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Use of genetic markers in the management of micropropagated *Eucalyptus* germplasm

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Abstract. Clonal propagation through rooted cuttings and micropropagation is widely used for large-scale *Eucalyptus* plantation program because of its ability to fix the desirable traits of mature plus trees. However, when a large number of clones are handled, variations and mixings are commonly confronted which may go undetected in the absence of prominent morphological descriptors. Material from germplasm resources are also used in breeding program. The economic implications of such inadvertent variations could be serious as considerable time and money is spent before the mistakes are detected. This paper reports the identification of mislabeling in *Eucalyptus* clones maintained through tissue culture, and the reestablishment of the identity of the mislabeled clones using genetic markers viz., RAPDs/AFLPs. The *in vitro* propagated *Eucalyptus* plants from two groups, group 1 derived directly from SMD7 (a candidate plus tree), and group 2 derived from coppice shoots of trees of group 1, were assessed for their genetic uniformity using RAPD markers. The small intra-group genetic variations of 0.02 in the second group were attributed to somaclonal variations induced during long culture periods. However, the genetic distances of 0.20 and 0.31 between SMD7 and the two micropropagated groups were too high to be attributed to somaclonal variations as axillary bud culture was used for micropropagation. To test the possibility of inadvertent mixing, RAPD profiles of the micropropagated groups were compared with that of other clones in the tissue-cultured *Eucalyptus* germplasm. The RAPD profiles of group 2 plantlets matched with that of another unrelated clone in the germplasm. The authenticity of this donor was further re-established using AFLP markers.

Introduction

The ability to achieve large genetic gains in a short time has made clonal propagation a widely accepted means for large-scale plantation programs of *Eucalyptus*. The main aim of clonal propagation is to retain the genetic integrity of the propagated plants with respect to the donor plus tree so that the desirable traits of the donor tree are retained. Macropropagation through rooted cuttings is the commonly used approach, yet many genotypes show recalcitrance to rooting, especially when non-juvenile shoots are used. Micropropagation may overcome the problem (Kretzschmar and Ewald, 1994). Besides, it can provide large numbers of propagules in a short time. These advantages have made *in vitro* techniques popular for large-scale propagation and germplasm conservation. Techniques like cryopreservation of germplasm entirely rely on

in vitro propagation. However, micropropagation through tissue culture can cause somaclonal variants especially when subjected to several *in vitro* transfers (Larkin and Scowcraft 1981; Meins 1983). In addition to somaclonal variation, mislabeling and mixing of clones in germplasm collections have also been reported (Keil and Griffin, 1994). Materials from germplasm banks are frequently used in breeding and tree improvement programs. The economic implications of such inadvertent variations and mixings of accessions could be serious as considerable time and money is spent before the mistakes are detected. This necessitates development of suitable strategies for assessing genetic uniformity and for identifying the variations. The need to test the genetic fidelity of tissue culture plants in tree species is important also because they are harvested on long rotations and the *in vitro* cultures are maintained through many subcultures. In this paper, we describe a case study of reestablishment of clonal identity of *Eucalyptus* using DNA fingerprinting.

Materials and methods

Plant material

To test the genetic fidelity of micropropagated plants, a candidate plus tree of *Eucalyptus tereticornis* Sm. (SMD7) selected from Sethumadai, Tamil Nadu, India, and its putative *in vitro* propagated plants (Table 1) already available in the germplasm collections of the Institute of Forest Genetics and Tree Breeding, Coimbatore, India, were used. The candidate plus tree, SMD7, was micropropagated through axillary branching and some of these *in vitro* regenerated individuals (henceforth collectively referred as group 1) were planted in the germplasm bank during 1992. In 1998, the explants from the coppice shoots of these group 1 trees were used to establish another axillary bud culture. Plantlets regenerated from this culture (henceforth collectively referred as group 2) were out planted on different dates over a period of fifteen months as detailed in Table 1. Multiple samples (up to six) were used to represent each outplanting date. Trees 1a, 1b, 1c, 1d, 1e, 1f of group 1 and Plantlets $2a_1$, $2a_2$, $2a_3$, $2b_1$, $2b_2$, $2b_3$, 2c, 2d, 2e, 2f of group 2 were used for evaluating the genetic uniformity *vis-a-vis* their respective donors. For some of the samples ($2b_1$, $2b_2$ and $2b_3$), the DNA isolation was carried out on plantlets randomly selected directly from the culture flasks. The experimental material being of widely differing age groups, and being grown under differing environmental conditions were not amenable for genetic uniformity analysis via morphological markers necessitating the use of genetic markers.

