Theor Appl Genet (1994) 89: 442–450

M. Keil · A. R. Griffin

Use of random amplified polymorphic DNA (RAPD) markers in the discrimination and verification of genotypes in *Eucalyptus*

Received: 27 January 1994 / Accepted: 8 March 1994

Abstract We carried out four separate studies using random amplified polymorphic DNA (RAPD) markers to analyse samples of *Eucalyptus* supplied by several different organisations. The objective was to examine the reproducibility of the RAPD technique and its ability to discriminate between individual genotypes for verification of clonal identities. We found that RAPD profiles that are unique to a genotype can be generated reliably and simply and that even closely related genotypes can be distinguished. In addition, in each of the four studies, we detected cases where the plant material studied had been mis-sampled or mislabelled (i.e. the RAPD profiles were not consistent with the identification numbers): (1) ramets of a *Eucalyptus grandis* clone were found to be derived from 2 different clones; (2) ramets labelled as 2 different *Eucalyptus* hybrid clones were found to be the same clone, owing to a mis-planted clonal hedge; (3) samples supplied as a single progeny of a controlled *E. nitens* cross were derived from two crosses involving different pairs of parents; (4) mislabelling was detected for ramets of 4 of a set of 10 clones of *E. grandis* and *E. camaldulensis*. For three of the four studies, the detection of genotype mis-identifications was unexpected, suggesting that labelling or sampling errors during the handling of plant material are a frequent occurrence, with potentially serious economic consequences.

Key words *Eucalyptus* · RAPD markers
DNA fingerprinting · Genotype mis-identifications
Breeding

Communicated by P. M. A. Tigerstedt

M. Keil (✉)
Shell Research Limited, Sittingbourne Research Centre,
Sittingbourne, Kent, ME9 8AG, UK

A. R. Griffin
Shell International Petroleum Company Limited,
Shell Centre, London, SE1 7NA, UK

Introduction

A growing proportion of the world's demand for wood or wood products is being met from tree plantations. Species of the genus *Eucalyptus* have become the most widely planted hardwoods in the world over the past 20 years, and major efforts are now directed towards the improvement of the planting stock via breeding and the selection and clonal propagation of elite genotypes (Eldridge et al. 1993). Realisation of the benefits resulting from such improvements will be impeded if errors occur during the sampling or labelling of plant material. Such errors are often difficult to detect by visual inspection of the plants. In recent years, isozyme markers have been applied increasingly to horticultural, fruit and forest trees to detect errors more reliably (Adams 1983; Menendez et al. 1986; Santi and Lemoine 1990; Tobolski and Kemery 1992; Wheeler and Jech 1992). From such studies, it appears that mis-labelling of clones may be common.

A disadvantage of isozyme markers is that they may be affected by environmental conditions and different stages of development (Kuhns and Fretz 1978; Falkenhagen 1985). In addition, the number of isozyme loci that can be analysed is limited, and discrimination of different genotypes is not always possible (Adams and Joly 1980; Eckert et al. 1981; Santi and Lemoine 1990; Tobolski and Kemery 1992). DNA-based markers have largely overcome these disadvantages and have been applied successfully to discriminate between individual genotypes in a wide range of plant and animal species (Epplen et al. 1991; Nybom 1991; Weising et al. 1991). The discrimination of individual genotypes by DNA markers is often referred to as "DNA fingerprinting".

Recently, a novel DNA-marker system, the random amplified polymorphic DNA (RAPD) technique, was developed (Williams et al. 1990). This technique, which is based on the use of short primers of arbitrary nucleotide sequence in the polymerase chain reaction (PCR), has a number of advantages over other DNA-based marker systems (reviewed by Rafalski and Tingey 1993). The RAPD tech-

nique and modifications thereof have been shown to be useful for a wide range of applications (Waugh and Powell 1992, Williams et al. 1993), including the DNA fingerprinting of plants and animals (Welsh et al. 1991; Caetano-Anollés et al. 1991; Hu and Quiros 1991; Wilde et al. 1992; Kaemmer et al. 1992; Torres et al. 1993). These studies demonstrate that it is possible to obtain RAPD profiles that are reproducible and unique to different genotypes, but no reports specifically address the use of RAPD markers for DNA fingerprinting in *Eucalyptus*.

We examined four independent sets of *Eucalyptus* germ plasm with the following specific objectives:

(1) to examine the reproducibility of RAPD profiles in samples of the same genotype, including samples of different developmental stages,

(2) to examine whether the amount of variation between the RAPD profiles of different genotypes is sufficient for their discrimination and

(3) to fingerprint a number of clones whose identity was reputedly uncertain.

Materials and methods

Plant material

The plant material analysed was supplied by several organisations, designated Organisations A through F. The plant material comprised leaves collected from a range of *Eucalyptus* clones and a putative full-sib family. When clones were analysed, between 1 and 8 ramets[1] per clone were studied. For some clones, the ortets were also analysed. After collection, the leaves were packed in polythene bags and transported to the UK by air mail, courier or as checked baggage, in a domestic styrofoam-lined picnic box containing domestic freezer blocks. The freezer blocks were separated from the leaves by layers of newspaper for insulation. After phytosanitary inspection at Sittingbourne, the leaves were wrapped in aluminium foil and frozen in liquid nitrogen until required. Leaves collected from plants grown at Sittingbourne were frozen directly after collection. Details of the plant material are given in Table 1.

