

# RAPD Fingerprint to Appraise the Genetic Fidelity of In Vitro Propagated *Araucaria excelsa* R. Br. var. *glauca* Plantlets

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**Abstract** Randomly amplified polymorphic DNA (RAPD) was used as a tool to assess the genetic fidelity of in vitro propagated *Araucaria excelsa* R. Br. var. *glauca* with explants taken from orthotropic stem along with their related mother plants after treatment with kinetin, 2iP, BA (0.02–0.26 mg/l) and TDZ (0.001–1 mg/l) to produce axillary shoots. TDZ and kinetin induced more shoot and higher length per explant. Results showed a total of 1,676 fragments were generated with 12 RAPD primers in micropropagated plants and their donor mother plants. The number of loci ranged from 6 in OPB 12–18 in OPY 07 with a size ranging from 250 bp in OPH 19–3500 bp in OPH 11. Cluster analysis of RAPD data using UPGMA (unweighted pair group method with arithmetic average) revealed more than 92% genetic similarities between tissue cultured plants and their corresponding mother plant measured by the Jaccard's similarity coefficient. Similarity matrix and PCoA (two dimensional principal coordinate analysis) resulted in the same affinity. Primers had shown 36% polymorphism. However, careful monitoring of tissue culture derived plants might be needed to determine that rooted shoots are adventitious in origin.

**Keywords** *Araucaria excelsa* R. Br. · Cytokinin · Genetic fidelity · Micropropagation · RAPD

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## Introduction

Critical progress has been made in vegetative propagation of coniferous species by in vitro techniques. Species of *Araucaria*, *Agathis*, and *Wollemia* (Araucariaceae) possess an apparently unique axillary structure consisting of undifferentiated axillary meristems that have neither leaf primordial nor vascular connections. However, if the orthotropic leader is lost or damaged, it can be replaced by buds or meristems (orthotropic epicormic shoot reiterate) lower on the trunk, which is the characteristic strategy for surviving environmental pressures such as drought and fires [1]. These shoots are adventitious in origin. Successful micropropagation, especially for difficult and recalcitrant species, is primarily dependent on the quality of the explant and plant growth regulator [2] used in the culture media albeit, some conifers such as hoop pine (*Araucaria cunninghamii* AIT.) and douglas fir (*Pseudotsuga menziesii* Mirb. Franco) are difficult to regenerate under in vitro conditions and are highly sensitive to high concentrations of cytokine in culture medium [3, 4]. Propagation of *Araucaria* using in vitro techniques has been reported by several researchers using different explant sources [3, 5, 6]. The most important part of any in vitro propagation system is mass multiplication of plantlets that are genetically homogenous and phenotypically uniform. Generally, in callus culture and through adventitious shoot formation some desirable characters may be lost because of somaclonal variation occurrence. Several approaches have been applied for identifying variants among micropropagated plants. Molecular markers have been shown to enhance breeding efforts in annual and perennial crops, since they are not altered by major environmental factors. Random amplified polymorphic DNA (RAPD), requires only a little volume of DNA without involving radioactivity test, and is

often preferred because of reduced complexity and the fact that RAPD is faster than RFLP. In addition, RAPD [7] has proven to be efficient in detecting genetic variation even in closely related organisms, such as two near isogenic lines [8]. Allelic variation among individuals is detected as the presence or absence of the amplification product, visualized as a band after PCR and electrophoresis [9]. Analysis of variation at the nuclear genome level using RAPDs has advantages over RFLPs, as a single primer produces several loci, covering a large portion of the genome [10]. However, uncertain homology of co-migrating fragments in gel electrophoresis and reproducibility have some limitation [11], that can be minimized by carefully adjusting the reaction and detection conditions [12]. Venkatachalam et al. [13], by ISSR and RAPD markers has shown that use of high level of cytokinins does not involve any genetic fidelity in all the banana plantlets analyzed. RAPD have been successfully applied to detect the genetic similarities or dissimilarities [14] in micropropagated materials in various plant species [15–18]. However, there are no reports on in vitro manipulations and somaclonal variation of this woody and hard-to-root species. The aim of this study was the possible finding of genetic variability in micropropagated plants derived from orthotropic stem explant in *Araucaria excelsa* R. Br.