Other clones maintained in the tissue culture germplasm since 1998 were EC 89-01-06, EC 89-20-02, EC 89-01-07, ET 89-10-05, ITC 1, ITC 3, ITC 4, ITC 6, ITC 7, ITC 10, ITC 71, ITC 99, ITC 128, ITC 130, ITC 132 and ITC 148. The donor trees for all these clones were also available in the field germplasm collections.

Table 1. Details of the plant material used for testing genetic fidelity.

Group	Tree/plantlets	Details	Year of out-planting
	Tree 1	Seed raised plus tree (SMD7)	1972
Group 1	Trees 1a, 1b, 1c, 1d, 1e and 1f	Tissue culture raised trees reportedly derived from tree 1	1992
Group 2[a]	Plantlets $2a_1$, $2a_2$, $2a_3$	Tissue culture raised plantlets reportedly derived from trees of group 1	March 2000
	Plantlets $2b_1$, $2b_2$, $2b_3$	Tissue culture raised plantlets reportedly derived from trees of group 1	October 2000
	Plantlet 2c	Tissue culture raised plantlet reportedly derived from trees of group 1	December 2000
	Plantlet 2d	Tissue culture raised plantlet reportedly derived from trees of group 1	January 2001
	Plantlet 2e	Tissue culture raised plantlet reportedly derived from trees of group 1	March 2001
	Plantlet 2f	Tissue culture raised plantlet reportedly derived from trees of group 1	May 2001

[a]Culture initiated in December 1998.

Tissue culture

The axillary bud culture protocol of Preetha et al. (1993) was followed. The axillary buds were used as explants for micropropagation. The explants were surface sterilized and placed on Murashige and Skoog (1962) medium with 0.1 mg/l benzyl adenine (BA) and 0.1 mg/l kinetin. Shoot multiplication was carried out in fresh medium after every 25–30 days. The number of shoots obtained averaged around 15–20 per flask. The shoots were transferred to White's liquid medium (White, 1934) supplemented with 1 mg/l indole butyric acid (IBA), 0.5 mg/l rutin and 0.5 mg/l quercetin for rooting. Roots emerged in 15–20 days, after which the plantlets were transplanted to root trainers with vermiculite media (Sumathi et al. 1999).

RAPD

DNA was isolated according to Murray and Thompson (1980). The standard protocol for RAPD (Williams et al. 1990) was followed. The 25 μl reaction mixture contained 25 ng of genomic DNA, 2.0 mM $MgCl_2$, 100 μM dNTPs, 5 pmol of primer and 1 Unit of Taq DNA polymerase in 1× enzyme assay buffer. The amplification conditions were an initial denaturation at 94 °C for 5 min followed by 40 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 °C 2 min, and a final extension step at 72 °C for 7 min. Seventeen 10-mers were used to screen the micropropagated plants. Agarose gels (1.5%) were run in 0.5× TBE (Tris base–boric acid–EDTA) buffer at 30 V for 2 h and stained with 0.5 μg/ml ethidium bromide and viewed over UV light.

