i>	Zimbabwe x Camaquã	Aracruz – ES
17	<i>E. grandis</i> x <i>E. urophylla</i>	unknown	Guaíba - RS
18	<i>E. grandis</i> x <i>E. urophylla</i>	unknown	Guaíba - RS
19	<i>E. grandis</i> x <i>E. urophylla</i>	unknown	Champion – SP
20	<i>E. grandis</i> x <i>E. urophylla</i>	unknown	Guaíba - RS

programmed as follows for this analysis phase: 1<sup>st</sup> step – 94 °C for 2 min; 2<sup>nd</sup> step – 94 °C for 1 min; 3<sup>rd</sup> step – 36 °C for 1 min; 4<sup>th</sup> step – 72 °C for 1 min; 5<sup>th</sup> step – 38-fold repetition of the 2<sup>nd</sup> step; 6<sup>th</sup> step – 72 °C for 7 min; 7<sup>th</sup> step – cooling down to 10°C.

The amplified samples were analyzed in 1.5% agarose gel using the running buffer TBE and 3 mL loading buffer. The electrophoretic run lasted approximately 2 h at a tension of 70V, sufficient for band separation. The PCR-amplified and electrophoretically separated fragments were compared to those of the DNA of known molecular size (1Kb Plus DNA Ladder), visualized under UV light and photographed by a Gel Doc 2000 (Bio Rad) digital capture system.

#### RAPD data analysis

In the gel evaluation, each band was treated as a single trait, where 1 denoted presence and 0 (zero) absence. A binary matrix of 0 and 1 was constructed on these underlying data. The presence of one common

band between the two compared genotypes was considered as similarity and the presence in one genotype and absence in the other as difference.

The genetic distance was calculated pairwise, considering only the polymorphic bands, using the arithmetic complement of the indices of similarity of Jaccard (JAC) and Sorenso-Nei and Li (SNL), according to the expressions proposed by Cruz (2001).

The genetic distances were represented in simplified form by means of dendrograms constructed based on the algorithms of the Unweighted Pair-Group Method Based on Arithmetic Means (UPGMA) and of the Single Linkage, using the software Genes (Cruz 2001). The cophenetic correlation coefficient was calculated to assess the consistency of clusters obtained by the hierarchical methods, according to Bussab et al. (1990) and Cruz and Carneiro (2003).

#### RESULTS AND DISCUSSION

Forty-five primers of the University of British Columbia were tested and 23 selected to study the

**Table 2.** Primers, with respective commercial names, nitrogenated base sequences, total number of fragments (TNF), number of polymorphic fragments (NPF) and percentage of polymorphism (P%) obtained in the RAPD analyses of 20 *Eucalyptus* spp. clones

Primers	Sequence 5'→3'	TNF	NPF	P%
UBC-204	TTCGGGCCGT	4	4	100.0
UBC-209	TGCACTGGAG	6	6	100.0
UBC-210	GCACCGAGAG	9	8	88.9
UBC-211	GAAGCGCGAT	10	10	100.0
UBC-212	GCTGCGTGAC	12	11	91.7
UBC-213	CAGCGAACTA	5	5	100.0
UBC-214	CATGTGCTTG	12	12	100.0
UBC-215	TCACACGTGC	9	9	100.0
UBC-219	GTGACCTCAG	6	5	83.3
UBC-222	AAGCCTCCCC	8	6	75.0
UBC-225	CGACTCACAG	9	7	77.8
UBC-226	GGGCCTCTAT	5	3	60.0
UBC-227	CTAGAGGTCC	6	5	83.3
UBC-230	CGTCGCCAT	5	5	100.0
UBC-231	AGGGAGTTCC	6	5	83.3
UBC-232	CGGTGCCATC	8	8	100.0
UBC-235	CTGAGGCAAA	8	8	100.0
UBC-237	CGACCAGAGC	6	6	100.0
UBC-239	CTGAAGCGGA	7	7	100.0
UBC-240	ATGTTCCAGG	8	8	100.0
UBC-241	GCCCCACGCG	6	5	83.3
UBC-243	GGGTGAACCG	7	7	100.0
UBC-244	CAGCCAACCG	5	5	100.0
Total		167	155	
Mean		7.26	6.74	92.8