## Materials and Methods

### Plant Materials and Micropropagation Procedure

Three year's old seedlings of *A. excelsa* R. Br. var. *glauca* were chosen for this study. Pieces of orthotropic stem were cut from the 8 cm upper half and prewashed in tap water for 3 h. Owing to high contamination rate observed nano silver [19, 20] was used to eradicate the internal bacteria with a concentration of 500  $\mu\text{m}/\text{ml}$  under reduced pressure (300 mm Hg in 5 min). Then, explants were treated with 70% ethanol for 3 min and 15% Clorox (containing 5.25% sodium hypochlorite) with 0.2% household detergent for at least 10–20 min for surface sterilization, and then rinsed six times with sterilized distilled water. The stem pieces were then cut into 7–9 mm long explant and placed with their proximal ends on MS [21] basal medium with 3.0% sucrose and 0.8% agar. Explants for shoot induction and proliferation were cultured on MS medium supplemented with 0, 0.02, 0.04, 0.06, 0.08, 0.10, 0.16, 0.21, and 0.26 mg/l kinetin and BA for 8 weeks. In a separate experiment, TDZ (0.001–1 mg/l) and 2iP (in the range of 0.02–0.26 mg/l) were used to evaluate the mean shoot number and length per explants after two subcultures. The explants were subcultured every fourth week. The pH of the media was adjusted to 5.8 by 0.1 N HCl before

autoclaving for 15 min at 121 °C and 1.5 kg/cm<sup>2</sup> pressure. Cultures were kept at 25 ± 2 °C temperature under cool white fluorescent light (30  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) with 16/8 h day/night photoperiods. Thirteen lines of in vitro micropropagated shoots (After 5–6 subcultures) were randomly chosen from micropropagated plants (affected by BA) for analysis of genetic stability. Ex vitro grown mother plants were used as the control.

### DNA Extraction and PCR Amplification

Total DNA was extracted from 100 mg leaves of 13 lines of micropropagated plants and their donor mother plants as described by Stange et al. [22], with many modifications. The RNA contamination in all samples was removed by digesting the extract with 1 U RNAase-A (Fermentas, Germany). The DNA quality and concentration were analyzed by electrophoresis and Nano drop (Thermo. ND1000. USA), respectively, followed by dilution to 8 ng/ $\mu\text{l}$  with TE buffer. Sixteen arbitrary primers each consisting of ten nucleotides, were purchased from MWG (Germany) and used in the amplification process. Primers were screened initially and 12 were selected for the present study (Table 1).

PCR was performed in a volume of 20  $\mu\text{l}$  containing 8 ng of template DNA, 10  $\mu\text{M}$  of decamer primers, 1 $\times$  reaction buffer, 1.75 mM MgCl<sub>2</sub>, 0.25 mM dNTP mix and 2 U Taq DNA polymerase (The optimized PCR conditions for RAPD analysis were consisted of an initial denaturation at 94 °C for 2 min followed by 45 cycles of 60s at 92 °C, 60s at 36 °C, and 2 min at 72 °C and finally terminated with an extension of 5 min at 72 °C in a DNA thermocycler (Bioer. Gene Pro, China). The PCR products were separated in 2% agarose gel containing 1  $\mu\text{g}/\text{ml}$  ethidium bromide, at a constant voltage (60 V) and the number of bands were recorded using a gel documentation system (GENE FLASH). The size of the amplification products was estimated using a 100 bp DNA ladder (Gene ruler DNA Ladder mix # SM 0331, Fermentas, Germany).

### Statistical Analysis

#### *Plant Tissue Culture Studies*

Shoot proliferation rate, shoot length, and visual quality were recorded after nearly 56 days. Each experiment was carried out as a completely randomized design with at least ten replications for each treatment. Data were analyzed using SPSS statistical software, and means were compared using Duncan's Multiple Range Test ( $P \leq 0.05$ ). For visual quality test, the proliferated shoots were ranked according to their color and abnormality [yellowing of the leaves, formation of malformed leaves and suppression of growth,