AFLP

AFLP Analysis System I kit from Life Technologies Inc. (based on Vos et al. 1995) was used. Five hundred nanograms of genomic DNA was digested using 5 Units each of *Eco*RI and *Mse*I for 3 h. Digestion was followed by heat inactivation of the restriction endonucleases at 70 °C for 15 min. Ligation of *Eco*RI and *Mse*I adapters to the digested DNA was carried out by incubating the reaction mixture containing 1 Unit of T4 DNA ligase at 20 °C for 3 h. Preselective amplification with primers having one extra selective nucleotide was carried out with the following cycle profile: a 30 s DNA denaturation step at 94 °C, a 1-min annealing step at 56 °C and 1 min extension step at 72 °C. The reaction was carried out for 20 cycles. The PCR products of the preamplification reaction were diluted 50 times and used for selective amplification using primers having 3 selective nucleotides at the 3′ end. Selective amplification reactions were performed with the following cycle profile: a 30 s DNA denaturation step at 94 °C, a 30 s annealing step and 1 min extension step at 72 °C. The annealing temperature in the first cycle was 65 °C, which was subsequently reduced each cycle by 0.7 °C for the next 12 cycles, and was continued at 56 °C for another 40 cycles. Primer pairs used were E_{AAG} and M_{CAA}, E_{ACG} and M_{CTA}, E_{ACC} and M_{CAG}, and E_{ACC} and M_{CAC}. All amplification reactions were carried out in a PTC 200 thermocycler (MJ Research Inc. USA). The AFLP reaction products were resolved in 6% sequencing gels containing 7.5 M urea. The gel was run at a constant power of 65 W. The bands were detected by silver staining (Caetano-Anollés and Gresshoff, 1994). Both the RAPD and AFLP gels were photographed using a Kodak EDAS 290 photodocumentation system. The RAPD and AFLP experiments were repeated at least twice and only the distinct, well-resolved and reproducible bands were scored. The scoring was done manually for their presence or absence using the Kodak ID utility.

Results

Genetic fidelity of micropropagated plants

All 17 RAPD primers showed polymorphisms among different groups of micropropagated plants and SMD7 (Figure 1). The size of the amplified products ranged from 300 to 3500 bp. Out of 183 bands scored, 123 were polymorphic between the different groups of plants. The 1/0 matrix was used to calculate similarity coefficients for the data pooled over all the primers using the numerical taxonomy and multivariate analysis system, NTSYSpc (Rohlf 1997). The dendrogram was constructed using Dice similarity coefficient and UPGMA (Unweighted Paired Group Method of Averages) clustering (Dice 1945). The dendrogram placed all the micropropagated plants into two clear groups while SMD7 (tree 1) clearly separated from the micropropagated plants

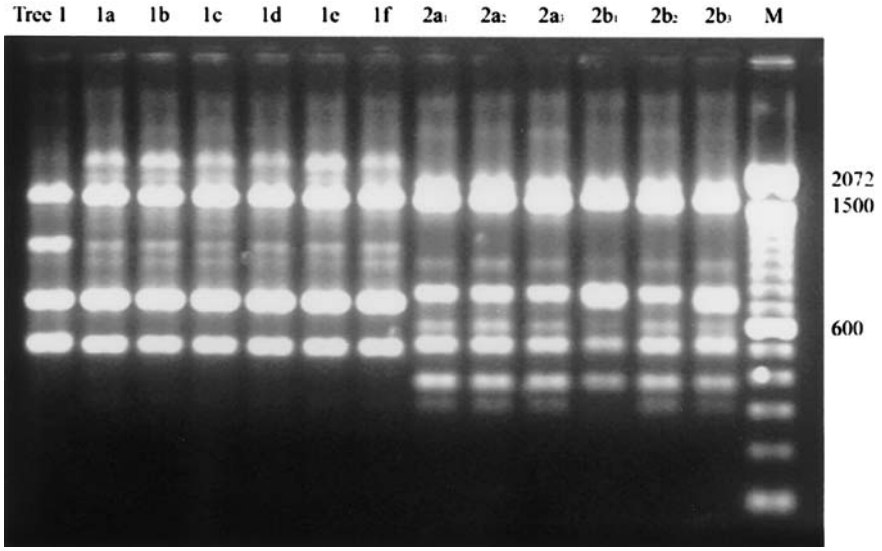

Figure 1. RAPD profiles of SMD7 (tree 1), group 1 (trees 1a, 1b, 1c, 1d, 1e and 1f) and group 2 (plantlets $2a_1$, $2a_2$, $2a_3$, $2b_1$, $2b_2$ and $2b_3$) using primer OPE-01. Lane M is 100 bp ladder.

