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Full Length Research Paper

Genetic diversity among four *Eucalyptus* species (myrtaceae) based on random amplified polymorphic DNA (RAPD) analysis

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Results obtained from random amplified polymorphic DNA (RAPD) analysis of four species of the genus *Eucalyptus* revealed that the number of fragments amplified for each of the five studied primer varied between 13 and 14 fragments and their size ranged from 95 to 1055 bp. The total number of bands was 67, total number of monomorphic bands was 32, total number of polymorphic bands was 35, and the polymorphism percentage was 52.2%. The results indicate that 16 positive and 13 negative markers were detected. The marker fragments size ranged between 175 to 630 bp for the negative markers and 235 to 945 bp for the positive markers.

Key words: *Eucalyptus*, random amplified polymorphic DNA (RAPD), genetic diversity, DNA fingerprinting PCR, dendrogram.

INTRODUCTION

Eucalyptus is a large genus of the Myrtaceae family which includes about 900 species and subspecies (Brooker and Kleingi, 2004). Although most of the plants are native to Australia, numerous species have been introduced to other parts of the world, including Egypt, as economic and ornamental trees in the forest, where the plants have become source of important fast-growing hardwood trees and *Eucalyptus* oil. The random amplified polymorphic DNA (RAPD) technique had been successfully used in a variety of taxonomic and genetic diversity studies (Alam et al., 2009), where it has several distinct advantages like, the cost per reaction is low, only a small amount of plant material is required for DNA extraction and the method does not require any prior knowledge of the sequence of the genome (Karatash and Agaoglu, 2008). Several studies have used RAPDs to assess levels and patterns of variation with different plants (Chalmers et al., 1992; Huff et al., 1993; Nesbitt et al., 1995; Esselman et al., 1999; Prathepha and Baimai, 1999; Navarro-Quezada et al., 2003; Nasser and Al-Khalifah, 2004; Chaturvedi and Nag, 2010; Salim et al., 2010). Recently, RAPD has been used for the estimation of genetic diversity in various endangered plant species (Wang et al., 2005; Lu et al., 2006, 2007; Zheng et al., 2008).

RAPD has frequently been used for the detection of genetic diversity in plants, genotyping, phylogenetic analysis, genetic relationships, etc. The advantages of this method are its rapidity, simplicity and lack of need for any prior genetic information about the plant. RAPD primers are able to distinguish taxa below the species level (Choo et al., 2009) because RAPD analysis reflects both coding and non-coding regions of the genome (Vanijajiva et al., 2005). However, some of the problems with RAPD are related to reproducibility, designing appropriate primers and amplification of RAPD-PCR products.

RAPD have been used earlier to study *Eucalyptus* and among its many utilities, emphasization of the characterization of diversity in *Eucalyptus grandis* and *Eucalyptus urophylla* for diallel cross design (Muro-Abad et al., 2001), estimation of genetic variability in

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Table 1. Primer sequences and codes used to study the variation among different species of *Eucalyptus*.

S/N	Primer code	Sequence (from 5`to 3`)
1	OP-B12	5´ CCTTGACGCA 3`
2	OP-C15	5` GACGGATCAG 3`
3	OP-D01	5` ACCGCGAAGC 3
4	OP-I15	5` AAG AGA GGG G3`
5	OP-L13	5` ACC GCC TGC T 3`

Eucalyptus microtheca (Li, 2000), identification of somaclonal variation in micropropagated *Eucalyptus* spp. clones (Laia et al., 2000; Lange et al., 1993), analysis of the genetic diversity in commercial *Eucalyptus* spp. clones (Rocha et al., 2002), construction of genetic maps, and quantitative trait loci (QTL) detection (Grattapaglia and Sederoff, 1994; Verhaegen et al., 1997), and to evaluate genetic variability between individuals (Marques et al., 1999).

The purpose of the present study was to assess the genetic diversity within four species of *Eucalyptus* grown in Egypt (*Eucalyptus citridora* Hook., *Eucalyptus camaldulensis* Dehn., *Eucalyptus gomphocephala* DC. and *Eucalyptus resinfera* Sm.) using RAPD markers and to compare their genotypes with each other.