**Table 1** Characterizations of markers used

Code name	Sequence	No. of total band (SD) <sup>a</sup>	Loci (SD)	Polymorphic band (SD)
OPA 11	CAATCGCCGT	78 (6.22)	8 (1.08)	3 (1.05)
OPA 18	AGGTGACCGT	186 (7.88)	15 (1.15)	7 (0.05)
OPB 01	GTGTCGCTCT	88 (0.58)	9 (0.00)	8 (0.00)
OPB 12	CCTTGACGCA	71 (0.49)	6 (0.00)	1 (1.05)
OPC 13	AAGCCTCGTC	98 (1.01)	7 (0.00)	1 (1.05)
OPH 11	CTTCCGCAGT	138 (11.52)	14 (.033)	11 (3.19)
OPH 18	GAATCGGCCA	150 (11.83)	11(3.23)	1 (1.57)
OPH 19	CTGACCAGCC	204 (8.44)	16 (2.88)	2 (1.07)
OPK 17	CCCAGCTGTG	210 (12.68)	17 (2.03)	7 (3.61)
OPX 11	GGAGCCTCAG	103 (10.09)	11 (2.19)	3 (0.57)
OPY 04	GGTCGCAATG	138 (8.02)	13 (3.92)	4 (0.57)
OPY 07	AGAGCCGTCA	212 (4.73)	18 (2.04)	6 (2.46)

<sup>a</sup> SD standard deviation. This table is according to three experimental and biological repeat

ranking from 1 (lowest abnormality) to 3 (highest abnormality)].

#### Marker Analysis

Well-resolved and consistently reproducible fragments ranging from 240 to 3500 bp were scored. Reproducible fragments were scored as 1 or 0 for presence or absence of the band on the gels. The bands of each gel were scored using the Lab work 4.6 software. The distance matrix and dendrogram were constructed using the NTSYS-pc 2.02 [23]. Genetic similarities between tissue culture-derived plants and their donor mother plants were measured by the Jaccard's similarity coefficient [24] with SIM-QUAL module. Similarity coefficients were used to construct a dendrogram using the UPGMA (unweighted pair group method with arithmetic average). The two dimensional principal coordinate analysis (PCoA) was conducted with the same program using the EIGEN and PROJ module. The polymorphism information content (PIC) described by [25], and probability of identity (PI) described by Pollefeys and Bousquet [26] were calculated as follows:

$$PIC = 2 \sum_{i=1}^{k-1} \sum_{j=i+1}^k P_i P_j (1 - P_i P_j)$$

$$PI = \sum_i P_i^4 + \sum_{i=1}^n \sum_{j>i}^n (2P_i P_j)^2$$

where  $P_i$  and  $P_j$  are the frequencies of the  $i$ th and  $j$ th alleles within each locus, respectively. This experiment was carried out based on three experimental and biological repeats and their means were used in data analysis. Each amplification was performed at least three times and RAPD experiments were conducted after nearly six subcultures.

## Results

### Effects of PGRs

The present investigation showed that axillary shoots could be regenerated from orthotropic stems of *Araucaria excelsa* R. Br. even in growth regulator free MS medium. From the two hormones tested, kinetin showed higher shoot proliferation and longer shoots than BA (Table 2). The addition of 0.02–0.26 mg/l kinetin to MS basal medium had not significant effect on mean number of shoots per explants (MNS/E) while concentrations more than 0.1 mg/l BA caused the formation of distorted axillary buds with limited growth but not in kinetin. Two to ten adventitious shoots on each explant started to elongate 2 months after culturing (Fig. 1). However, the MNS/E increased up to 4.00 and 4.33 in 0.06 and 0.1 mg/l BA and kinetin, respectively. In the medium supplemented with 0.16 mg/l kinetin the length of proliferated shoots raised to 10.03 mm. Among different PGRs lead to highest mean shoot number and length per explants, TDZ and kinetin were better than others (Table 3).

### Genetic Fidelity

In order to confirm the dependability of micropropagated plants for maintaining genetic fidelity, RAPD fingerprinting of 13 lines selected from micropropagated *A. excelsa* R. Br. and their corresponding mother plants were carried out. Figure 2 shows the profiles obtained with primers used. From the 16 arbitrary RAPD primers initially screened, 12 produced clear and scorable bands (Table 1).

