opportunity for analysing what is arguably one of the most significant and contentious issues around the release of transgenic trees – gene introgression. Large scale field trials of transgenic trees transformed with benign marker genes (eg. green fluorescent protein) could provide a powerful experimental model for studying pollen movement and gene flow but only if the trees are permitted to continue past the point of sexually maturity.

Thus far, the U.S. is the only country to have authorized the environmental release of a transgenic tree. In 1997 APHIS deregulated the transgenic Sunset papaya lines 55-1 and 63-1 which were developed to resist infection by papaya ringspot virus (PRV). The viral coat protein gene from a mild strain of PRV was introduced into the genome of Sunset papaya via *Agrobacterium*-mediated transformation as part of a genetic construct that also included the *nptII* and *uidA (gus)* marker genes. More information about this release, which is limited to a very small geographic area, is available in APHIS' published decision document (USDA, 1996b).

At present, there are no other petitions for the deregulation of transgenic trees pending. *Tables 1* and *2* show that, unlike traditional agricultural crops, the time line in moving from development through evaluation, and perhaps approval is significantly expanded for tree species. Just how long is long enough to accrue sufficient data for an environmental safety assessment remains controversial.

Conclusion

Canada and the United States have robust, science-based regulatory systems that have proven effective in providing for the environmental safety of field trials and unconfined releases of transgenic plants. The case-by-case approach to product evaluation is well suited to accommodate the unique challenges posed by the assessment of transgenic tree species. Both regulatory systems have the flexibility to effectively evaluate the environmental safety of transgenic trees and remain responsive to scientific development and innovation.

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Random Amplified Polymorphic DNA (RAPD) Analysis of Genotypic Identities in *Eucalyptus* Clones

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Summary

Vegetative micropropagation is usually applied in *Eucalyptus* in order to obtain clones for improvement on plant propagation for commercial purposes. One problem of this technique is somaclonal variation, which serves as a source of undesirable genetic variation, in a propagation of previously selected clones. To analyze the genotypes, *Eucalyptus* clones hybrids obtained by vegetative micropropagation were evaluated by RAPD markers. Fifteen arbitrary 10-mer primers were successfully used to amplify DNA of four clones obtained in different subcultures from callus to adult plants. During the analysis of clone "A" polymorphism was observed in the pattern of fragments of amplified DNA among subcultures, producing 39 polymorphic and 23 monomorphic bands. The genetic distance varied from 0 to 37% within this clone. For clones "B", "C" and "D" no polymorphism was observed in all plants in different ages. These results suggest the existence of sample exchange or somaclonal variation in clone "A" and showed that RAPD markers are an efficient tool for the early analysis of genotypes in *Eucalyptus* clones.

Key words: Random amplified polymorphic DNA, RAPD, genotypic identity, *Eucalyptus* clones, somaclonal variation.

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Introduction

Species of *Eucalyptus* are widely cultivated specially in tropical areas where they are used to produce charcoal, cellulose pulp, essential oil and in civil engineering applications (TIBOK et al., 1995; ANDRADE and BRASILEIRO, 1997). *Eucalyptus* productivity can be increased by plant breeding programs. However, due to the length of time from juvenile to adult phase, the selection of superior genotypes of *Eucalyptus* requires in average five to seven years. Vegetative propagation has been used to clone superior genotypes reducing the time required to obtain highly profitable forests (ELDRIDGE et al., 1993).

Among those vegetative propagation techniques, plant tissue culture is often employed in *Eucalyptus*, since it is very efficient in rejuvenation, facilitating vegetative propagation. The clones considered not viable from conventional branching propagation, can be used commercially employing tissue culture (XAVIER et al., 1997) and also provide seedlings for clonal tests, seeds orchard, clonal and microclonal gardens (ELDRIDGE et al., 1993; XAVIER et al., 1997).

Various species of plants regenerated by callus formation may present a genetic variability known as somaclonal variation (LARKIN and SCOWCROFT, 1981; DAN and STEPHENS, 1995; BOZORGIPOUR and SNAPE, 1997; GODWIN et al., 1997; HASHMI et al., 1997). This variation can be used for increasing genetic variability in breeding programs, since it does not demand sophisticated procedures or a long period of time; however, it is not recommended for propagation of previously selected clones.

