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ANALYSIS OF THE RESTRICTION FRAGMENT LENGTH POLYMORPHISM OF *Eucalyptus urophylla, E. grandis* AND *E. citriodora*

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ABSTRACT

Restriction fragment length polymorphysm analysis was performed to assess the level of genetic variability three eucalypt species (*Eucalyptus citriodora, E. grandis* and *E. urophylla*) and to select genetic markers for the establishment of a linkage map. Homologous and heterologous probes were hybridized to total DNA, digested with the restriction enzymes Eco RI and Hind III. Very high levels of intra and inter-specific polymorphism were detected.

INTRODUCTION

The genus *Eucalyptus* (Myrtaceae), comprises more than 600 species and has its center of diversification in Australia, with a few native species in Papua, Timor and some adjoining islands of the Lesser Sunda group (Pryor, 1976). Because of their fast development and the wide range of conditions in which the various species can grow, the genus has been a popular choice of introduction worldwide. Eucalypts have been planted mainly in tropical and subtropical areas, and have become the most widely planted hard wood timber in the world (FAO, 1985). Eucalypts were introduced into Brazil at the beginning of this century, and have been extensively planted in several regions of the country.

Generally, breeding programs based on intra and interspecific crossing and clonal propagation of selected trees have been limited by the length of the reproductive

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cycle, the cross-incompatibility of the species, and a high degree of heterozygosity within species (Pederick, 1979; Brooker and Kleinig, 1983; Moran and Bell, 1987; Malan, 1988; Sampson *et al.*, 1988).

The identification of genetic markers of eucalypts would provide valuable tools for breeding programs. To date, restriction fragment length polymorphism (RFLP) analysis has provided a number of useful genetic markers for many species, from woody trees (Riemenschneider *et al.*, 1988) to humans (Beckmann and Soller, 1987). RFLP markers are inherited in a codominant fashion, are not affected by the environment, and are limited only by the degree of homology existing among the organisms of interest and by the number of restriction enzymes available (Tanksley *et al.*, 1989).

There was very little understanding of the basic genetics of eucalypts until the advent of isozyme research (Moran and Bell, 1983). The results obtained have provided some insight to the genetic variability within each species and also about the genetic similarity among them.

RFLP analysis was performed to generate genetic markers for *E. citriodora, E. grandis* e *E. urophylla*, the species most extensively cultivated in Brazil. Such genetic markers could used for constructing linkage maps or in studies of correlation to specific economic traits, or for other aspects of breeding programs for these species.

MATERIALS AND METHODS

Plant material

The species of eucalypts used in this study were *Eucalyptus citriodora* Hook (Provenance Gregory Springs, Australia), *E. grandis* Hill ex. Maiden (Provenance Cofsharbour, Australia), *E. urophylla* S.T. Blake (Provenance Buritizeiro, Timor). Leaves were collected from four to six month old trees grown from seeds collected in two different regions of the state of Minas Gerais, Brazil: Turmalina for *E. urophylla*, and Timóteo for *E. grandis* and *E. citriodora*. The seeds were supplied by the steel company Acesita Energética S.A.

Young leaves were collected from ten individual plants in the field, immediately freeze dried, pulverized in a blender, and stored at -20°C. For the construction of the genomic library the DNA was extracted from leaves of a plant miantained in the greenhouse.

DNA isolation, digestion, and Southern blotting

DNA was extracted using the CTAB extraction method (Lichtenstein and Draper, 1985), modified as follows in order to remove contaminants: for an extraction of

0.5 g of freeze dried material, 0.4 ml of 1M NaCl was added to the DNA pellet after precipitation with buffer in 15 ml Falcon tubes. The tubes were then placed in a refrigerator for two hours. The DNA was transferred to an Eppendorf tube, thoroughly redissolved at 60°C for 10 minutes, 0.1 ml of 5M NaCl was added to the solution, and the mixture was centrifuged in a microcentrifuge at full speed for 10 minutes. The supernatant was precipitated with two volumes of cold 100% ethanol. The pellet was washed with 70% ethanol and resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA pH 8.0). The above method was improved by adding 1% N-laurosylsarcosine to the extraction buffer of *E. grandis* and 2% to the extraction buffer of *E. citriodora* and *E. urophylla*.