(Figure 2). Trees 1a, 1b, 1c, 1d, 1e and 1f clustered together (group 1) while plantlets $2a_1$, $2a_2$, $2a_3$, $2b_1$, $2b_2$, $2b_3$, clustered together (group 2). The genetic distance between SMD7 and group 1 was 0.20 as compared to 0.31 between SMD7 and group 2, and 0.11 between group 1 and group 2. RAPD analysis of SMD7 (tree 1), trees 1a, 1d, plantlets $2a_1$ and $2b_1$ using 12 more primers again separated these five plants in three clear clusters (data not shown).

AFLP analysis was done on the representative individuals from each group and four additional individuals from group 2 (Table 1). A total of 9 individuals [SMD7 (tree 1), trees 1a, 1d, plantlets $2a_1$, $2b_1$, 2c, 2d, 2e, and 2f] were used for this study. Out of 119 bands scored for four primer pairs (E_{AAG} and M_{CAA}, E_{ACG} and M_{CTA}, E_{ACC} and M_{CAG}, E_{ACC} and M_{CAC}), 58 were polymorphic. The dendrogram obtained using AFLP data gave a genetic distance of 0.17 between SMD7 and group 1 and 0.25 between SMD7 and group 2 (Figure 4).

Intra-group genetic uniformity in micropropagated plants

Amongst the group 1 trees, out of 132 bands scored for 17 RAPD primers, only one band, $OPE02_{500}$ was polymorphic. This band was present only in tree 1d. The cluster analysis revealed a small variation of 0.01 within group 1 trees (Figure 2). Amongst the group 2 plantlets (plantlets $2a_1$, $2a_2$, $2a_3$, $2b_1$, $2b_2$, $2b_3$), out of 125 bands scored for 17 primers, three bands $OPE02_{575}$, $OPC15_{3000}$ and $OPE13_{950}$ were polymorphic. $OPE02_{575}$ was absent in $2a_2$ and $2b_3$ but

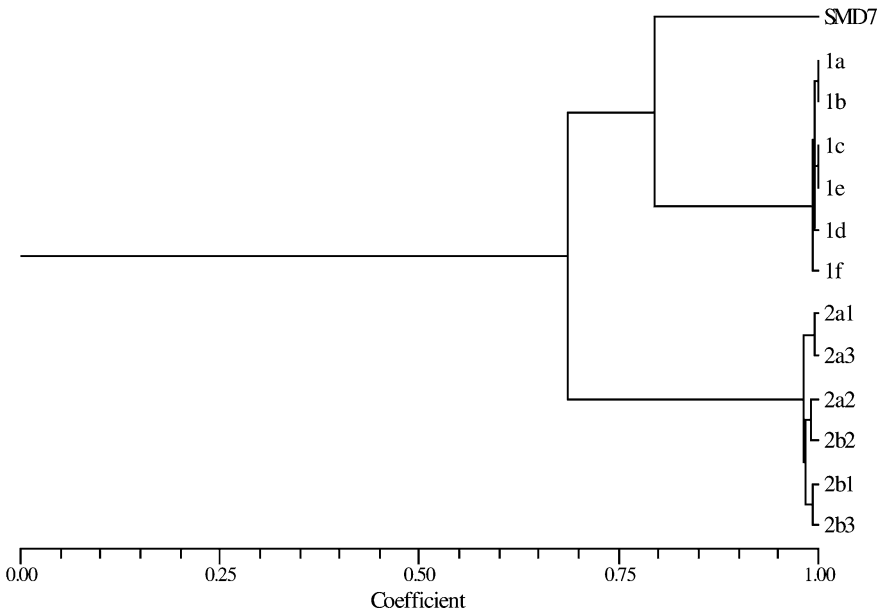

Figure 2. Dendrogram showing genetic distances between SMD7 and the micropropagated plants. Data obtained from 17 RAPD primers were pooled and used for generating the dendrogram using Dice similarity coefficient and UPGMA clustering.