Micropropagated *A. excelsa* R. Br. and their corresponding mother plants produced a total of 1,676 bands

**Table 2** Effects of BA and kinetin on mean number of shoots per explant (MNS/E) and mean length of shoots per explants (MLS/E) in *Araucaria excelsa* R. Br

BA (mg/l)	MNS/E	MLS/E (mm)	Abnormality <sup>a</sup>	Kinetin (mg/l)	MNS/E	MLS/E (mm)
0.0	3.40 ab <sup>b</sup>	1.48 ab	0 b	0.00	2.77 a	5.48 ab
0.02	2.71 ab	1.88 ab	0 b	0.02	2.62 a	5.55 ab
0.04	3.16 ab	1.65 ab	0 b	0.04	3.00 a	3.85 b
0.06	4.00 a	5.02 a	0 b	0.06	3.66 a	4.03 b
0.08	2.75 ab	2.25 ab	0 b	0.08	3.00 a	6.05 ab
0.10	2.12 ab	1.52 ab	0.75 ab	0.10	4.33 a	5.81 ab
0.16	2.90 ab	2.37 ab	1.60 ab	0.16	3.25 a	10.03 a
0.21	2.70 ab	1.40 ab	3.25 a	0.21	3.12 a	7.55a b
0.26	0.87 b	0.90 b	1.75 ab	0.26	3.22 a	8.50 ab

<sup>a</sup> Ranking from 1 (lowest abnormality) to 3 (highest abnormality)

<sup>b</sup> Means with common letters are not significantly different at  $P \leq 0.05$ , according to Duncan's Multiple Range Test (DMRT)

**Fig. 1** High shoot proliferation in MS medium supplemented with 0.08 mg/l BA after 38 days (*left*) and 0.16 mg/l kinetin after 56 days (*right*)



**Table 3** Effects of different PGRs on mean number of shoots per explant (MNS/E) and mean length of shoots per explants (MLS/E) in *Araucaria excelsa* R. Br. after 40 days

Treatment	MNS/E	MLS/E (mm)
Control	2.27 b <sup>a</sup>	3.48 bc
TDZ	3.81 a	5.63 ab
2iP	2.95 ab	4.76 ab
Kinetin	3.30 ab	6.50 a
BA	2.70 b	2.20 c

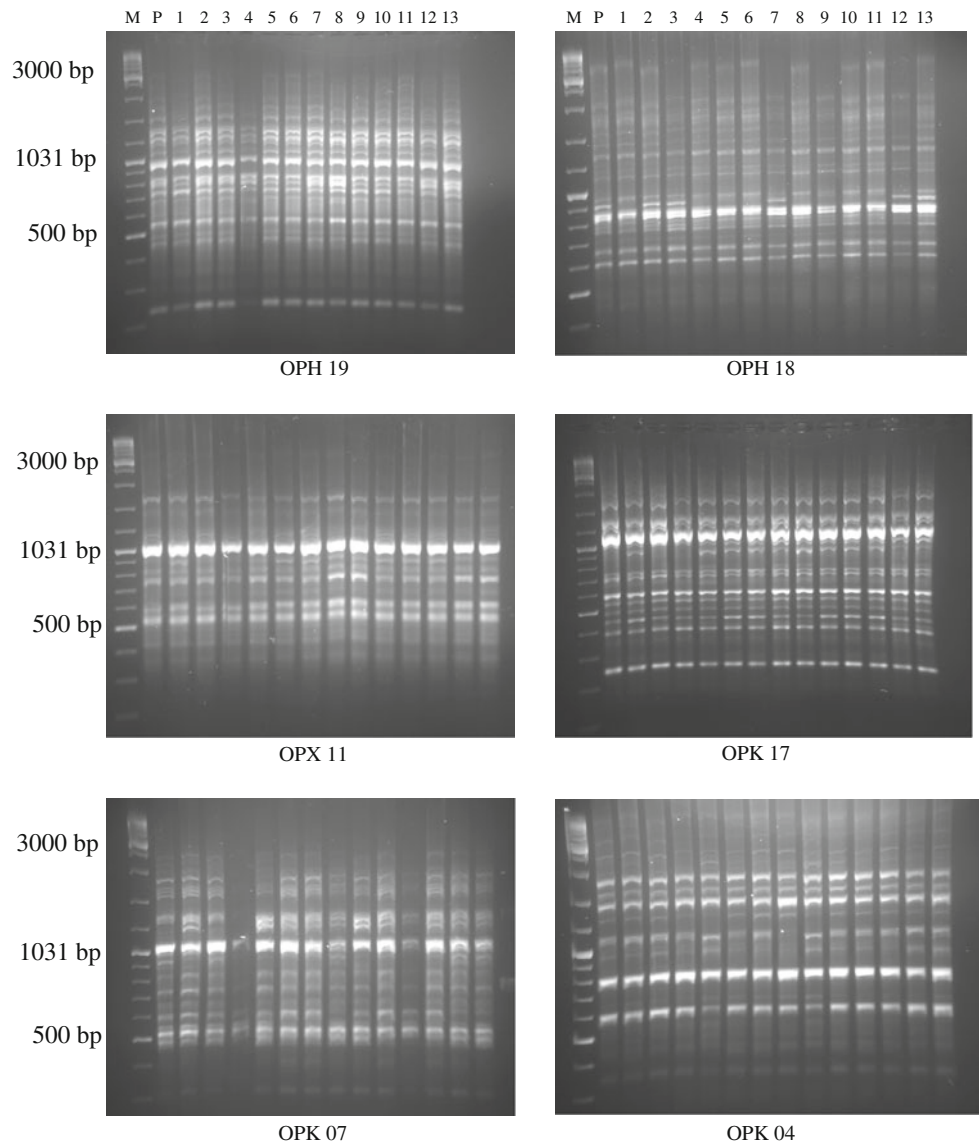
<sup>a</sup> Means with common letters are not significantly different at  $P \leq 0.05$ , according to Duncan's Multiple Range Test (DMRT)