Somaclonal variation, which occurs during the period of cell differentiation in the regeneration process (MUNTHALI et al., 1996), can be caused by changes in chromosome number or structure (e.g. deletions, rearrangements); DNA amplification; DNA modification (e.g. methylation); activation of transposable elements and cytoplasmic genome rearrangement (SABIR et al., 1992). It will cause various alterations in the amount or organization of genomic DNA (LARKIN et al., 1984). Some changes in the genome can not be observed at morphological or physiological levels because different gene structures may not alter the biological activity enough to modify the phenotype (SABIR et al., 1992). The phenotypic changes resulting from somaclonal variation that appear only in adult plant in *Eucalyptus* cause enormous economic losses (GRATTAPAGLIA and SEDEROFF, 1994). However, a broad range of clonal variation found in *E. grandis* (LANGE et al., 1993) and the appearance of various colours in callus of tissue culture connected to polyploidy (TIBOK et al., 1995), suggest a need for precise individual identification of clones of this species during the process of micropropagation.

Molecular markers can be used in the juvenile phase of the plant before mature characteristic appear, allowing for early selection. Among molecular markers, the RAPD (Random Amplified Polymorphic DNA) technique has been used to verify the existence of polymorphism in many plants regenerated by tissue culture (ISABEL et al., 1993; HEINZE and SCHMIDT, 1995; GALLEGO et al., 1997; PICCIONI et al., 1997).

The objective of this study is to analyze the genotypic identity in *Eucalyptus* clones to detect different genotypes by RAPD technique.

Material and Methods

Plant material

Hybrids of *Eucalyptus* were obtained from cultivated areas in São Paulo State, Brazil. Four clones (A, B, C and D) obtained in a serial process, were analyzed in different and non-consecutive subcultures, from callus to harvesting plant, about seven years old (*Table 1*). The callus were obtained in a micropropagation laboratory and the full expanded leaves were harvested in commercial areas or from a nursery.

The clone "A" was analyzed in eight (AA to AH) different subcultures, "B" in five (BA to BE), "C" and "D" were analyzed in two (CA to CB and DA to DB, respectively) different subcultures. Replicates were employed in each subculture, as following: three for subcultures of adult plants (e.g. AA1, AA2 and AA3) and two for subcultures for explants in the callus phase (e.g. AH1 and AH2). In the numbering system used, the first letter corresponds to clone, the second to subculture and the number to replicate. The donor plants were used as a control (subculture zero) and their pattern of amplified DNA fragments were compared to that of other plants from the same clone in different subcultures.

DNA extraction

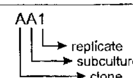
The DNA was extracted from young leaves, fully expanded, which were fresh or stored at –80 °C following the procedure described by DOYLE and DOYLE (1987), modified by GRATTAPAGLIA and SEDEROFF (1994).

Amplification conditions

Amplification reactions were performed in a total volume of 25 µl, which contained 10 mM Tris-HCl pH 8.0; 50 mM KCl; 2.5 mM $MgCl_2$; 0.1 mM each of deoxynucleotides (dATP, dCTP, dGTP and dTTP); 0.4 µM primer (Operon Technologies Alameda, CA, USA); 25 ng genomic DNA; and 1 unit of *Taq* DNA polymerase (Promega, Madison, USA). The amplifications were run in a thermal cycler (PTC – 100™ MJ Research, Inc.) programmed for 40 cycles, each cycle consisted of a denaturation step of 15 sec at 94 °C; an annealing step of 30 sec at 35 °C and an elongation step of 1 min at 72 °C. After 40 cycles and a final extension for 7 min at 72 °C, the temperature was decreased to 4 °C until the samples were removed. Fifteen 10-mer primers of known random sequence were employed in this

Table 1. – Identification, local and subculture of *Eucalyptus* clones.