DNA from leaves of ten plants of each species was digested overnight at 37°C with EcoRI and HindIII (Pharmacia), according to manufacturer instructions. Electrophoresis was carried out on 0.8% agarose gels in 1x TAE (Maniatis *et al.*, 1987) for about 16 hours at 20 mA, using approximately 10 μg per lane. After electrophoresis, the DNA was transferred to Zeta probe nylon membranes (Bio-Rad), using a vacuum transfer apparatus (LKB, 2016 VACUMGENE) and bound to the membrane by baking at 80°C for 2 hours.

Source and preparation of probes for library construction

Total DNA from a select individual was digested with Pst I and gel fractionated. Fragments between 1 and 2 kb were purified using the Genecleen kit (Bio 101), and ligated into dephosphorylated Pst I cut pEMBL. Strain XL1-blue of *E. coli* was transformed with the plasmids according to Chung and Miller (1988) and grown on LB plates with ampicillin, the inducer IPTG, and the indicator dye X-gal (Messing, 1983). White colonies were selected and grown overnight on LB media with ampicillin. The plasmids were extracted by the alkaline lysis minipreparation method (Birnboim, 1983) and subjected to gel electrophoresis in order to confirm the presence of eucalypt DNA inserts.

Probe preparation, hybridization, and autoradiography

Two labelling methods were used. Probes were labelled either with ^{32}P-dCTP, according to the usual nick translation procedure of Maniatis *et al.* (1987), or with digoxigenin-dUTP, using the digoxigenin labeling and detection kit of Boehringer Mannheim, according to manufacturer instructions.

For the radioactive probes, prehybridizations (6-12 hours) and hybridizations (overnight) were performed at 65°C in 1M NaCl, 50 mM Tris pH 7.5, 1% SDS, and 5-10% Na dextran. After hybridization, filters were washed twice for 5 min in 2x SSC, 0.5%

SDS at room temperature, and twice for 30 min each with 0.25 x SSC, 0.1% SDS at 65°C. The filters were then exposed to Kodak X-Omat or Hyperfilm (Amersham) at -70°C for 5 to 10 days, using Dupont Cronex Lightning-plus intensifier screens.

Detection of the digoxigenin labeled probes was performed using the chemiluminescent substrate detection procedure with AMPPD (disodium 3-(4-methoxyspirol {1,2-dioxetane-3,2-tricyclo-[3.3.1.1]decan}-4-yl) phyenil phosphate) (Kreike *et al.*, 1990; Allefs *et al.*, 1990), also according to manufacturer instructions (Tropix, Inc.). For signal detection, the membrane and the film were placed in a cassete at room temperature. The film was developed after 30 to 120 minute exposure, depending on the number of times the membrane had been used. In general, membranes were hybridized with probes three times.

RESULTS

Probes from a random DNA library were hybridized to membranes with two sets of DNA samples from ten plants, one set digested with Eco RI and another with Hind III (Figure 1). More than 17% of probes that detected polymorphic bands did so with both of these restriction enzymes. Although the enzymes Eco RI and Hind III are recommended to cleave non methylated plant DNA (Helentjaris *et al.*, 1985), analysis of polymorphism with the restriction enzyme indicated that Hind III produces more polymorphic probes (Table I).

Table I - Distribution of useful polymorphic probes in *Eucalyptus urophylla* (URO), *E. citriodora* (CIT), and *E. grandis* (GRA), according to the restriction enzyme.

Enzyme	URO	CIT	GRA	GRA/URO	CIT
			(%)		
Eco RI	36	36	36	37	28
Hind III	45	58	31	37	36
Eco RI/Hind III	19	17	33	26	36

The DNA probes were divided into five groups according to the hybridization pattern detected (Figure 1, Table II): I) one to four bands per lane showing no variation among the ten samples; II) more than four bands indicating polymorphism; III) repetitive sequence showing a complex restriction fragment pattern; IV) one or two bands per lane showing a very strong signal, characteristic of organelle DNA (Liou, 1990); V) could not be interpreted due to technical problems.