with an average of 140 bands per primer. The number of loci ranged from six in OPB 12–18 in OPY 07 with a size ranging from 250 bp in OPH 19–3500 bp in OPH 11. OPH 11 produced 11 polymorphic bands but OPH 18, OPB 12, and OPC 13 produced least one polymorphic band. Nearly 64% monomorphic profiles produced in all 12 primers.

Indeed, polymorphism information content (PIC) and probability of identity (PI) showed that the OPB 12 was more practicable than other primers in this experiment due to more monomorphic band (Table 4). The similarity matrix established through the analysis of all the bands obtained showed more than 92% similarity between micropropagated plants and their corresponding mother plants that is also distinct in RAPD dendrogram (Table 5; Fig. 3).

As can be observed in Fig. 2 the regenerated plants shared the same banding patterns as those of the donor plants providing orthotropic stem, implying that they possibly were genetically identical to each other. Cluster analysis of RAPD using UPGMA showed that regenerated plants compared to their respective donor mother plants had high affinity (92%), and the affinity was more than 90% among micropropagated plants. The PCoA (Fig. 4) depicts genetic status and association of micropropagated plants and their corresponding mother plants more accurately.

**Fig. 2** DNA fingerprinting of micropropagated *A. excelsa* R. Br. and their corresponding mother plants grown on MS medium supplemented with BA. RAPD profiles followed decamer primers. Lane *M* represents the Gene Ruler DNA Ladder mix # SM0331, Lane *P* represents mother plant, lanes *1–13* represents the micropropagated plants



**Table 4** Polymorphic information content (PIC), Probability identity (PI) and discriminating power ( $D = 1 - PI$ ) of primers used

Primer	PIC (SD) <sup>a</sup>	PI (SD)	D (SD)
OPA 11	0.805 (0.047)	0.053 (0.019)	0.947 (0.020)
OPA 18	0.923 (0.007)	0.009 (0.001)	0.998 (0.001)
OPB 01	0.864 (0.000)	0.027 (0.000)	0.972 (0.000)
OPB 12	0.775 (0.000)	0.068 (0.000)	0.931 (0.000)
OPC 13	0.839 (0.000)	0.037 (0.000)	0.962 (0.000)
OPH 11	0.912 (0.025)	0.013 (0.005)	0.987 (0.006)
OPH 18	0.856 (0.081)	0.035 (0.026)	0.964 (0.027)
OPH 19	0.926 (0.014)	0.008 (0.002)	0.990 (0.003)
OPK 17	0.929 (0.014)	0.008 (0.002)	0.991 (0.003)
OPX 11	0.877 (0.052)	0.025 (0.015)	0.974 (0.016)
OPY 04	0.899 (0.041)	0.017 (0.010)	0.982 (0.011)
OPY 07	0.933 (0.018)	0.008 (0.003)	0.991 (0.004)