No	Clone	Local	Subculture	No	Clone	Local	Subculture
1	AA1	Plot 44	Donor	24	BA1	Plot 44	donor
2	AA2	Plot 44	Donor	25	BA2	Plot 44	donor
3	AA3	Plot 44	Donor	26	BA3	Plot 44	donor
4	AB1	Unknown	22°	27	BB1	Plot 142	Unknown
5	AB2	Unknown	22°	28	BB2	Plot 142	Unknown
6	AB3	Unknown	22°	29	BB3	Plot 142	Unknown
7	AC1	Unknown	39°	30	BC1	Plot 94-95	Unknown
8	AC2	Unknown	39°	31	BC2	Plot 94-95	Unknown
9	AC3	Unknown	39°	32	BC3	Plot 94-95	Unknown
10	AD1	Unknown	68°	33	BD1	Plot 12	Unknown
11	AD2	Unknown	68°	34	BD2	Plot 12	Unknown
12	AD3	Unknown	68°	35	BD3	Plot 12	Unknown
13	AE1	Nursery	73°	36	BE1	Laboratory	87°
14	AE2	Nursery	73°	37	BE2	Laboratory	87°
15	AE3	Nursery	73°	38	CA1	Laboratory	28°
16	AF1	plot 12	Unknown	39	CA2	Laboratory	28°
17	AF2	plot 12	Unknown	40	CB1	Plot 79	donor
18	AF3	plot 12	Unknown	41	CB2	Plot 79	donor
19	AG1	plot 79	Unknown	42	CB3	Plot 79	donor
20	AG2	plot 79	Unknown	43	DA1	Laboratory	28°
21	AG3	plot 79	Unknown	44	DA2	Laboratory	28°
22	AH1	Laboratory	88°		DB1	Plot 12	donor
23	AH2	Laboratory	88°				



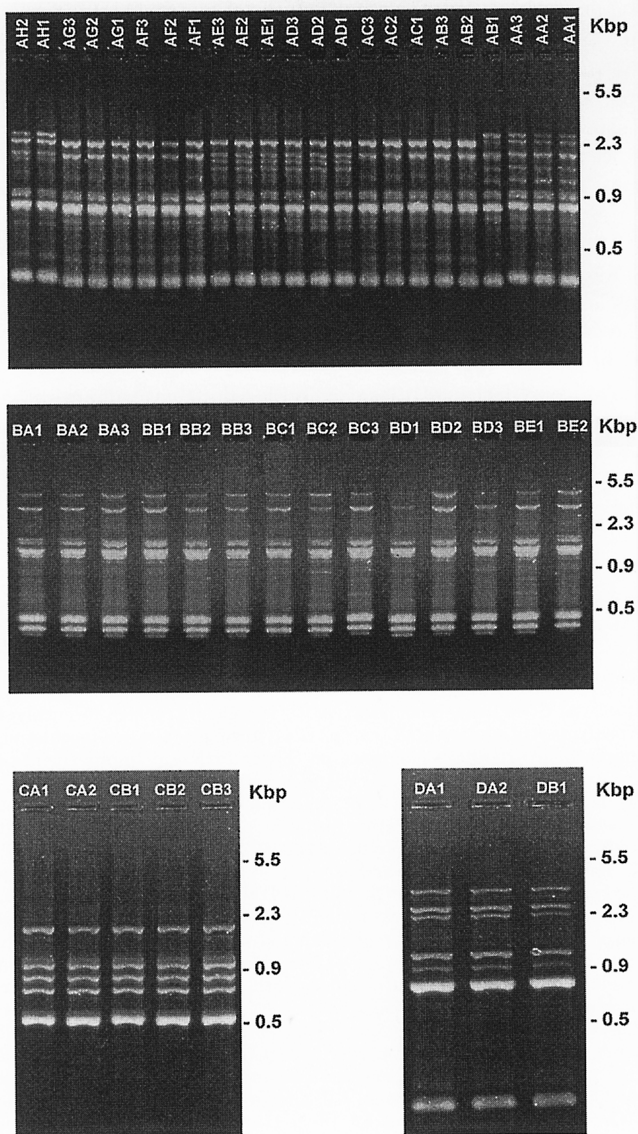


Figure 1. – Gel electrophoresis of random amplified polymorphic DNA (RAPD) fragments obtained with primer OPB04 for 4 clones of *Eucalyptus*. Clones identification are in *table 1*. Fragment sizes are indicated in kilobase pairs.

study. The RAPD amplifications were repeated three times with each primer. The amplification products were separated by electrophoresis in 1.5% agarose gels containing 0.25 µg/ml of ethidium bromide. After the electrophoresis, the images were captured and stored using a photo-documentation system (Eagle Eye II, Stratagene).