DNA isolation

Approximately 300 mg of leaf material of each sample was ground in liquid nitrogen to a fine powder using pestle and mortar or a freezer mill (Retsch Micro Mixer Mill MM2). After addition of 1 ml of DNA extraction buffer [500 m*M* NaCl, 100 m*M* TRIS-HCl (pH 8.0), 50 m*M* ethylene diamine tetraacetic acid (EDTA), 1% (w/v) polyvinylpyrrolidone, 1% (w/v) sodium dodecyl sulphate (SDS)] the samples were incubated at 60 °C for 15 min. After centrifugation for 5 min in a microcentrifuge at approximately 13,000 *g* (all subsequent centrifugation steps were carried out in a microcentrifuge at the same centrifugal force), the supernatant was extracted once with 0.5 ml phenol/chloroform/isoamylalcohol (25 : 24 : 1, v : v : v). After a second centrifugation for 5 min, the aqueous phase was transferred into a new microcentrifuge tube, and the nucleic acids precipitated by adding 600 µl of icecold isopropanol. The samples were incubated at –20 °C for 30 min and then centrifuged for 10 min. The supernatant was discarded, and the nucleic acid pellet dissolved in 300 µl TE-buffer (Sambrook et al. 1989). For further purification of the DNA, this solution was mixed with 300 µl of CTAB buffer 1 [2% (w/v) cetyl trimethyl ammonium bromide (CTAB), 100 m*M* TRIS-HCl (pH 8.0), 20 m*M* EDTA, 1.4 *M* NaCl] and then extracted with 600 µl of chloroform/isoamylalcohol (24:1, v:v). After centrifugation for 5 min, the aqueous phase was transferred into a new microcentrifuge tube, and the nucleic acids precipitated by the addition of CTAB buffer 2 [1% (w/v) CTAB, 50 m*M* TRIS-HCl (pH 8.0), 10 m*M* EDTA]. After 30 min at room temperature, the nucleic acid precipitate was collected by centrifugation for 10 min. The supernatant was discarded, and the pellet dissolved in 300 µl of a 1 *M* cesium chloride solution containing 10 µg/ml Ribonuclease A. After incubation at 37 °C for 30 min, the DNA was precipitated again by the addition of 600 µl absolute ethanol. The DNA precipitate was collected by centrifugation for 10 min, the supernatant discarded and the pellet washed in 500 µl 70% ethanol. After re-centrifugation, the DNA pellet was finally dissolved in 100 µl TE. The quality of the DNA was assessed by agarose gel electrophoresis, and the quantity determined by measuring the optical density at a wavelength of 260 nm.

Generation of RAPD profiles

RAPD reactions were performed in a volume of 25 µl containing 1 unit of *Taq* DNA Polymerase, 2.5 µl of 10× concentrated reaction buffer (both supplied by Northumbria Biologicals, Cramlington, UK), 0.1 m*M* of each dATP, dGTP, dTTP and dCTP, 400 n*M* primer and 25 ng genomic eucalyptus DNA. The primers were obtained from Operon Technologies (Alameda, Calif.; primer kits A and B) or synthesised at Sittingbourne using an Applied Biosystems DNA synthesiser (model 381A; primers RAPD-01 to RAPD-06). The sequences of primers RAPD-01 to RAPD-06 were (in 5′ to 3′-direction): GGATCTCGAC (RAPD-01), GCGTTCCATG (RAPD-02), CAAGCCAGGA (RAPD-03), GGATCTCGA (RAPD-04), CAGTTGCGA (RAPD-05), ATCGGAAGG (RAPD-06). The final concentration of $MgCl_2$ in each reaction was 1.5 m*M*. Genomic DNA was omitted in control reactions to determine whether any of the bands seen with genomic DNA were due to contamination. Each reaction was overlaid with an equal volume (25 µl) of mineral oil. The samples were subjected to a total of 45 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C, using the fastest possible temperature transitions. The RAPD reactions were performed in either a Perkin Elmer Cetus DNA thermal cycler or a Biometra Trio thermal cycler (we generally do not observe any differences in RAPD profiles when comparing the two cyclers using the same DNA samples). After completion of the thermal cycles, 10 µl of type III gel loading buffer (Sambrook et al. 1989) was added to each sample. Samples of 15 µl were then loaded onto 1.5% agarose gels. Electrophoresis was carried out at 7.5 V/cm for 2.5 h in half concentrated TBE buffer (Sambrook et al. 1989) containing ethidium bromide (0.3 µg/ml). Afterwards, the gels were photographed on an ultraviolet transilluminator, using Polaroid 667 film.

Analysis of RAPD data

The presence or absence of RAPD bands was scored by visual inspection of the gel photographs from at least two independent experiments. The data were transformed into a 1 (present) or 0 (absent) matrix over all genotypes and fragment positions scored. Genetic distance values were calculated on the basis of the Jaccard coefficient (Anderberg 1973) using the formula: $D_{ij}=1-(B_{ij}/M_{ij})$. D_{ij} is the distance between genotypes i and j, B_{ij} is the number of bands common to i and j and M_{ij} is the total number of bands scored in i and j. A genetic distance value of 0 indicates that no differences in the RAPD profiles were observed. A genetic distance value of 1 indicates that no bands were shared between 2 RAPD profiles. Cluster analysis was carried out using Ward's minimum variance analysis (Ward 1963) provided by the CLUSTER procedure of the SAS statistical analysis software (SAS Institute Inc. 1989).