genetic divergence, generating a total of 167 markers (bands). Of these; 155 were polymorphic (Table 2); 16 primers did not amplify and six did not present a quality that could be exploited in this study. The 23 selected primers presented polymorphism, where UBC-214 presented the highest number (12) and UBC-226 the lowest number of polymorphic bands (3) and of polymorphism percentage (60%). The number of generated fragments varied from four (UBC-204) to 12 (UBC-212 and UBC-214), with a mean of 7.26 per primer; the percentage of polymorphism was high (92.8%), with a mean of 6.74 polymorphic bands (Table 2). Caixeta et al. (2003) studied 44 genotypes of *Eucalyptus* spp and detected polymorphism of 76.52% with initiators of Operon Technologies.

The number of polymorphic bands required to

represent the genetic distance between accesses adequately, as well as to promote an appropriate clustering of the same based on RAPD markers has been the objective of other studies, with varied results due to the study species and the very set of evaluated genotypes. Nevertheless, regardless of the disagreeing results, the use of a limited number of bands has been criticized. Through resampling, Nienhuis et al. (1995) affirmed that the genetic distances between the genotypes of *Phaseolus lunatos* L. were practically stabilized when based on 100 polymorphic bands. Fanizza et al. (1999) worked with 10 *Vitis vinifera* genotypes and concluded that at least 400 polymorphic bands would be necessary for the establishment of stable clusterings and that with the use of less than 100 to 150 bands the clusterings were completely altered.

Teixeira-Cabral et al. (2002) studied the genetic diversity in 40 *Coffea* spp genotypes and observed that the formation of the main groups was not influenced when 73, 151, 314, or 348 polymorphic bands were used; however, this number affected the formation of subgroups based on a lower divergence level. Picoli et al. (2004) concluded that at least 393 bands are necessary to obtain consistent estimates of the genetic diversity in *Eucalyptus*. Based on a literature review that included 78 articles on the use of RAPD to study genetic divergence, Dias et al. (2004) observed that the number of RAPD markers varied from 27 to 932, with a mean of 160 markers. According to these authors, this number (160) is small and should be raised to obtain more consistent analyses. Costa et al. (2006) detected pronounced genetic divergence among and within *Capsicum* spp species, based on 77 polymorphic RAPD bands, but ascribed the non-distinction of the accesses of *C. baccatum* var. *baccatum* and *C. b.* var. *pendulum* to the low number of polymorphic bands or to constraints of the molecular technique. Considering the discussed aspects, the 155 polymorphic bands obtained here with the 23 primers, although below the minimum number of bands recommended by some authors, were considered sufficient to estimate the genetic divergence in the 20 clones studied here.

The mean distance between the genotype pairs was 0.56 for the arithmetic complement of Jaccard and 0.39 for that of Sorenso-Nei and Li. This is due to the fact that the dissimilarity by the SNL coefficient attributed a higher weight to the presence of bands (1-1) in two genotypes, reducing the divergence among accesses. Clones 7 and 17 stood out as the most divergent, and clones 11 and 14 were the most similar, for both coefficients (data not shown). Working with other genotypes and using Jaccard's complement, Keil and Griffin (1994) found a mean distance of 0.45 between *E. grandis* clones; Gaiotto et al. (1997) observed 0.40 for *E. urophylla*; Leite et al. (1997) 0.67 for *E. grandis*; Pigato and Lopes (2001) 0.36 for *E. urophylla*; and Leite et al. (2002) observed a mean distance of 0.68 for *E. urophylla* as well.

The dendrograms based on the matrices of genetic dissimilarity by JAC and SNL (Figures 1 and 2) are represented, respectively, by the algorithms of the Single Linkage and UPGMA. The clones were ranked similarly in the four dendrograms, though with greater dissimilarity by Jaccard's arithmetic complement. There