MATERIALS AND METHODS

Plant materials

Fresh leaves of four adapted *Eucalyptus* species were collected in February 2009 from various localities in Egypt. These species represent the more dominant cultivated plants for ornamentals, medicinal importance and street trees in Egypt. All selected trees were healthy, uniform in growth and without visible symptoms of diseases or insects infection. Voucher specimens deposited in the QNA Herbarium indicate voucher numbers (Proposed abbreviation).

DNA extraction

Total genomic DNA was isolated from young trees and fresh leaf samples were collected separately from 10 trees for each specie. All leave samples were saved in ice box and quickly transported to laboratory. Plant tissues were ground under liquid nitrogen to a fine powder, then bulked DNA extraction was performed using DNeasy plant Mini Kit (QIAGEN).

Polymerase chain reaction

DNA amplification protocol was performed as described by Williams et al. (1990) with some modifications. PCR amplification was performed using five random ten-mer arbitrary primers (OP-B12, OP-C15, OP-D01, OP-I15, and OP-L13) (Operon Biotechnologies, Inc.). Their sequences are shown in Table 1. The DNA amplifications were conducted in 25 µl reaction volume containing the following reagents: 2.5 µl of dNTPs (2.5 mM), 2.5 µl $Mgcl_2$ (2.5 mM), and 2.5 µl of 10 × buffer, 3.0 µl of primer (10 pmol), 3.0 µl of template DNA (25 ng/µl), 1 µl of *Taq* polymerase (1U/ µl) and 10.5 µl of sterile dd H_2O. The reactions were performed in an automated thermal cycle (model Techno 512) programmed for one cycle at 94°C for 4 min followed by 45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C. The reaction was finally stored at 72°C for 10 min.

Amplified products were size-fractioned using 100 bp ladder marker (Fermentas) by electrophoresis in 1.5% agarose gels in TBE buffer at 120 V for 1 h. The bands were visualized by ethidium bromide under UV florescence and photographed.

Data analysis

The photographs of gels were used to score data for RAPD markers. RAPDs behave as dominant markers (Shiran et al., 2007), thus, they tend to be a bistate (present-absent) type of scoring. Each DNA fragment amplified by a given primer was considered as a unit character and the RAPD fragments were scored as a binary variable (1) for presence and (0) for absence of each of the primer accession combinations.

Agarose gel photos were scanned by the Gene Profiler 4.03 computer software program that uses automatic lane and peak finding for detecting the presence of banding patterns, and calibrating them for size and intensity. A binary data matrix recording the presence (1) or the absence (0) of bands was made. The software package MVSP (Multi-Variate Statistical Package) was used and genetic similarities were computed using the Dice coefficient of similarity of Nei and Li (1979):

$$\text{Similarity} = \frac{2 * n11}{(2 * n11) + n01 + n10}$$

Where, n11 is the number of common bands for the two compared samples; n10 is cases where the bands were visible only in the first sample and n01 is when bands were visible in the other sample only (Dice, 1945).

Unique bands detected in a particular genotype but not in others were used as positive DNA markers. The absence of a common band for a given genotype was referred to as a negative specific marker. The polymorphic fragment of electrophoretic DNA, generated by RAPD- PCR amplification with five primers, were used to identify and characterize the relationship between the species.

Cluster analysis was carried out on similarity estimates using the unweighted pair-group method with arithmetic average (UPGMA) software. These methods were carried out using MVSP software programs as in Youssef (2004). The results were then represented as a dendrogram for allover primers.

RESULTS AND DISCUSSION

Detection of genetic variation using RAPD-PCR analysis

Results obtained from RAPD analysis of four species of the genus *Eucalyptus*, revealed that the number of fragments amplified for each primer varied between 13 and 14 fragments and their size ranged from 95 to 1055 (Tables 3 to 7). The total number of bands for all five primers was 67, the total number of monomorphic bands was 32, the total number of polymorphic bands was 35,

Table 2. Primers names, polymorphic, monomorphic bands and polymorphism percent detected by RAPD analysis in camphor cultivars discrimination.