[1] The original stockplant of a clone is called 'ortet', whereas the plants derived from cuttings that are taken from the ortet are referred to as 'ramets'

Table 1 List of plant material analysed

Study 1 Leaf samples obtained from cuttings of *E. grandis* clones raised at Sittingbourne

Clonal I.D. number	Number of ramets	Origin	Supplying organisation	Country of origin	Number of primers used
1	4 (nos. 1.1–1.4)	Clonal hedge [a]	A	South Africa	20
1	2 (nos. 1.5–1.6)	Coppice regrowth [b]	B	South Africa	20
2	6 (nos. 2.1–2.6)	Clonal hedge [a]	A	South Africa	10
2	2 (nos. 2.7–2.8)	Coppice regrowth [b]	B	South Africa	10
3	1	Clonal hedge [a]	A	South Africa	9
4	1	Clonal hedge [a]	A	South Africa	9

[a] Small coppice shoots [b] Crown of 2- to 4-year-old coppice regrowth

Study 2 Leaf samples from 10 hybrid clones, sampled directly in the field

Clonal I.D. number	Variety [a]	Origin	Number of ramets	Supplying organisation	Country of origin	Number of primers used
5–8	PF1	Clonal hedge	1/clone	C	Congo	36
9–11	Uro-grandis	Clonal hedge	1/clone	C	Congo	36
12–14	HS2	Clonal hedge	1/clone	C	Congo	36
13 [b]	HS2	5 clonal hedges 13A–13E	3/clonal hedge	C	Congo	10
14 [b]	HS2	2 clonal hedges 14A–14B	3/clonal hedge	C	Congo	10

[a] The varieties are derived from the following hybrids: *E. alba* × *E. urophylla* (PF1); *E. urophylla* × *E. grandis* (uro-grandis); *E. tereticornis* × *E. grandis* (HS2)
[b] Repeated sampling

Study 3 Leaf samples obtained from 19 individuals of a putative full-sib family of *E. nitens* plus their parents

I.D. number	Origin	Supplying organisation	Country of origin	Number of primers used
M	Male parent ortet	D	Australia	20
F	Female parent ortet	D	Australia	20
1–19	Ortets from progeny trial	D	Australia	20

Study 4 Leaf samples obtained from 10 clones of *E. grandis* and *E. camaldulensis*.

a) Samples analysed of the 10 clones

Clonal I.D. number	Species	Affix of samples analysed	Number of primers used
15	*E. grandis*	O; E; 1; 2; 3	20
16	*E. grandis*	E	43
17	*E. grandis*	O; E; 1; 2; 3	20 (O: 43)
18	*E. grandis*	O; E; 1; 2; 3	20
19	*E. grandis*	O; E; 1; 2; 3	20 (O: 43)
20	*E. camaldulensis*	E	43
21	*E. camaldulensis*	E	43
22	? [a]	E	20
23	*E. camaldulensis*	E	20
24	*E. grandis*	O; 1; 2; 3	20

[a] The exact genetic background of this clone is not known to us, but the morphology of the leaves resembles that of *E. saligna* or *E. tereticornis*

b) Cultural history of the samples taken for each of the clones nos. 15–24

Sample number (affix)	Origin	Supplying organisation	Country of origin
O	Field-grown ortet	F	USA
E	Ex-tissue culture-ramet	E	USA
1	Field-grown ramet of 1st propagation cycle [a]	F	USA
2	Field-grown ramet of 2nd propagation cycle [a]	F	USA
3	Field-grown ex-tissue culture-ramet	F (originally E)	USA

[a] Ramets of the first propagation cycle are derived from the ortet, ramets of the second propagation cycle are derived from the first propagation cycle ramets

Results

Study 1

To study the reproducibility of RAPD markers, we compared the RAPD profiles obtained from multiple ramets of each of 2 *E. grandis* clones. Figure 1a shows the results obtained with 5 primers for 6 ramets of clone no. 1. The RAPD profiles of the 6 ramets were indistinguishable, using a total of 20 primers. Indistinguishable profiles were also obtained for 6 ramets of clone no. 2, using a total of 10 different primers. However, for 2 additional ramets of this clone, all primers gave different RAPD profiles. Results from clone no. 2, obtained using 4 primers, are shown in Fig. 1b.

The different RAPD profiles obtained for ramets of clone no. 2 were not a result of the different developmental stage of the plant material from which these ramets originated (small coppice shoots vs. crown shoots of 2- to 4-year-old trees; see Table 1: No difference in the RAPD profiles were observed for ramets of clone no. 1 that originated from comparable material as the ramets of clone no. 2 (Fig. 1a). In addition, we compared the RAPD profiles of leaves of juvenile and mature morphology that had been collected from each of three *E. nitens* trees and one *E. globulus* tree that displayed leaves of both morphologies. We could not detect significant differences when the RAPD profiles of leaves of the two different developmental stages, collected from the same genotype, were compared (data not shown).