Data analysis

Intra-clone data were analyzed comparing the pattern of amplified DNA fragments of each plant to the other plants from the same clone, in different ages. The comparison was based on presence (1) or absence (0) of a determined band (DNA fragments) among plants belonging to a determined clone. The presence of one band in one plant and its absence in other plant indicates that there are difference between them.

The genetic distances were estimated by pair-group, using the coefficient of NEI and LI (1979). This coefficient was then used to construct dendrograms by UPGMA method (Statistica for Windows – StatSoft, Inc., 1995).

Results

RAPD analysis showed the same pattern of amplified DNA fragments for all plants in different subculture of clones "B", "C" and "D" (*Figure 1*). For these clones the amplification resulted in 54 monomorphic bands and no polymorphic band.

RAPD analysis for clone "A" (*Figure 1*) producing 39 polymorphic and 23 monomorphic bands with polymorphisms occuring both inter- and intrasubcultures. The genetic distances (*Table 2*) varied from 0% to 37% within this clone. The dendrogram (*Figure 2*) shows the genetic relationship among different subcultures.

The analysis of amplification products showed that in subculture 22 of clone "A" there is a plant (AB1) with the same pattern of bands as in donor plants (AA1 to AA3) (*Figures 1* and *2*). The genetic distance between plant AB1 from this subculture and plants AB2 and AB3 from the same subculture was 23%, while the distance between AB1 and donor plants (AA1 to AA3) was 8%.

In the analyzed subcultures of clone "A", the RAPD technique detected six changes in the DNA pattern (*Figures 1* and *2*), as follows: first, with donor plants (AA1 to AA3); second, it happened to replicate AB1 of subculture 22, which isolated

Table 2. – Matrix genetic distances (%) among plants of clone "A" based on RAPD analyses. Clones identification are in *table 1*.

	AH1	AH2	AG1	AG2	AG3	AF1	AF2	AF3	AE1	AE2	AE3	AD1	AD2	AD3	AC1	AC2	AC3	AB3	AB2	AB1	AA1	AA2	AA3
AH1	0																						
AH2	0	0																					
AG1	16	16	0																				
AG2	16	16	0	0																			
AG3	16	16	0	0	0																		
AF1	16	16	0	0	0	0																	
AF2	16	16	0	0	0	0	0																
AF3	16	16	0	0	0	0	0	0															
AE1	35	35	36	36	36	36	36	36	0														
AE2	35	35	36	36	36	36	36	36	0	0													
AE3	35	35	36	36	36	36	36	36	0	0	0												
AD1	35	35	36	36	36	36	36	36	0	0	0	0											
AD2	35	35	36	36	36	36	36	36	0	0	0	0	0										
AD3	35	35	36	36	36	36	36	36	0	0	0	0	0	0									
AC1	20	20	6	6	6	6	6	6	37	37	37	37	37	37	0								
AC2	20	20	6	6	6	6	6	6	37	37	37	37	37	37	0	0							
AC3	20	20	6	6	6	6	6	6	37	37	37	37	37	37	0	0	0						
AB3	20	20	6	6	6	6	6	6	37	37	37	37	37	37	0	0	0	0					
AB2	20	20	6	6	6	6	6	6	37	37	37	37	37	37	0	0	0	0	0				
AB1	21	21	24	24	24	24	24	24	31	31	31	31	31	31	23	23	23	23	23	0			
AA1	18	18	23	23	23	23	23	23	30	30	30	30	30	30	27	27	27	27	27	8	0		
AA2	18	18	23	23	23	23	23	23	30	30	30	30	30	30	27	27	27	27	27	8	0	0	
AA3	18	18	23	23	23	23	23	23	30	30	30	30	30	30	27	27	27	27	27	8	0	0	0