present in all other plantlets of group 2. $OPC15_{3000}$ was absent only in $2b_3$. $OPE13_{950}$ was present in plantlets $2b_1$ and $2b_3$ but absent in other plantlets of group 2. A small variation of 0.02 within group 2 trees was revealed by cluster analysis (Figure 2).

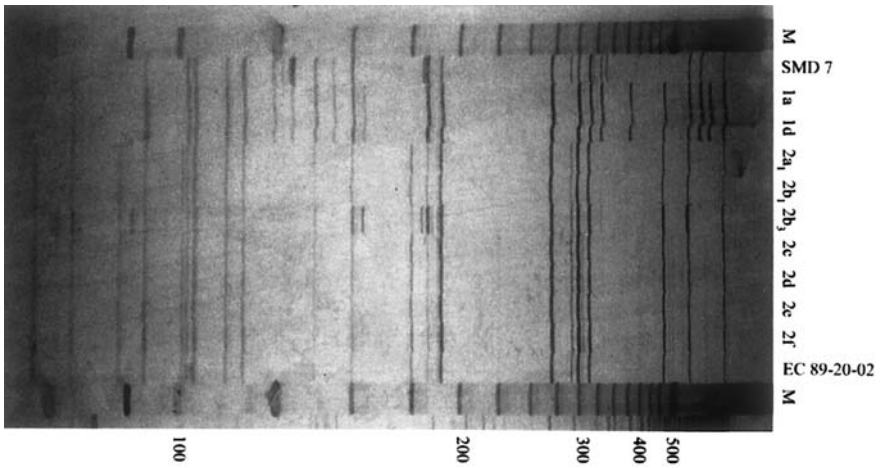

Figure 3. AFLP analysis of SMD7, group 1 (trees 1a and 1d) and group 2 (plantlets $2a_1$, $2b_1$, $2b_3$, 2c, 2d, 2e and 2f) with primer pair EACC and MCAC. Lane M is 25 bp ladder.

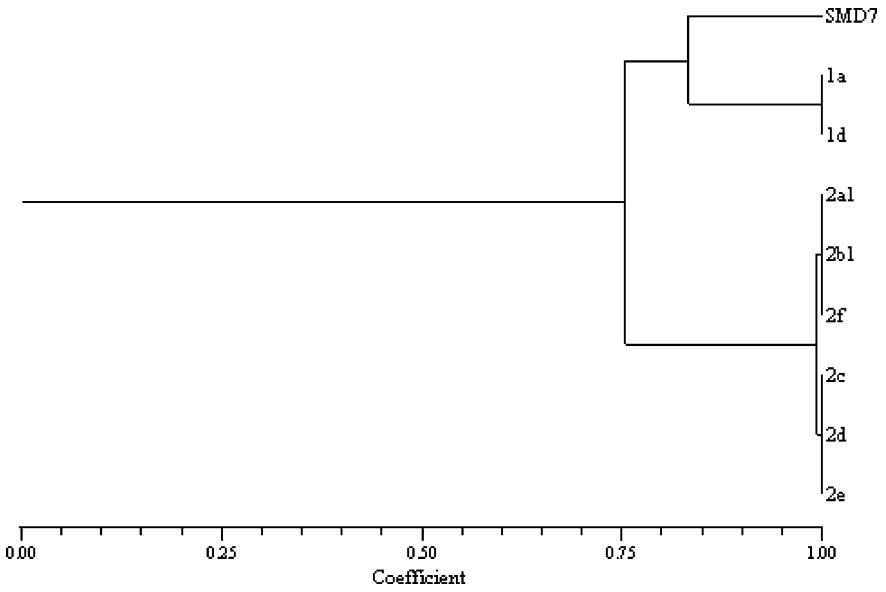

Figure 4. Dendrogram showing genetic distances between SMD7 and the micropropagated plants. Data obtained from 4 AFLP primer pairs were pooled and used for generating the dendrogram using Dice similarity coefficient and UPGMA clustering.