The differences observed among the RAPD profiles of the ramets of clone no. 2 (ramets nos. 2.1–2.6 vs. nos. 2.7–2.8) translated into a genetic distance value of 0.410 (Table 2). We concluded that the ramets labelled as clone 2 are derived from more than 1 ortet.

Fig. 1a, b RAPD profiles of ramets of *Eucalyptus grandis* clones nos. 1 and 2. The primers that were used to obtain the profiles shown are indicated at the *top* of the photographs. The size of selected DNA fragments of a size standard (*Pst*I- digested phage Lambda DNA) is indicated in basepairs (bp). **a** Clone no. 1: ramets nos. 1.1 (*1*), 1.2 (*2*), 1.3 (*3*), 1.4 (*4*), 1.5 (*5*), 1.6 (*6*). No reproducible differences were observed between the RAPD profiles of these ramets. **b** Clone no. 2: ramets nos. 2.1 (*1*), 2.2 (*2*), 2.3 (*3*), 2.4 (*4*), 2.5 (*5*), 2.6 (*6*), 2.7 (*7*), 2.8 (*8*). In the case of primer A-06, only the result obtained with ramets 2.5–2.8 is shown. Note differences in the RAPD profiles of ramets nos. 2.1–2.6 compared to ramets nos. 2.7–2.8, indicated by *asterisks*. The ramets nos. 1.5 and 1.6 (**a**) and nos. 2.7 and 2.8 (**b**) originated from crown shoots of 2- to 4-year old trees, whereas all other ramets originated from small coppice shoots

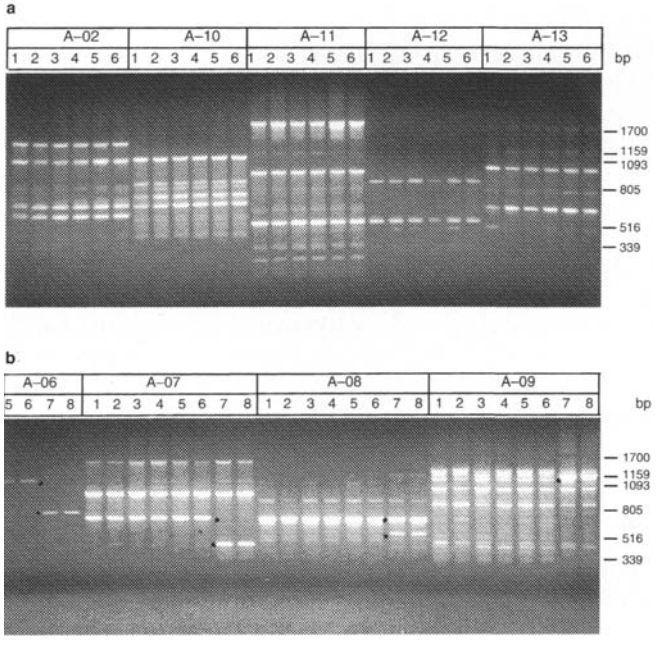

Table 2 Genetic distance values obtained for comparisons between ramets of *E. grandis* clone no. 2 and 2 unrelated *E. grandis* clones, nos. 3 and 4

	2.1[a]	2.7[b]	3	4
2.1[a]	0.00	0.41	0.48	0.37
2.7[b]		0.00	0.45	0.54
3			0.00	0.44
4				0.00

[a] Representative for ramets nos. 2.1–2.6
[b] Representative for ramets nos. 2.7 and 2.8

Study 2

To investigate in more detail whether we could generate RAPD profiles unique to different *Eucalyptus* clones, one sample of each of 10 hybrid clones was analysed using a total of 36 different RAPD primers (Table 1). An example of the RAPD profiles, obtained using primer A-08, is shown in Fig. 2. The genetic distance values, calculated for each pairwise comparison, ranged from 0.24 to 0.71 for 8 of the 10 clones analysed. Thus, the variation of the RAPD profiles was sufficiently high to allow the distinction of 8 of the 10 clones. In addition, when further processed using cluster analysis, the genetic distance values reflected the genetic background (three hybrid taxa) of the clones analysed (Fig. 3).

Of the 36 primers used, 8 revealed at least one polymorphism for each pairwise comparison of the 8 clones. Each 1 of these primers would therefore be sufficient for the discrimination between the 8 clones (e.g. primer A-08; see Fig. 2). Of the other 28 primers, combinations of 4 or 5 primers (in individual reactions) would be required for the discrimination of the 8 clones.

The RAPD profiles of 2 samples (clones nos. 13 and no. 14) were indistinguishable. There are two possible explanations for this result: (1) RAPD profiles unique to individual genotypes cannot always be obtained or (2) the samples analysed of the 2 clones were of the same clonal identity and, hence, were mis-labelled. To investigate the second possibility, we analysed additional samples of clones nos. 13 and 14 that were collected from several clonal hedges (Table 1). For 2 of the 10 primers used (A-06 and B-14), no differences could be observed in the resulting