Primer number	Sequences	Total amplified band	Monomorphic band	Polymorphic band	Unique band	Polymorphism (%)
OP-B12	5` TTC GAG CCA G 3`	13	9	4	4	30.7
OP-C15	5` CTCACCGTCC 3`	14	3	11	11	78.5
OP-D01	5` CTCACCGTCC 3`	13	9	4	4	30.7
OP-I15	5` CAGCACCCCA 3`	14	6	8	5	57.14
OP-L13	5` GGGTGGGTAA3`	13	5	8	5	61.5
Total		67	32	35	29	52.2

Table 3. Survey of RAPD markers using primer OP-B12.

Band number	Molecular weight	*E. citriodora*	*E. camaldulensis*	*E. gomphocephala*	*E. resinifera*
1	850	1	0	0	0
2	680	1	1	1	1
3	640	1	0	0	0
4	590	1	0	0	0
5	550	1	1	1	1
6	500	1	0	0	0
7	445	1	1	1	1
8	370	1	1	1	1
9	325	1	1	1	1
10	295	1	1	1	1
11	240	1	1	1	1
12	205	1	1	1	1
13	150	1	1	1	1
Total		13	9	9	9

Presence= 1, while absence= 0.

and the polymorphism percentage was 52.2%. The characterization of the fragments generated by the array of the five primers is surveyed in Tables 3 to 7. The description of each primer, and generated bands are summarized further.

Primer OP-B12

The results of RAPD–analysis obtained by primer OP-B12 are illustrated in Figure 1 and Table 3. The number of amplified fragments generated by this primer was 13, which ranged in size between 150 and 850 bp, and the number of bands among species of the genus *Eucalyptus* ranged between 9 and 13. Table 2 shows that the polymorphic percentage (30.7%) for species of the genus *Eucalyptus*. Results in Figure 1 and Table 3 demonstrated that the fragments of molecular weight (150, 205, 240, 295, 325, 370, 445, 550, and 680 bp) were common in all four species of *Eucalyptus*.

Primer OP-C15

Data in Figure 2 and Table 4 illustrate results of RAPD-analysis obtained by this primer. The number of amplified fragments generated by this primer was 14, which ranged in size between 95 and 945 bp, and the number of bands among species of the genus *Eucalyptus* ranged between 8 and 10. Table 2 shows that the polymorphic percentage was 78.5% for species of the genus *Eucalyptus*. Results in Figure 2 and Table 4 demonstrated also that the fragments of molecular weight (95, 205 and 220 bp) were common in all four species of *Eucalyptus*.

Primer OP-D01

This primer contains 70% (G + C) as in Figure 3 and Table 5. The genus *Eucalyptus* and the total number of bands generated by this primer was of 13, which ranged in size between 145 and 690 bp, and the number of

Table 4. Survey of RAPD markers using primer OP-C15.

Band number	Molecular weight	*E. citriodora*	*E. camaldulensis*	*E. gomphocephala*	*E. resinifera*
1	945	1	0	0	0
2	885	1	0	0	0
3	800	1	0	0	0
4	725	1	0	0	0
5	630	0	1	1	1
6	560	1	0	0	0
7	525	0	1	1	1
8	360	0	1	1	1
9	270	0	1	1	1
10	250	0	1	1	1
11	220	1	1	1	1
12	205	1	1	1	1
13	175	0	1	1	1
14	95	1	1	1	1
Total		8	9	9	10

Presence= 1, while absence= 0.

Table 5. Survey of RAPD markers using primer OP-D01.

Band number	Molecular weight	*E. citriodora*	*E. camaldulensis*	*E. gomphocephala*	*E. resinifera*
1	690	0	0	1	0
2	560	1	1	1	1
3	520	1	1	1	1
4	435	0	0	0	1
5	395	0	0	0	1
6	360	1	1	1	1
7	330	1	1	1	1
8	305	1	1	1	1
9	250	1	1	1	1
10	230	1	1	1	1
11	195	1	1	1	1
12	175	0	1	1	1
13	145	1	1	1	1
Total		9	10	11	13

Presence= 1, while absence= 0.

bands among species of the genus *Eucalyptus* ranged between 9 and 13. Table 2 shows that the polymorphic percentage for species of the genus *Eucalyptus* was 30.7%. Results in Figure 3 and Table 5 demonstrated that the fragments of molecular weight (145, 195, 230, 250, 305, 330, 360, 520 and 560 bp) were common in all four species of *Eucalyptus***.**