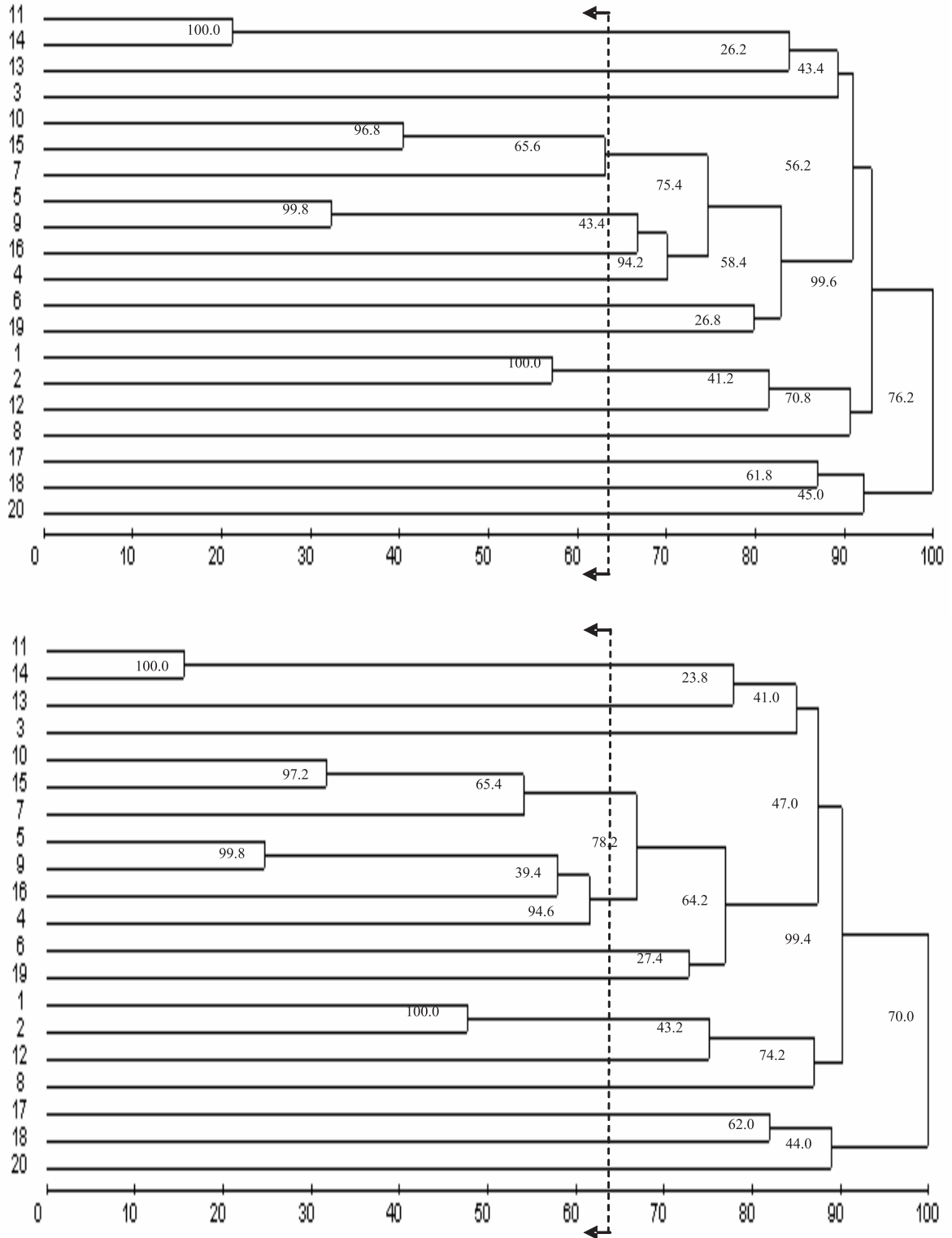
was also a small difference in the ranking of the clones and the group number and formation, when considering the two clustering methods, which can be attributed to the properties of the procedures. According to Arriel et al. (2004), it is not possible to determine which method is more precise without information on the genetic relation of the genotypes.

When a percentage of divergence of 65% is adopted, diverse heterotic clusterings are formed (Figures 1 and 2). For the UPGMA, 15 groups were formed based on the complement of Jaccard (Figure 1), of which 11 consisted of one clone each (20, 18, 17, 8, 3, 12, 13, 6, 19, 16, and 4); the 12<sup>th</sup> group was formed by clones 11 and 14; the 13<sup>th</sup> by clones 10, 15 and 7; the 14<sup>th</sup> by clones 1 and 2; and the 15<sup>th</sup> by clones 9 and 5. When the complement of the SNL coefficient was used, 13 groups were formed with nine single-clone groups; the difference of this clustering in relation to the previous was that in this case clones 16 and 4 joined 9 and 5, forming one single group.