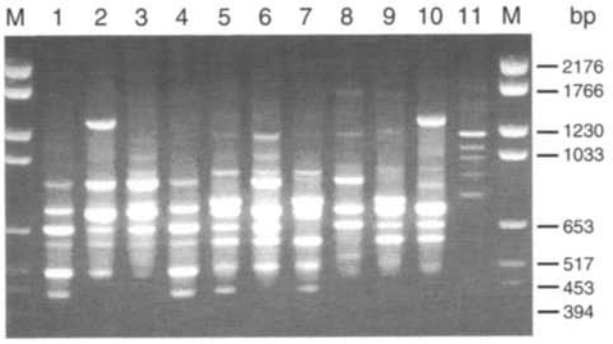

Fig. 2 RAPD profiles of ramets supplied as 10 different eucalyptus hybrid clones: nos. 14 (*lane 1*), 8 (*lane 2*), 5 (*lane 3*), 13 (*lane 4*), 10 (*lane 5*), 6 (*lane 6*), 11 (*lane 7*), 12 (*lane 8*), 9 (*lane 9*), and 7 (*lane 10*). The profiles shown were obtained using the primer A-08. *Lane 11* contains the amplification products of a control reaction to which no template DNA was added. The size of DNA fragments of a size standard (molecular weight marker VI, Boehringer Mannheim; *lane M*) is indicated in basepairs (bp). Note that the samples supplied as clones nos. 13 and 14 (*lanes 1* and *4*, respectively) cannot be distinguished. The other eight samples can be distinguished using this primer

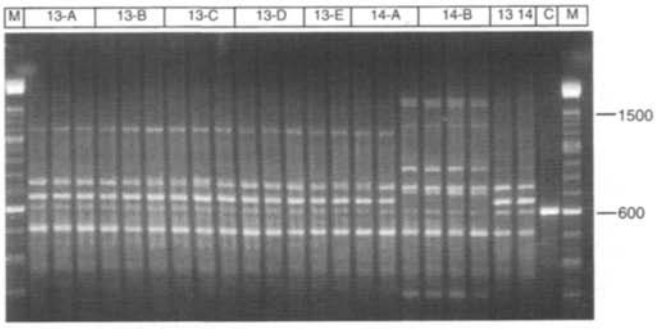

Fig. 4 RAPD profiles of multiple ramets of clones nos. 13 and 14. Samples of the ramets were collected from clonal hedges 13-A to 13-E (clone no. 13), and 14-A to 14-B (clone no. 14). The lanes designated *13* and *14* contain the RAPD profiles of the samples supplied as clones nos. 13 and 14, respectively, that were analysed initially and found to be indistinguishable. *Lane C* contains the amplification products of a control reaction to which no template DNA was added. The RAPD profiles shown were obtained using the primer A-08. Note the mixture of RAPD profiles for the samples collected from the clonal hedge 14-A. DNA size standard (*lane M*): 100-bp ladder (Gibco/BRL); the 1500-bp and 600-bp bands are indicated

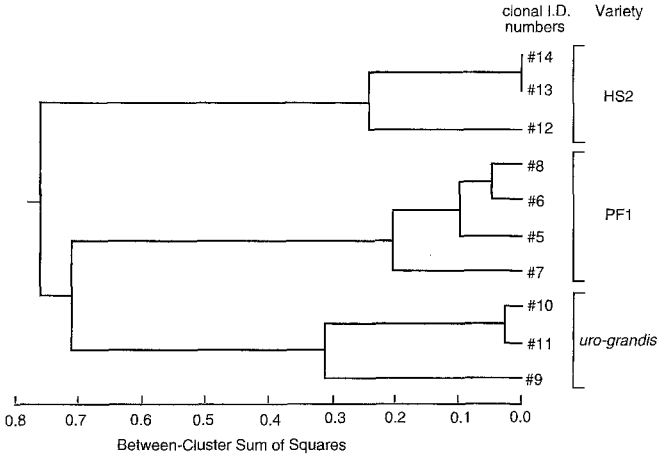

Fig. 3 Dendrogram showing the genetic relationship, determined using cluster analysis of genetic distance values, of samples supplied as 10 eucalyptus hybrid clones (clones nos. 5–14). The results are based on RAPD profiles obtained using a total of 36 primers. The dendrogram accurately reflects the genetic background of the samples analysed, at the taxon level. In addition, the dendrogram shows that the samples labelled as clones nos. 5–12 could be distinguished from each other, whereas discrimination between the samples labelled as clones 13 and 14 was not possible.

RAPD profiles. For the other 8 primers, two different classes of RAPD profiles were obtained. One class was representative of all 15 samples labelled as clone no. 13 and 2 samples labelled as clone no. 14. The other class of RAPD profiles was representative of 4 further samples labelled as clone no. 14 (Fig. 4). The comparison of the two classes of RAPD profiles resulted in a genetic distance value of 0.30.

These data suggest that the two classes of RAPD profiles are representative of the 2 clones nos. 13 and 14. The fact that 2 samples labelled as clone no. 14 have the same RAPD profiles as all samples labelled as clone no. 13 indicates that these two samples are derived from clone no. 13. The 2 samples of mistaken clonal identity were collected from the clonal hedge 14-A. The third sample that was collected from this clonal hedge had the same RAPD profiles as the 3 samples of clone no. 14 that were collected from hedge 14-B (Fig. 4). It appears that the clonal hedge 14-A, which should contain only trees of clone no. 14, in fact contains a mixture of both clones, nos. 13 and 14, and therefore was mis-planted. The sample labelled as clone no. 14 that was analysed initially and found to be indistinguishable from the sample labelled as clone no. 13 was collected from the same clonal hedge (14-A).