Primer OP-I15

RAPD analysis by using primer OP-I15 are illustrated in Figure 4 and Table 6. The total number of bands generated by this primer was of 14, which ranged in size between 225 and 1055 bp, and the number of bands among species of the genus *Eucalyptus* ranged between 9 and 10. The polymorphic percentage was 57.14% as shown in Table 2 for species of the genus *Eucalyptus*. Results in Figure 4 and Table 6 demonstrated also that the fragments of molecular weight (225, 250, 630, 745, 845 and 980 bp) were common in all four species of *Eucalyptus.*

Primer OP-L13

RAPD analysis by using oprimer OP–L13 are illustrated

Table 6. Survey of RAPD markers using primer OP-I15.

Band number	Molecular weight	*E. citriodora*	*E. camaldulensis*	*E. gomphocephala*	*E. resinifera*
1	1055	0	0	1	1
2	980	1	1	1	1
3	845	1	1	1	1
4	745	1	1	1	1
5	630	1	1	1	1
6	530	0	1	1	1
7	480	1	0	0	0
8	445	0	1	1	1
9	420	1	0	0	0
10	400	0	0	1	1
11	335	1	1	1	0
12	285	0	1	1	0
13	250	1	1	1	1
14	225	1	1	1	1
Total		9	10	10	10

Presence= 1, while absence= 0.

Table 7. Survey of RAPD markers using primer OP-L13.

Band number	Molecular weight	*E. citriodora*	*E. camaldulensis*	*E. gomphocephala*	*E. resinifera*
1	750	0	0	1	1
2	685	1	1	1	1
3	600	1	1	1	1
4	540	1	1	1	1
5	475	1	1	1	1
6	405	0	1	1	1
7	290	1	0	0	0
8	260	0	1	1	1
9	235	1	0	0	0
10	210	0	0	1	1
11	200	1	1	1	0
12	180	0	1	1	0
13	150	1	1	1	1
Total		8	9	11	9

Presence= 1, while absence= 0.

in Figure 5 and Table 7. The total number of bands generated by this primer was 13, which ranged in size between 150 and 750 bp, and the number of bands among species of the genus *Eucalyptus* ranged between 8 and 11. The polymorphic percentage was 61.5% as shown in Table 2 for species of the genus *Eucalyptus*. Results of reaction obtained by this primer demonstrated that fragments of size 150, 475, 540, 600 and 685 bp were common in all four species of *Eucalyptus.*

The most effective primer was OP-C15 which produced 14 bands. The primer gave the following ratios of polymorphic bands: 78.5% for OP-C15, 61.5% for OP-L13, 57.14% for OP-I15 and 30.7% for OP-B12 and OP-D01. The highest ratio of polymorphism was 78.5% (OP-C15) while the lowest ratio was 30.7% (OP-B12 and OP-D01). All the five primers used in the present study produced high degree of polymorphism.