AFLP data on $2a_1$, $2b_1$, 2c, 2d, 2e and 2f using four primer pairs (E_{AAG} and M_{CAA}, E_{ACG} and M_{CTA}, E_{ACC} and M_{CAG}, E_{ACC} and M_{CAC}) showed almost similar banding patterns among the micropropagated plants. No polymorphism was detected within the group 1 trees, out of 94 bands scored for four primer pairs. Amongst the group 2 plantlets, out of 67 bands scored for three primer pairs, only one band, $E_{AAG}M_{CAA}415$, was polymorphic. This band was present only in plantlets $2a_1$, $2b_1$ and 2f. Twenty-eight additional bands were scored using primer pair E_{ACC} and M_{CAC} on group 2 plantlets including plantlet $2b_3$. Only two bands, $E_{ACC}M_{CAC}177$ and $E_{ACC}M_{CAC}155$, were found polymorphic and were present only in $2b_3$ (Figure 3). The dendrogram showed that the group 2 plantlets, $2a_1$, $2b_1$ and 2f were identical and separated from the plantlets 2c, 2d, and 2e by a genetic distance of 0.01 (Figure 4).

Determination of the identity of micropropagated plants

The extent of variations observed between SMD7, group 1 and group 2 were very high and therefore the possibility of mixing/mislabeling of clones either during explant collection or during culture was explored. Therefore, RAPD profiles of all the 17 clones in the tissue cultured *Eucalyptus* germplasm maintained since 1998 (refer to Materials and methods) were compared with representative individuals from group 1 (tree 1a) and group 2 (plantlet $2a_1$). For this purpose, DNA was isolated from clones maintained as trees in the germ-

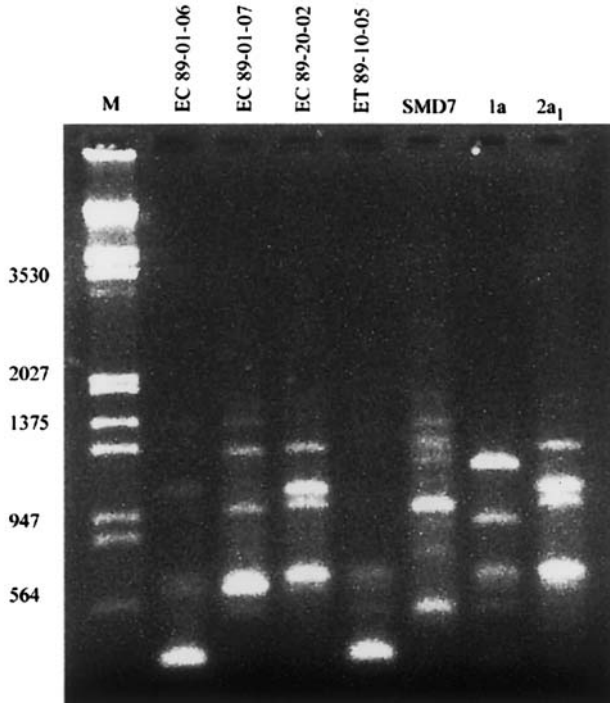

Figure 5. RAPD analysis with primer OPB-04 to compare the doubtful clones (1a and $2a_1$) with other clones maintained in tissue culture germplasm viz. EC 89-01-06, EC 89-01-07, EC 89-20-02, ET 89-10-05 and SMD 7. The profiles of $2a_1$ matched exactly with that of EC 89-20-02. Lane M is lambda *Hind*III/*Eco*RI digest (lane 1). Molecular sizes are shown in base pairs.