Study 3

The results described above demonstrate that *Eucalyptus* clones that presumably are not closely related can be distinguished using the RAPD technique. To examine whether closely related genotypes can also be distinguished, we analysed samples of 19 individuals of the progeny and both parents of a controlled cross of *E. nitens*. For this analysis, we selected 20 primers that revealed polymorphic RAPD profiles of the parents. The analysis of the RAPD profiles obtained for this family revealed two interesting features. First, 26% of all 105 bands scored in the RAPD profiles of this family deviated significantly from the expected segregation ratios (data not shown). Second, a further 29% of all bands were present in part of the progeny but could not be detected in either parent (non-parental bands) (Fig. 5). Only the RAPD profiles obtained with 1 primer (B-14) did not contain any non-parental bands. The non-parental bands occurred in a subset of 10 individuals of the progeny. Southern blot analyses using 2 of the non-parental bands as DNA probes revealed that these bands were derived from repetitive plant DNA sequences (data

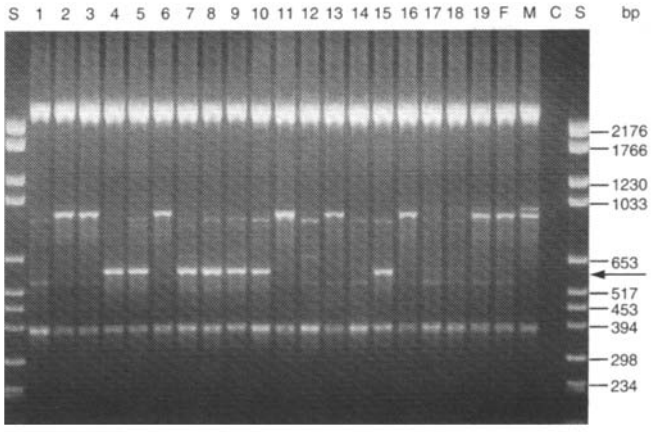

Fig. 5 RAPD profiles of samples supplied as 19 individuals of the progeny (*lanes 1–19*) and both female (*F*) and male (*M*) parents of a controlled cross of *Eucalyptus nitens*. The RAPD profiles shown were obtained using the primer B-01. *Lane C* contains the amplification products of a control reaction to which no template DNA was added. The sizes of DNA fragments of a size standard (*lane S*; molecular weight marker VI, Boehringer Mannheim) are indicated in basepairs (bp). The *arrow* indicates a band that is present in a subset of the progeny samples but that cannot be detected in either parent (non-parental band)

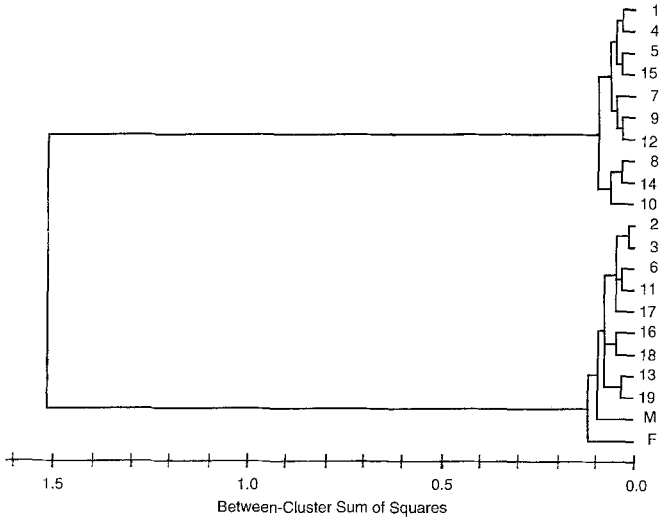

Fig. 6 Dendrogram showing the genetic relationship of samples supplied as 19 individuals of the progeny (*1–19*) and both female (*F*) and male (*M*) parents of a controlled cross of *Eucalyptus nitens*. The dendrogram was obtained by cluster analysis of genetic distance values calculated from RAPD data obtained by using 20 different RAPD primers. The dendrogram clearly shows that a subset of the samples supplied as the progeny is genetically distinct from all other samples, including those supplied as the parents

not shown). This demonstrates that the non-parental bands are not a result of microbial contamination.

We calculated genetic distance values for all samples analysed and used these to perform cluster analysis. A dendrogram of the relative genetic relationships of the samples is shown in Fig. 6. The dendrogram reveals a clear separation of the samples into two genetically distinct groups of individuals. Within each group, the individuals are highly related to each other. According to the dendrogram, one of the groups is genetically distinct from both parents. These results indicate that the samples analysed represent 2 progenies from two different crosses. Examination of the genetic distance values within the 2 progenies showed that all individuals could be distinguished from each other: the genetic distance values within each of the 2 progenies ranged from 0.11 to 0.39. The lowest genetic distance value of 0.11 was obtained for the comparison of progeny samples nos. 2 and 3: out of a total of 67 bands scored in the RAPD profiles of these 2 samples, 7 were not shared between them. These differences were revealed by 7 of the 20 primers used. The comparison of individuals from 1 progeny with those of the other progeny and its parents resulted in genetic distance values ranging from 0.51 to 0.71. The comparison of one parent with the other resulted in a genetic distance value of 0.54.