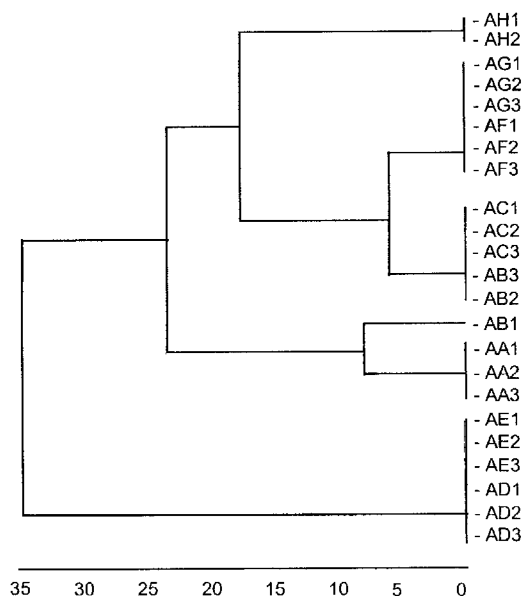


Figure 2. – Cluster analysis of clone "A" based on RAPD data. The dendrogram was generated from genetic similarity coefficients obtained from presence and absence of DNA bands and is based on the unweighted pair-group method using arithmetic average (UPGMA). Clones identification are in *table 1.*

from the others, third in two replicates (plants) of subculture 22 (AB2 and AB3) and three replicates of subculture 39 (AC1 to AC3); fourth, subcultures 68 (AD1 to AD3) and 73 (AE1 to AE3); fifth, it was detected in plants from plots 12 (AF1 to AF3) and 79 (AG1 to AG3); and finally, the replicates of callus (AH1 and AH2) where a different pattern of bands, according to previous ones, was detected.

Discussion

Micropropagation, among many other clonal propagation methods in *Eucalyptus*, is widely used allowing the rejuvenation of clones which would not be viable by other asexual propagation processes (XAVIER et al., 1997). Despite the fact that there are reports about the occurrence of somaclonal variation in regenerants by forming callus in other species (DAVEY et al., 1971; CHATURVEDI and MITRA, 1975; SELBY and COLLIN, 1976; BROWN et al., 1993; RUSKORTEKAAS et al., 1994), in *Eucalyptus*, studies, comparing donor plants to their clones at different subcultures, has not yet been conducted.

Among other applications, RAPD technique has been used to analyze somaclonal variation, while it can also detect single base mutations and deletions at the level of the primer target or insertions / deletions within the amplified fragments (GALLEGO et al., 1997).

RAPD analysis did not detect any polymorphism for clones "B", "C" and "D". This indicates that, despite being micropropagated for over two years and 87 subcultures, clone "B" according to RAPD characterization, presented all subcultures similar to the donor plants. The same pattern was observed in clones C and D, that were in the 28° subculture. In contrast, when clone "A" was analyzed, the RAPD technique detected polymorphism in the DNA of plants from different subcultures, from the earliest (clones AB) to the latest (clone AH) (*Figures 1* and *2*).

The genetic variation found in clone "A" can be explained by: 1) change of clones in the field; 2) change of clones while manipulated in the micropropagation laboratory; or 3) changes in the sequence DNA of the clone, because of successive subculturing processes or even by other manipulation conditions (RANI et al., 1995).

Swapping seedlings of *Eucalyptus* in the field during planting, as well as the swapping of calli during multiplication in tissue culture is common in the forestry industry. However since all the subclones were obtained as a result of a serial process, and since clone "B" was cultivated by the same process as "A", for 87 subcultures, it is likely that the variation observed reflects somaclonal variation.

Another indication of the occurrence of somaclonal variation in clone "A" is the fact that when clone "A" was compared with the other clones in the same gel, using the same primer, several differences were observed (not shown).

The compilation of these molecular data with quantitative data of productivity and quality of the wood for cellulose of these clones, and with morphological and genealogical information would lead to a better characterization of these eucalypt clones.