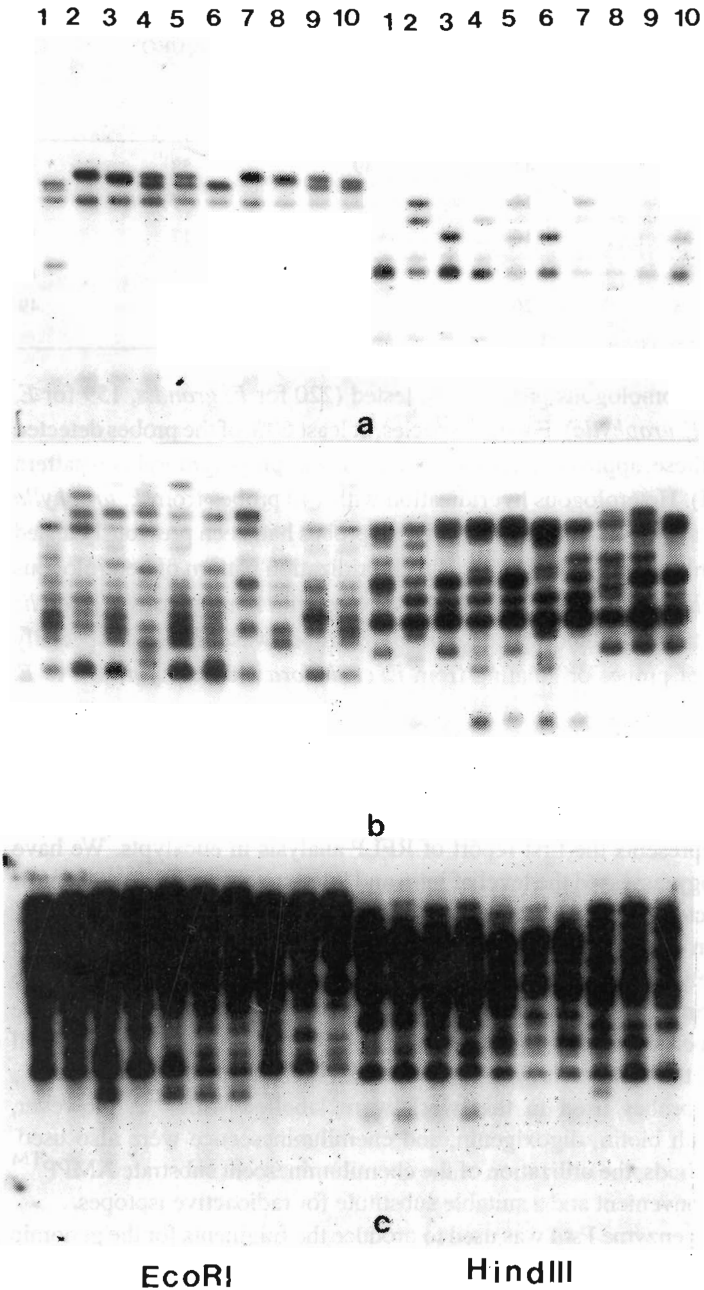

Figure 1 - Different patterns of restriction polymorphisms in a) *E. urophylla*, homologous probe of group I; b) *E. grandis*, heterologous probe of *E. urophylla* of group II; c) *E. citriodora*, homologous probe of group III. The numbers indicate ten different individuals, whose DNAs were cleaved with the restriction enzymes Eco RI or Himd III.

Table II - Distribution of the homologous probe group in *E. urophylla* (URO), *E. citriodora* (CIT), and *E. grandis*, and of the heterologous group in *E. grandis* (GRA).