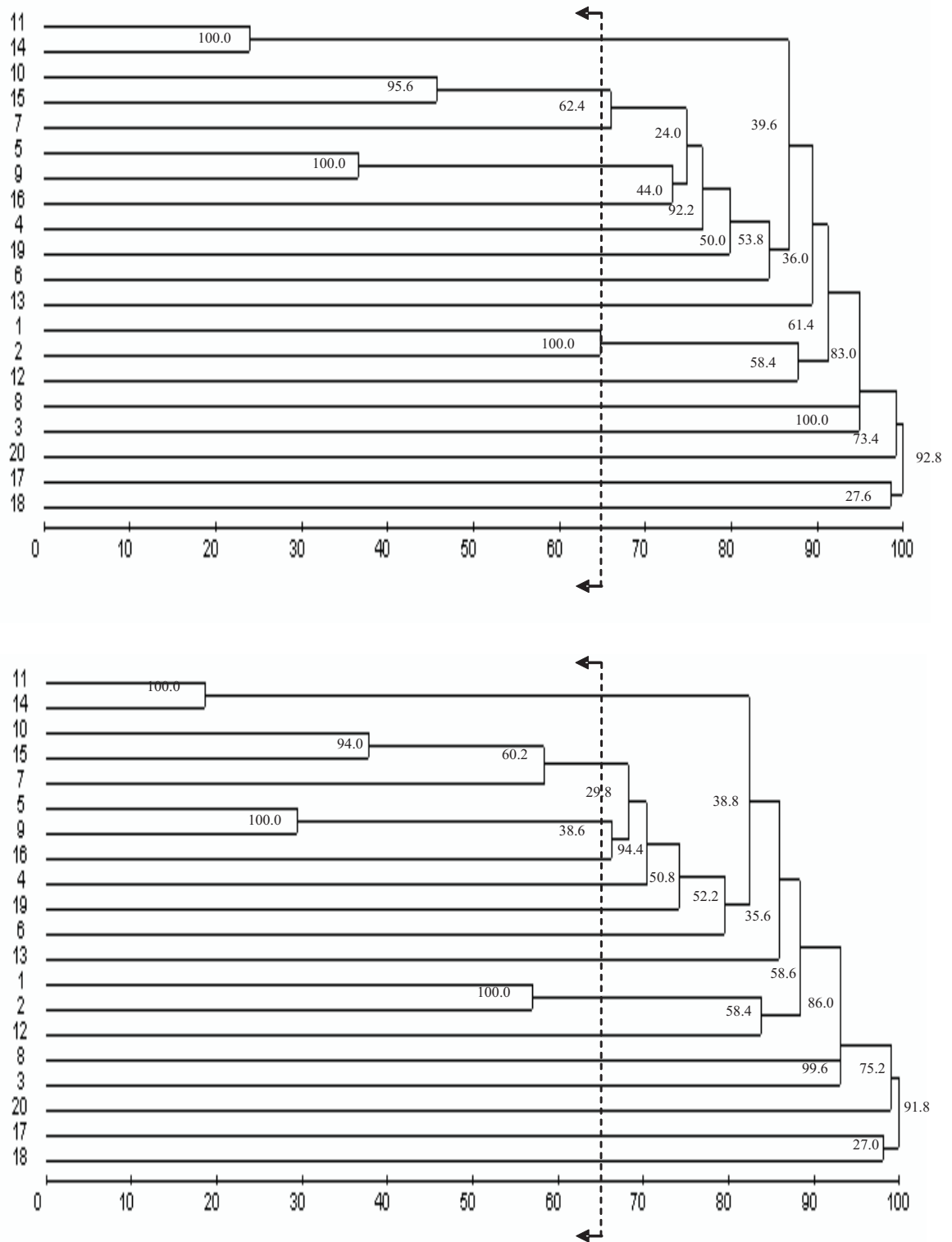
For the Single Linkage method (Figure 2) the formation of 17 groups was observed based on Jaccard's complement, similar to the formation by UPGMA, where clone 7 formed a group of its own and clones 1 and 2, which formed one group by UPGMA, were not grouped here. The 15 groups formed based on the coefficient of SNL were in line with those formed based on the complement of Jaccard in the UPGMA.