Specific DNA markers

Unique DNA fragments with different sizes were detected

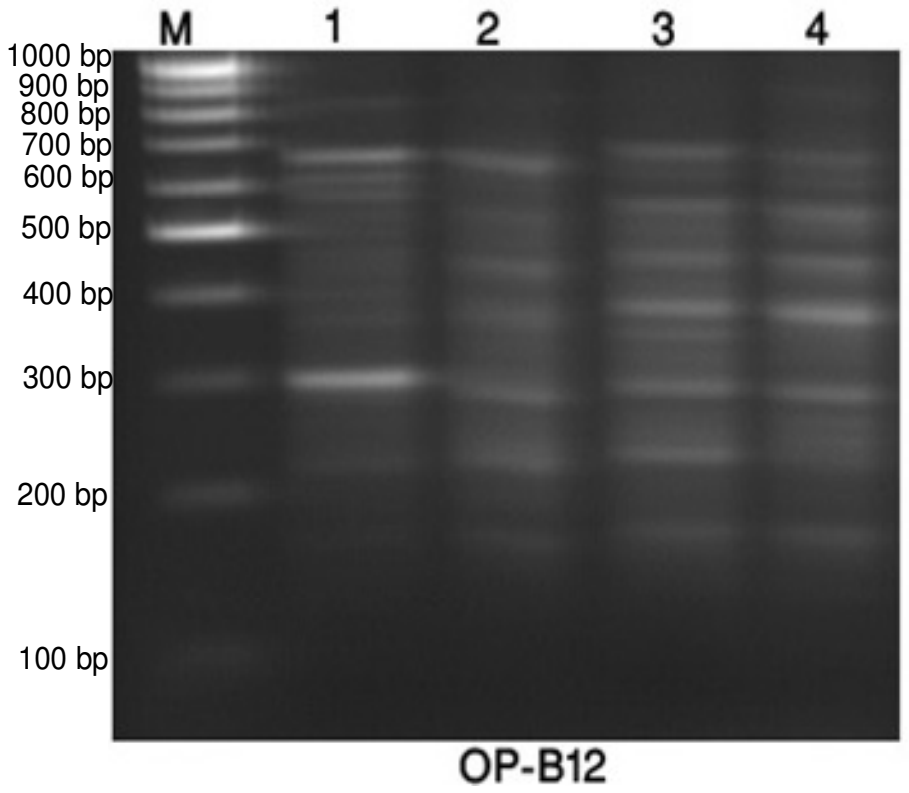

Figure 1. Agarose gel electrophoresis of RAPD products by OP-B12 primer or Agarose gel electrophoresis of RAPD profiles in four species of the genus *Eucalyptus* (1-4) generated by OP-B12 primer, where lane 1) *E. citriodora*, lane 2) *E. camaldulensis*, 3 lane) *E. gomphocephala* and lane 4) *E. resinifera*.

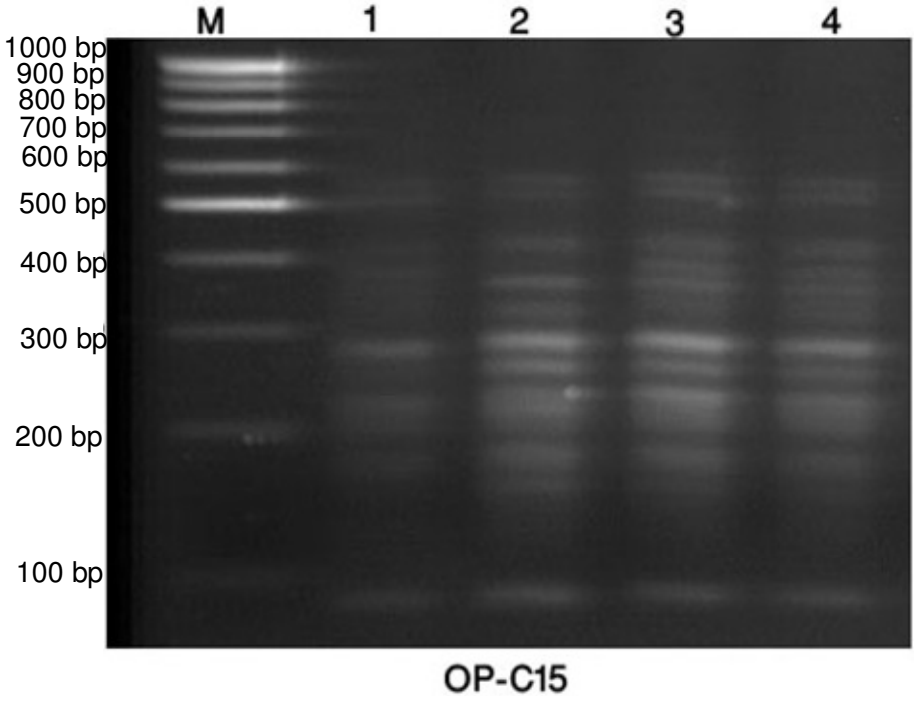

Figure 2. Agarose gel electrophoresis of RAPD products by OP-C15 primer.