plasm bank, which were used as explant donors for these 17 clones. The RAPD profiles of the group 2 plantlet $2a_1$ obtained using 9 RAPD primers were found to be identical to that of the clone EC 89-20-02 (Figure 5 and Figure 6). RAPD profiles of group 2 plantlets ($2a_1$, $2a_2$, $2a_3$, $2b_1$, $2b_2$, $2b_3$, 2c, 2d, 2e, 2f) obtained using an additional primer OPE-13, also showed similar results Figure 7). These 10 primers had shown differences between SMD7 (tree 1), group 1 and group 2. AFLP analysis with primer pair E_{ACC} and M_{CAC} revealed that the AFLP profiles of plantlets belonging to group 2 (Plantlet $2a_1$, $2b_1$, $2b_3$, 2c, 2d, 2e, 2f) were similar to that of EC 89-20-02. However, the AFLP profiles of group 2 plantlets varied with that of SMD7 and group 1 (Figure 3).

Discussion

The aim of the present study was to determine the genetic fidelity of micropropagated *Eucalyptus*. Studies using both RAPD and AFLP showed variations between SMD7 and the micropropagated plants. All the 17 RAPD primers showed polymorphisms among different groups of micropropagated

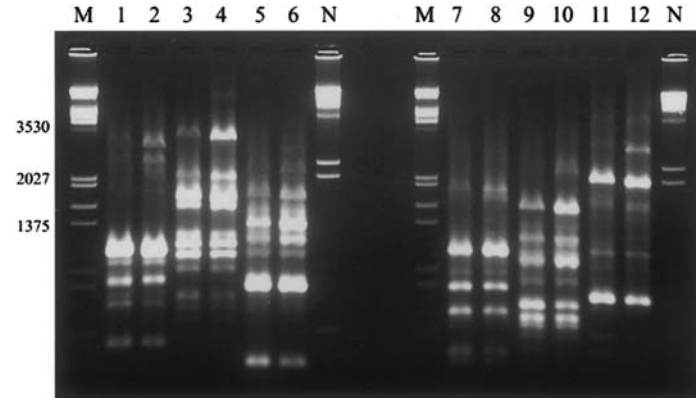

Figure 6. RAPD analysis with six primers to compare plantlet 2a1 (lanes 1, 3, 5, 7, 9, 11) with clone EC 89-20-02 (lanes 2, 4, 6, 8, 10, 12). Lane M is lambda *Hind*III/EcoRI digest. Lane N is lambda *Hind*III digest.

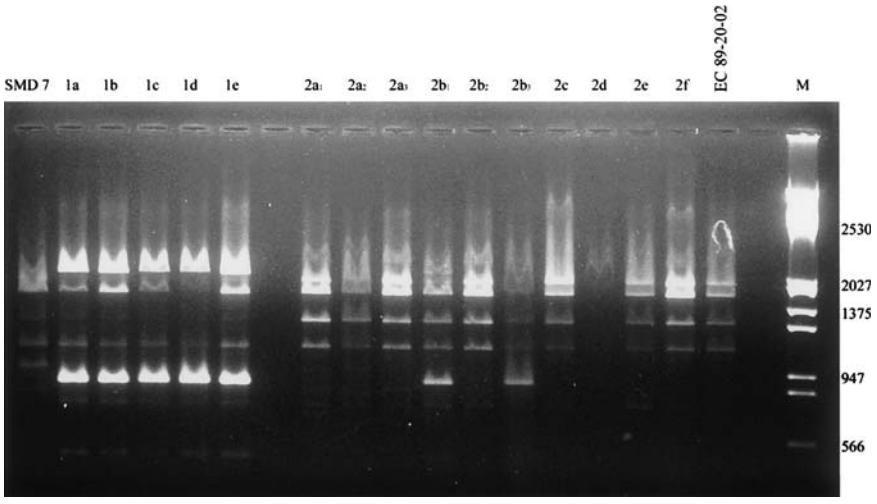

Figure 7. RAPD profiles of SMD7 (tree 1), group 1 (trees 1a, 1b, 1c, 1d and 1e), group 2 (Plantlets $2a_1$, $2a_2$, $2a_3$, $2b_1$, $2b_2$, $2b_3$, 2c, 2d, 2e and 2f) and EC 89-20-02 using primer OPE-13. Lane M is lambda *Hind*III/*Eco*RI digest.