Study 4

Recently, a Shell-affiliated company purchased micropropagated ramets of 10 *Eucalyptus* clones from a commercial laboratory (Organisation E). Some doubts existed regarding the true clonal identity of this plant material. For a number of the clones (Table 1) we were able to obtain material from the ortets together with samples from field-grown first and second cycle macro-propagated ramets, and ramets that had originated from earlier tissue culture by Organisation E. All this supplementary material was obtained from an independent organisation (F). Twenty different RAPD primers were used for this analysis; where differences between RAPD profiles were expected but not detected using 20 primers, the analysis was extended to a total of 43 RAPD primers. A representative result, obtained by using the primer A-07, is shown in Fig. 7.

The following observations were made (summarised in Table 3):

(1) Within each of the clones nos. 17, 18, 19 and 24, no reproducible differences were detected between the RAPD

Fig. 7 RAPD profiles of samples supplied as ortets and ramets of 10 clones (nos. 15–24) of *Eucalyptus grandis* and *E. camaldulensis*. The labels of the plant material, as supplied to us (Table 1), are indicated at the *top* of the photograph. The RAPD profiles shown were obtained using primer A-07. DNA size standard: 100-bp ladder (*lane M*; Gibco/BRL); the positions of the 600-bp and 1500-bp fragments are indicated. *Lane C* amplification products of a control reaction to which no template DNA was added

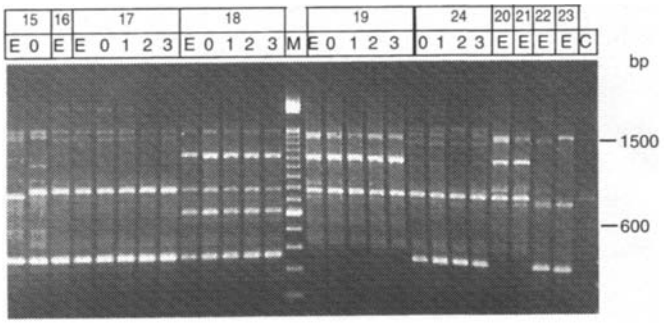

Table 3 True clonal identities of ramets labelled as clones nos. 15–24, as determined by RAPD analysis

Ramet I.D. numbers[a]	True clonal identity
15/E	Unkown[b]
16/E	Clone no. 17
17/E	Clone no. 17
18/E	Clone no. 18
19/E	Clone no. 19
20/E	Clone no. 19 (?)[c]
21/E	Clone no. 19 (?)[c]
22/E	Clone no. 22 (?)[d]
23/E	Clone no. 23 (?)[d]
24/E	n.d.

[a] I.D. numbers as described in Table 1. Only ramets purchased from Organisation E are listed; all ramets obtained from Organisation F had RAPD profiles indistinguishable from the ortets with the same I.D. number

[b] RAPD profiles differed from those of all other samples analysed

[c] RAPD profiles almost indistinguishable from those of clone no. 19

[d] No comparison made to ortet of this clone; clonal identity therefore not verified

profiles of all samples analysed, including those of the ortets. This indicates that the samples were labelled correctly.

(2) The RAPD profiles of the samples nos. 15/0 (ortet of clone no. 15) and 15/E (purchased ramets of clone no. 15) differed for 18 of the 20 primers used. A genetic distance value of 0.59 was calculated for the 2 samples. This indicates that the ramets that were purchased as clone no. 15 were derived from a different clone of unknown origin.

(3) No reproducible differences could be detected between the RAPD profiles of the sample no. 16/E and the profiles of all samples of clone no. 17 (including the ortet-sample of this clone) using 43 RAPD primers. We concluded that the clonal identity of the ramets purchased as clone no. 16 is that of clone no. 17.

(4) The RAPD profiles of the samples nos. 20/E and 21/E, obtained using 43 primers, were almost indistinguishable from those of all samples of clone no. 19. We obtained genetic distance values of only 0.005 (no. 20/E vs. no. 21/E), 0.018 (no. 21/E vs. no. 19), and 0.023 (no. 20/E vs. no. 19). These values are considerably lower than those that we obtained for full-siblings in *E. nitens*. Since clones nos. 19, 20 and 21 are not related to each other, we concluded that samples nos. 20/E and 21/E were mis-labelled.

(5) The RAPD profiles of the 2 samples nos. 22/E and 23/E were different from those of all other samples analysed, as would be expected for different, unrelated clones. The genetic distance values of each pairwise comparison ranged from 0.59 to 0.72. As we did not analyse reference ortet samples for these clones, we are unable to determine whether they are correctly identified.

Discussion

A number of different approaches for using primers of arbitrary sequence in the polymerase chain reaction have been developed with a view to analyse genetic variation (Williams et al. 1990; Welsh et al. 1991; Caetano-Anollés et al. 1991). We have evaluated the random amplified polymorphic DNA (RAPD) approach (Williams et al. 1990), which usually employs primers ten nucleotides in length, for DNA fingerprinting of *Eucalyptus*.

High reproducibility is an essential requirement for the suitability of a marker system for genetic fingerprinting. In our experience, RAPD markers generally fulfil this requirement. Repeated experiments using the same DNA samples show very little or no variation. In addition, our results show that indistinguishable DNA profiles can be obtained for different ramets of the same clone. In our experience, a consistent and high quality of DNA is essential for reproducibility. In this respect, our DNA isolation protocol gave superior results compared to other, published procedures.