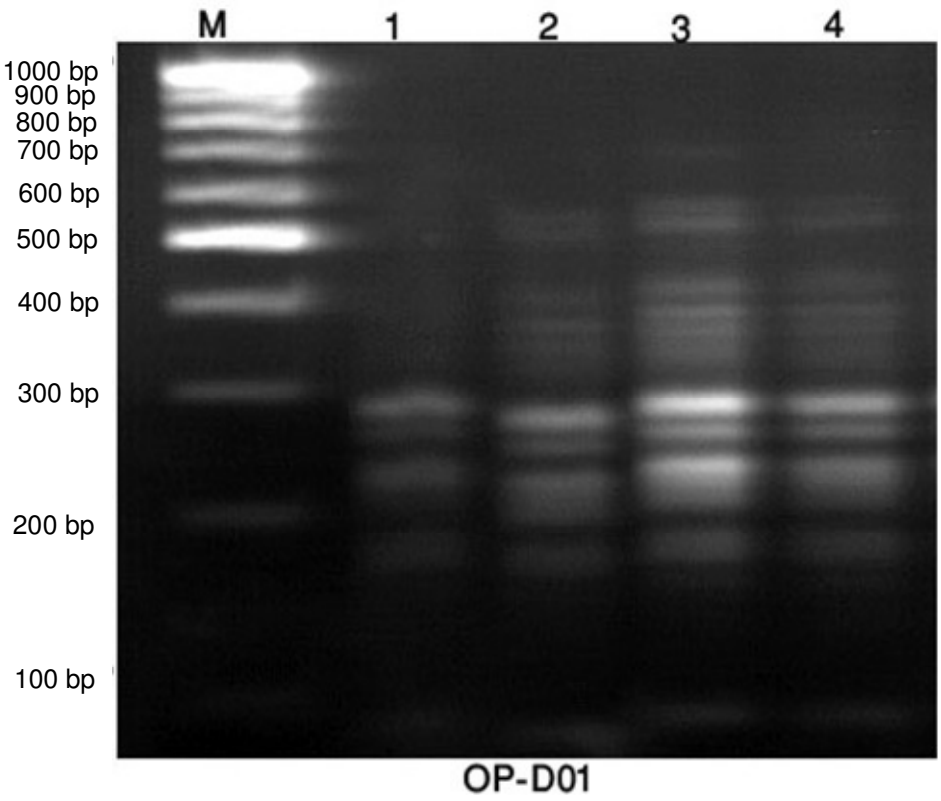

Figure 3. Agarose gel electrophoresis of RAPD products by OP-D01 primer.

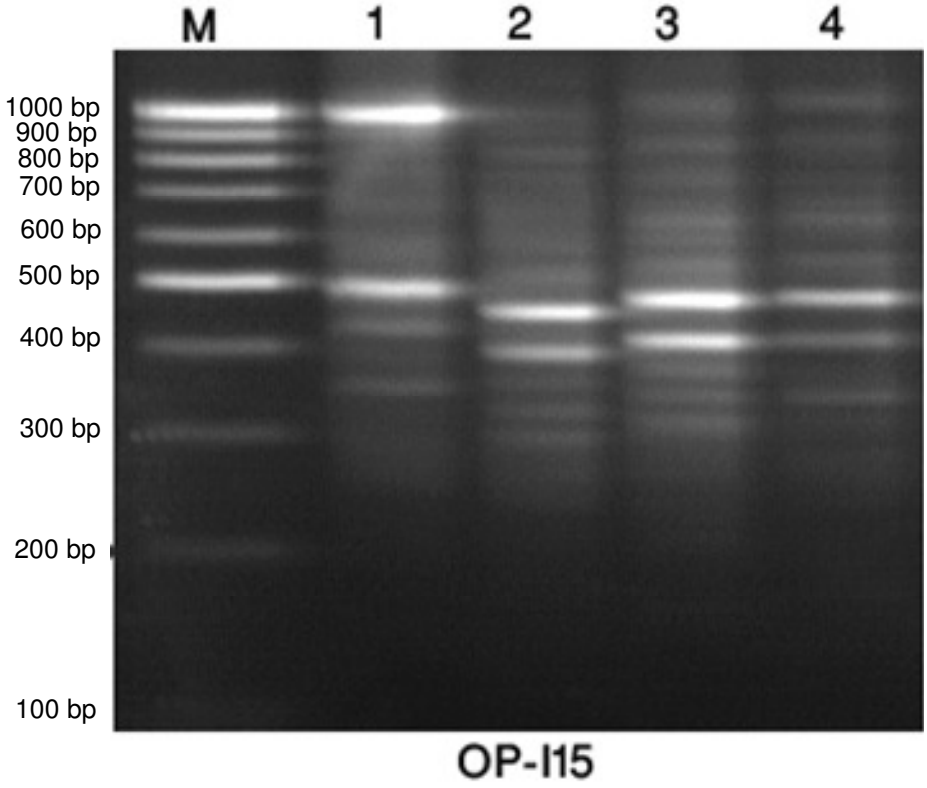

Figure 4. Agarose gel electrophoresis of RAPD products by OP-I15 primer.