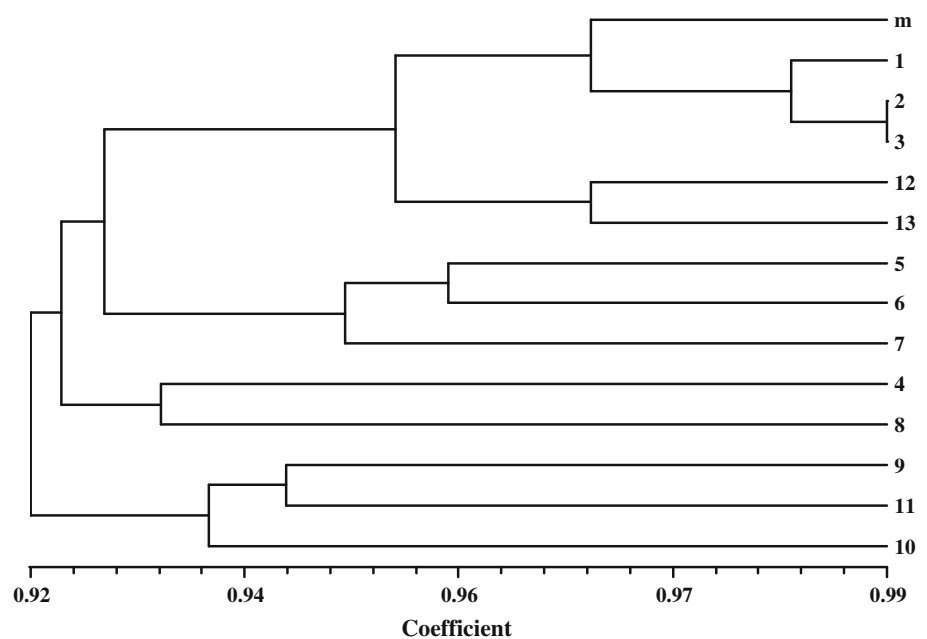
<sup>a</sup> SD standard deviation. This table is according to three experimental and biological repeat



**Table 5** Similarity matrixes of the 13 micropropagated *A. excelsa* R. Br. and their corresponding mother plant based on Jaccard's similarity coefficient (Jaccard [24])

	M	1	2	3	4	5	6	7	8	9	10	11	12	13	
M	1.000														
1	0.965	1.000													
2	0.965	0.983	1.000												
3	0.971	0.988	0.994	1.000											
4	0.937	0.931	0.920	0.926	1.000										
5	0.920	0.938	0.926	0.932	0.932	1.000									
6	0.911	0.929	0.918	0.923	0.911	0.954	1.000								
7	0.916	0.922	0.934	0.934	0.910	0.940	0.950	1.000							
8	0.904	0.910	0.904	0.904	0.928	0.916	0.913	0.930	1.000						
9	0.887	0.912	0.906	0.906	0.893	0.906	0.922	0.915	0.908	1.000					
10	0.925	0.937	0.920	0.926	0.920	0.914	0.905	0.910	0.903	0.931	1.000				
11	0.916	0.934	0.922	0.928	0.922	0.941	0.932	0.918	0.893	0.940	0.934	1.000			
12	0.959	0.941	0.941	0.947	0.936	0.924	0.910	0.926	0.914	0.904	0.918	0.932	1.000		
13	0.945	0.950	0.96	0.956	0.916	0.922	0.901	0.930	0.930	0.914	0.922	0.930	0.967	1.000	

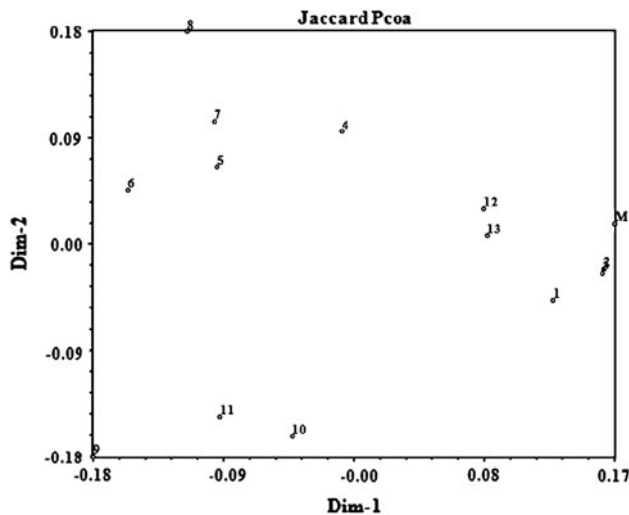
Lanes 1–13 represent the micropropagated plants and *M* represents their donor mother plants