Probe group	URO	CIT	GRA	GRA/URO	CIT
			(%)		
I	49	47	49	58	34
II	12	12	3	7	6
III	25	16	20	17	10
IV	6	5	-	-	10
V	8	20	18	17	49

A total of 600 homologous probes were tested (220 for *E. grandis*, 159 for *E. citriodora*, and 230 for *E. urophylla*). For each species, at least 60% of the probes detected polymorphism, and of those, approximately 80% detected a simple polymorphism pattern (type I probe) (Table II). Heterologous hybridization with 140 probes from *E. urophylla* and *E. citriodora* were also tested in *E. grandis*. These probes had been previously tested on their species and ranked in groups I and II. The hybridization pattern of heterologous probes from *E. urophylla* was very similar to that detected with homologous *E. grandis* probes, whereas the pattern obtained with *E. citriodora* probes was different, especially due the fact that 46% of probes originating from *E. citriodora* did not hybridize to *E. grandis* (Table II).

DISCUSSION

This paper represents the first report of RFLP analysis in eucalypts. We have adapted the methodology, assessed the level of intra and inter-specific polymorphism for three species, and selected probes for further research, aiming to develop a linkage map.

The extraction of clean DNA from plant tissues is usually a difficult task due to the presence of a variety of secondary metabolism compounds and carbohydrates. The abundance of terpenes, phenols and carbohydrates, some of which have physical-chemical properties similar to those of nucleic acids, may cause methodological problems. We adjusted the methodology to the work with species of *Eucalyptus*.

Most of the probes used in this work were labelled with ^{32}P. However, alternative methods with biotin, digoxigenin, and chemiluninescence were also used. Among these latter methods, the utilization of the chemiluminescent substrate AMPP^{TM} seems to be the most convenient and a suitable substitute for radioactive isotopes.

The restriction enzyme Pst I was used to produce the fragments for the genomic library. This enzyme was chosen due to its sensitivity to methylation in the 5'-cytosine

of its recognition site (5'-CTGCAG-3'). As coding regions of the genome are believed to be usually non-methylated, digestion with this enzyme was expected to reduce the number of repetitive sequences detected (Burr *et al.*, 1988; McCough *et al.*, 1988). The fact that a large number of repetitive sequences was still detected may be due to probes which were fragments of gene families commonly found in plants.

The probes used in this work have been classified into five groups according to the number of bands per lane and their frequency in ten individuals. Due to the simple pattern detected by probes from group II, these were selected for further analysis to determine whether they could be used in breeding programs. An adequate selection of parents within a highly heterologous population and early selection of offsprings are recommended. the highly polymorphic probes from group III are suitable for fingerprint analysis, and thus are of significant interest for the tracing of progenitors of elite trees.

The largely allogamous reproductive system of eucalypts is certainly a strong factor determining the high heterozygosity found within many species (Pederick, 1979; Malan, 1988; Sampson *et al.*, 1988). More than 70% of all homologous probes detected polymorphism (groups I, II, and III), in agreement with the phenotypic variability usually observed in progenies. Due to the high degree of polymorphism found within the three species, just two restriction enzymes provide enough markers for these species. However, even though only two enzymes were used, the present results indicate a lack of enzyme specificity and a frequent occurrence of polymorphic bands with both enzymes for the same probe. This suggest that their high variability may also be due to the occurrence of deletions and insertions (Liou, 1990).

The use of heterologous probes revealed a greater similarity between the genetic material of *E. grandis* and *E. urophylla* than between *E. citriodora* and *E. grandis*, for which 49% of the heterologous probes did not hybridize. The centers of origin of the two former species are far apart: *E. grandis* is native of Australia, whereas *E. urophylla* has its center of origin in Timor and Flores.

Although the primary objective of the present study was to generate genetic markers for facilitating the breeding programs for these species, the data are of broader interest and have provided some insight about the degree of DNA sequence homology among these species, and also about the nature of their high level of genetic variability.

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RESUMO

Foi efetuada uma análise do polimorfismo de comprimento de fragmentos de DNA, clivado com enzimas de restrição, para avaliar o nível de variabilidade genética dentro de três espécies de eucalipto (*Eucalyptus citriodora, E. grandis* e *E. urophylla*) e selecionados marcadores genéticos para estabelecimento de mapa de ligação. Sondas homólogas e heterólogas foram hibridizadas com o DNA total, digerido com as enzimas de restrição Eco RI e Hind III. Foi detectado elevado polimorfismo tanto inter-específico como intra-específico.

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