The consistency of the clusterings evaluated by the cophenetic correlation coefficient was 0.8 for Single Linkage and of 0.91 for UPGMA, based on the arithmetic complement of Jaccard, and 0.86 and 0.89, respectively, for the Single Linkage and UPGMA, when the complement of the coefficient of Sorenso-Nei and Li was used. According to Bussab et al. (1990) and Dias (1998), values of cophenetic correlation of over 0.80 indicate good cluster consistency. Both clustering methods can therefore be considered effective in the separation of eucalypt clones, with a slight superiority of the UPGMA.

In the four situations under study, the clones 20 and 8 were isolated throughout, forming one group each, evidencing that these genotypes are quite distinct from the others, and also that these have greater mean genetic distances than the other clones. Both clones have different origins from the others; clone 8 is a pure species (*E. urophylla*) native to Timor, and clone 20 a hybrid of unknown origin. Clones 17 and 18 were close



**Figure 1.** Dendrograms obtained by the UPGMA clustering method constructed based on the arithmetic complement of the Jaccard (top) and Sorenso-Nei and Li (bottom) coefficients among 20 *Eucalyptus* spp. clones, considering the molecular RAPD markers (500 bootstrap replicates)



**Figure 2.** Dendrograms obtained by the Single Linkage clustering method constructed based on the arithmetic complement of the Jaccard (top) and Sorenso-Nei and Li (bottom) coefficients among 20 *Eucalyptus* spp. clones, considering the molecular RAPD markers (500 bootstrap replicates)

to each other, but distant from the others, and with a less pronounced distance in relation to clone 20. These two genotypes also presented greater mean genetic distances than the general mean, and the distance between them was 0.48 for the coefficient of Jaccard and 0.32 of Sorenso-Nei and Li (data not shown). The origin of this material is unknown but the provenance is the same (Table 1), unlike the provenance of most other accesses, except for clone 20, which approaches clones 17 and 18 in both clustering methods. A comparison of the two similarity coefficients shows a similar group formation, with small alterations. Cruz and Carneiro (2003) mention that the two methods are similar since both do not consider the absence of bands as factor of similarity. Basically, the complement of Jaccard's coefficient can be considered the more interesting one, since it shows a higher degree of genetic divergence, which, associated to the molecular technique we used, represents a less conservative criterion in the detection of genetic divergence.

The relatively high genetic diversity in the study material can be ascribed to the fact that most clones are interspecific hybrids and that, even for *Eucalyptus*, the genotypes possibly have a high degree of heterozygosis. Crosses of the accessions evaluated here can be recommended with clones that belong to distinct groups and with greater genetic distance so that the recombination of contrasting genotypes could produce a greater heterotic effect and the maintenance of a broader genetic base.

The comparison of the clustering methods evidenced that the molecular RAPD markers were effective in the characterization of the genetic divergence in eucalypt clones.

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## Divergência genética entre clones de *Eucalyptus* spp. por marcadores moleculares RAPD

**RESUMO** - A divergência genética entre 20 clones de *Eucalyptus* spp. foi avaliada utilizando técnicas multivariadas a partir de 167 marcas RAPD, das quais 155 polimórficas e 12 monomórficas. As medidas de distâncias genéticas foram obtidas pelo complemento aritmético de Jaccard e de Sorenso-Nei e Li e avaliadas pelos métodos hierárquicos de agrupamento do Vizinho Mais Próximo e das Médias Aritméticas Não-Ponderadas (UPGMA). Independente do coeficiente de dissimilaridade, a maior divergência ocorreu entre os clones 7 e 17 e a menor entre os clones 11 e 14. O agrupamento dos clones foi pouco alterado conforme o procedimento empregado, em que, adotando-se o mesmo porcentual de divergência, o UPGMA identificou dois grupos a menos para o coeficiente de Sorenso-Nei & Li. Os clones evidenciaram considerável divergência genética entre si, estando esta, em parte, associada à origem dos materiais estudados. O algoritmo de agrupamento do UPGMA associado ao complemento aritmético de Jaccard apresentou maior consistência no agrupamento formado.

**Palavras-chave:** Dissimilaridade, análise de agrupamento, eucalipto, RAPD.

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