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Contribution to the Karyomorphology of Some Species of the Genus *Quercus*

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Summary

In order to contribute to the knowledge of the taxonomic relationships between some species of the *Quercus* genus, the karyomorphology of 4 species (*Quercus ilex* L., *Q. suber* L., *Q. robur* L. and *Q. pubescens* WILLD.) was carried out. The somatic chromosome number 2n = 24 was found in all taxa examined. An evident differentiation in karyotype structure of *Q. ilex* compared to other previous studied species has been found. *Quercus suber*, *Q. robur* and *Q. pubescens* possess moderately asymmetrical karyotypes. *Q. suber* differs from other species of the subgenus *Cerris* on the basis of karyological data showing similarities with *Q. robur* and *Q. pubescens*.

Key words: karyotype, *Quercus ilex*, *Q. pubescens*, *Q. robur*, *Q. suber*.

Introduction

The genus *Quercus* including about 300 species with many varieties and natural hybrids widespread in many regions of the world is one of the most complex taxonomic groups. The systematic relationships in this genus are still not completely clear (NIXON, 1993) although in the literature many data concerning morphological information (SPELLENBERG, 1992; BACILIERI et al., 1995), chemo-taxonomical characters (KNOPS and JENSEN, 1980), enzymes (GUTTMANN and WEIGT, 1989) and DNA (BODENES *et al.*, 1997) studies, ribosomal RNA genes (BELLAROSA *et al.*, 1990) and molecular cytogenetic analysis are available (ZOLDOS *et al.*, 1999). Nevertheless, data on oak chromosomes are considered still insufficient (OHRI and AHUJA, 1990; ZOLDOS *et al*, 1999). D'EMERICO *et al.* (1995) report some data about the karyomorphologies of 8 species of *Quercus* pointing out morphological similarities but also differences in intrachromosomal and interchromosomal asymmetry indices.

In this report, in order to contribute to the knowledge of the relationships between the species belonging to the genus *Quercus,* we have continued the karyological investigation (D'EMERICO *et al.*, 1995), with traditional Feulgen methodology, to describe the karyotype of *Quercus ilex*, *Q. pubescens*, *Q. robur*, *Q. suber.*

Materials and Methods

Acorns of *Quercus ilex* were collected at various sites in Apulia; those of *Q. suber*, *Q. pubescens* and *Q. robur* in Latium (Italy).

For cytological examination of somatic chromosomes, actively growing root tips were pre-treated in a 0.3% colchicine at 20 °C for 2 h, and then fixed for 5 min in a 5 : 1 : 1 : 1 (volume ratio) mixture of absolute ethanol, chloroform, glacial acetic acid, and formalin (BATTAGLIA, 1957a). Hydrolysis was carried out at 20 °C in 5.5 N HCl for 20 min (BATTAGLIA, 1957b), and then stained with SCHIFF's reagent. Squashes were made in a drop of 45% acetic acid. Chromosomes of 5 to 6 plates were measured in 3 trees of each species.

The nomenclature used for describing karyotype composition followed LEVAN *et al.* (1964). Karyotype morphometric characters were evaluated by calculating haploid complement length and the indices: SYi, which describes the average symmetry of the karyotype (GREILHUBER and SPETA, 1976), A_1, which represents the degree of asymmetry in arm length within the chromosome of the genome, and A_2, expressing the variation in chromosome lengths (ROMERO ZARCO, 1986), as used in D'EMERICO *et al.* (1996).

Results

Karyotype morphometric characters, haploid complement and karyotype formulae are given in *table 1*.

The somatic chromosome number 2n = 24 was found in all taxa examined (D'EMERICO *et al.*, 1995; and references therein).

Q. robur possesses a moderately asymmetrical karyotype, consisting of 14 metacentric and 10 submetacentric chromosomes. Two chromosome pairs (1 and 3) bear evident secondary constrictions on the short arms; pair 7 presents a secondary constriction on the long arm; pair 11 has a satellite on the long arm (*Fig. 1a*).

The detailed karyotype morphology of *Q. pubescens* is reported. The karyotype of this species consists of 18 metacentric and