**Fig. 3** RAPD dendrogram based on the Jaccard similarity indices, showing the genetic distance among regenerated *A. excelsa* R. Br. plants (1–13) in comparison to their donor mother plants (*M*)

## Discussion

Comparing the explants cultured on MS medium supplemented with BA, kinetin and the explants cultured on MS medium containing both BA and NAA, it was found that few variation exist between different experiments. However, development of adventitious shoots was faster after the kinetin treatment compared to the others. It was noted that the medium containing higher concentrations of cytokinin, particularly BA gave rise to the lowest regeneration response, which may be ascribed to toxic effects of

high concentration of cytokinins. Generally, low concentration of cytokinin was more effective for the induction of shoots in *A. excelsa* R. Br. The present results are in agreement with earlier reports in which lower concentration of cytokinin was more effective for induction of shoots [3]. Explants in the medium supplemented with kinetin and TDZ had a mean shoot number and length per explant higher than BA and 2iP after two subcultures. The results suggested that plantlets regenerated in *in vitro* culture were similar to *A. excelsa* R. Br. seedlings in the earlier stages of growth with an asymmetrical pattern. Asexual propagation



**Fig. 4** Association among micropropagated *A. excelsa* R. Br. plants (J1–J13) and corresponding mother plants (M) as revealed by the two dimensional principal coordinate analysis (PCoA) from RAPD data sets

can induce somaclonal variation [27]. Piola et al. [15] reported that chromosome instability and somaclonal variation are frequently associated with plant regeneration from unorganized callus producing adventitious buds rather than cultures derived from axillary meristems. It has been reported that genetic fidelity of tissue cultured clones mainly depends on genotype [28], number of subculture passages, culture condition and maintenance [29]. Clonal fidelity is a major consideration in commercial micropropagation using in vitro culture methods. Plantlet regeneration in conifers occurs by somatic embryogenesis and organogenesis, with the former being the preferred route [30]. More recently Bonga et al. [31] have shown that, despite major advances in clonal propagation of woody species, somatic embryogenesis or organogenesis is still difficult [32] for many conifers; however, there are gaps in our knowledge in some areas which allow for a degree of skepticism. The paucity of knowledge controlling somatic embryogenesis, the synchrony of somatic embryo development and low frequency true to type embryonic efficiency are responsible for its reduced commercial application in forest trees [2]. Therefore, production of axillary buds from orthotropic stems in *A. excelsa* R. Br. is of major importance because direct plant production may exhibit greater genetic stability than those produced from callus. Overall, it is important to ensure that the regenerants are genetically true-to-type to their corresponding mother plant with respect to genetic stability. In order to know if there is any abnormality in the micropropagated plant, RAPD as a marker that has been revealed to be a potential marker for distinctive genetic variation [15–17], was employed for this purpose, which confirmed that all

micropropagated plants had a high affinity to their donor plants. However, 8% variations in cultures might be due to long subculturing and production of adventitious shoot formation. Topophysis is the effect of position of the propagule in the source plant on the phenotype of the progeny plant [33], that it is very important in *A. excelsa* R. Br. plants which are desirable as ornamentals. Tissue cultured derived plantlets from lateral shoots of *A. excelsa* R. Br. produced plagiotropic form [34]. However, this study with RAPD markers proposed that tissue culture conditions did not induce high somaclonal variation in *A. excelsa* R. Br. Major disadvantage of RAPD technology is that the results are often difficult to reproduce between or even within laboratories, but for this particular application the reproducibility issues are not as pressing because analyses will always be carried out with reference to an internal standard that here is a mother plant. A very low level of genetic divergence observed in cultured *A. excelsa* explants, could be connivance in commercial production even though these low variations exist in seed production of *A. excelsa*. Further studies with microarray or cDNA AFLP are needed to confirm these results. Albeit 12-months-old derived plants had an upright stem, their general forms were as the same as their seedlings with alternative foliage.

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