plants and SMD7. The dendrogram placed all the micropropagated plants into two clear groups while SMD7 (tree 1) was clearly separated from the micropropagated plants. The genetic distance between SMD7 and group 1 was 0.20 as compared to 0.31 between SMD7 and group 2, and 0.11 between group 1 and group 2. Results obtained from AFLP analysis on the representative samples from each group and additional four samples from group 2 were similar. Besides these major variations, minor intra-group variations were also

observed. The cluster analysis revealed a small variation of 0.01 within group 1 trees and 0.02 within group 2 trees. As these intra-group variations are very small, these may be attributed to somaclonal variations occurring due to long-term culture. Similar variations have been reported in micropropagated plants. Based on RFLP analysis, Muller et al. (1990) reported fourfold increase in the level of variation on doubling the culture period of rice callus tissues from 28 to 67 days. Rani et al. (1995) attributed the variations in RAPD profiles of micropropagated *Populus deltoides* generated through axillary branching to somaclonal variations.

DNA polymorphisms in micropropagated plants are possible either due to somaclonal variations or by mechanical mixing and mislabeling. In the present study, as axillary branching was used for micropropagation, the variations of 0.20, 0.31 and 0.11 observed between the groups were too high to be attributed to somaclonal variations. Keil and Griffin (1994) found major RAPD variations of up to 0.59 in clonal identification studies of microproapagated *Eucalyptus* which they attributed to mislabeling. Therefore, we explored the possibility of mixing/mislabeling of clones either during explant collection or during culture and finding the legitimate donor. For this purpose, RAPD profiles of all the 17 clones in the tissue cultured *Eucalyptus* germplasm maintained since 1998 were compared with the dubious micropropagated plantlets of group 1 and group 2. Both RAPD and AFLP analysis revealed that plantlets belonging to group 2 were identical to a *Eucalyptus* clone EC 89-20-02. The clone EC 89-20-02 is unrelated to SMD7 (tree 1) as revealed by DNA fingerprinting studies. As none of the micropropagated plants matched with SMD 7, a strong case for mislabeling during micropropagation of group 1 and group 2 plants was established. However, the identity of group 1 trees could not be established. It may be noted that the group 1 trees were planted in 1992 and many of the contemporaneous clones were not available in the germplasm collection for comparison.

Conclusions

We conclude that there has been an inadvertant mislabeling of clones during early stages of micropropagation of both the groups. This type of accidental mislabeling is common where large number of genotypes are maintained in culture conditions (Keil and Griffin, 1994). This study shows that DNA markers are useful tools for identification and verification of genotypes especially during establishment and maintenance of germplasm collections and propagation units such as clonal multiplication area (CMA) and clonal seed orchards (CSO). This study therefore reinforces the need for maintaining the original germplasm backed with DNA fingerprint identities as a reference to verify the authenticity of clones. DNA fingerprint profiles with a proper database strategy can be effectively used for maintenance and verification of clonal identity (Mathish et al. 2001). The present study used RAPD and AFLP

markers to solve the problem of mislabeling detected during genetic quality testing of micropropagated *Eucalyptus* tereticornis. More useful markers such as microsatellites are being developed (Brondani et al. 1998; van der Nest et al. 2000) for various species of *Eucalyptus*, which have been shown to have better discriminating power than RAPD and AFLP Belaj et al. 2003). However, for detection of somaclonal variations, arbitrary markers such as RAPDs, ISSRs and AFLPs may be more efficient due to their higher multiplex ratios. These markers may also be useful in identification of clones in species for which microsatellite markers are yet to be developed.

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