With an average of 4–5 bands per primer, we obtained less complex DNA profiles using the RAPD approach compared to the other approaches employing random primers where profiles consisting of over 100 bands have been observed (Caetano-Anollés et al. 1991). Less complex DNA profiles significantly facilitate the scoring of individual bands in the profiles. Despite the presence of fewer bands per profile, our results show that the variation of RAPD profiles between individual *Eucalyptus* genotypes is sufficiently high to allow them to be distinguished. Even when highly related *Eucalyptus* genotypes (full-siblings) were compared, genetic distance values ranging from 0.11 to 0.39 were obtained, indicating that all full-siblings analysed could be distinguished from each other. However, more extensive studies analysing full-siblings from a wider range of crosses would be needed to establish whether highly related *Eucalyptus* genotypes can always be distinguished with confidence using RAPD markers. For other samples, genetic distance values up to 0.71 were obtained, indicating that the respective genotypes compared were relatively unrelated. The genetic distance values appear to correctly reflect the genetic background of the samples analysed, as demonstrated by our results obtained using cluster analysis.

In one of the four studies that we carried out, anecdotal reports and ramet morphology led us to suspect that the attributed clonal identity of some of the plant material analysed was incorrect. Our findings that ramets of 4 of 10 clones obtained from Organisation E were mis-labelled confirmed these suspicions. For the samples analysed of 2 of the clones (nos. 16 and 17), we could not detect any differences in the RAPD profiles obtained using a total of 43 primers. Clone no. 16 was supposed to be derived from open-pollinated progeny of clone no. 17. In view of the high genetic heterogeneity of eucalyptus, and the fact that we could distinguish full-siblings, the chances that the 2 clones share the same 43 RAPD profiles are extremely re-

mote. It is therefore reasonable to conclude that the 2 samples analysed are derived from the same clone. The samples analysed of 3 additional clones (nos. 19/O, 20/E, 21/E) were found to be almost indistinguishable, although they were expected to be unrelated genotypes. It cannot be excluded that 2 samples highly related to clone no. 19 were mistaken as clones nos. 20 and 21. However, it is more likely that samples of clone no. 19 were mis-labelled as clone nos. 20 and 21. As the samples analysed of clones nos. 20 and 21 were derived from micro-propagated plants, the differences observed between these samples and clone no. 19 could be a result of somaclonal variation. It has been shown previously that somaclonal variation can be detected by RAPD markers (Brown et al. 1993).

We also detected cases of genotype mis-identifications in each of the other three studies, where we did not expect to find such errors. In one of these studies, we detected non-parental bands at a high frequency in samples supplied as the progeny of a controlled *E. nitens* cross. The occurrence of non-parental RAPD bands in progenies, at varying frequencies, has been reported before (Riedy et al. 1992; Pellissier Scott et al. 1992). Mutations, contamination and PCR artefacts have been suggested as possible reasons for the occurrence of these bands. In our study, however, the occurrence of non-parental bands almost certainly is a result of mis-identification of two progenies, as demonstrated by cluster analysis.

A survey of controlled crosses of Douglas-fir and loblolly pine by Adams et al. (1988) using isozymes led them to the conclusion that errors had occurred in a high proportion of the crosses. In most cases, the errors were a result of pollen-contamination or the application of mis-labelled pollen. This can be excluded as the reason for the error in the *E. nitens* cross that we have analysed, as the resulting progenies would be expected to be genetically similar to the female parent. Our results indicate that one subset of the progeny was genetically distinct from both the male and female parents, which would be expected if errors occurred during the labelling or handling of seed or seedlings, leading to mis-planted progeny stands. Alternatively, an error could have occurred during the collection of plant material for this study.

Mis-planting or mis-labelling of clones appears to be a common occurrence in forestry operations (Adams 1983; Harju and Muona 1989; Wheeler and Jech 1992). Any errors in the identification of genotypes are clearly undesirable, but economic impact will depend upon where these occur within the cycle of breeding and benefit capture. Errors in the assumed pedigree, or family structure, of progenies could bias estimates of genetic parameters and of breeding values. However, unless extremely frequent, they are unlikely to have a substantial effect on the gain realised in the plantation crop.

In the select production population, the risk from errors in the identification of any one genotype will be proportional to its contribution to the operational planting stock. A mis-labelled ramet may be inconsequential if it is planted in a clonal seed orchard, but be the cause of major loss if used as the source of mother plant stock in a clonal programme. Many companies planting eucalyptus clones now use less than 10 clones in any one season, and before long such clones will gain added value through the application of genetic modification technology; the implications are obvious.

It is recommended that the ortet of each select clone be fingerprinted and that this reference library be used in conjunction with an ongoing sampling programme for quality control in the plant production system.

Acknowledgements We would like to thank Organisations A to F for supplying us with the plant material used in these studies. We gratefully acknowledge the assistance of Dr. C. Burridge and Mrs. M. Smart in setting up the SAS software for cluster analysis. We also thank Dr. J. Purse for his critical comments and suggestions during the preparation of this manuscript. The plant material was imported into the UK under permission granted by the Ministry of Agriculture, Fisheries and Food [import license numbers PHF80D/29(110) and PHF80D/59(91)].

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