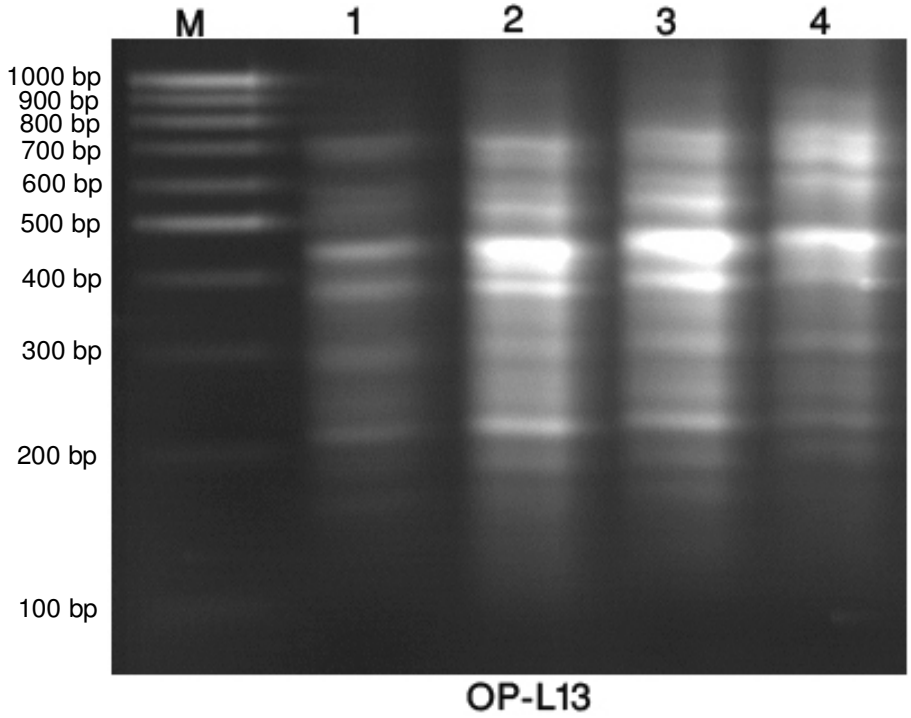

Figure 5. Agarose gel electrophoresis of RAPD products by OP-L13 primer.

Table 8. Genetic similarity values calculated from the DNA fragments amplified from the different genotypes of four species of *Eucalyptus* using five RAPD primers.

Similarity matrix	*E. citriodora*	*E. camaldulensis*	*E. gomphocephala*	*E. resinifera*
E. citriodora	1.00	-	-	-
E. camaldulensis	0.717	1.00	-	-
E. gomphocephala	0.68	0.948	1.00	
E. resinifera	0.659	0.893	0.929	1.00

in particular genotype but not in the others. The presence or absence of such DNA fragments in a particular genotype could be used as positive or negative specific DNA markers for such genotype and might be helpful in genotype identification and discrimination. In the present investigation, 16 positive and 13 negative markers were detected by the tested primers (Table 8). The marker fragments size ranged from 235 to 945 bps for the positive markers and from 175 to 630 bps for the negative markers.

In primer OP-B12, 4 positive markers with fragments of molecular weight 850, 640, 590, and 500 bps were detected in *E. citriodora*. In primer OP-C15, 5 positive markers (560, 725, 800, 885, 945 bps) were observed in *E. citriodora*; in addition, 6 negative (175, 250, 270, 360, 525, 630 bps) markers were also observed in *E. citriodora*.

In primer OP-D01, 3 positive markers (690) was observed in *E. gomphocephala* and 395, 435 in *E. resinifera*; in addition, one negative marker (175) was observed in *E. citriodora*. In primer OP-I15, 2 positive markers (420, 480) were observed in *E. citriodora*. 3 negative markers (445, 530) were shown in *E. citriodora* and a marker (335) was recorded in *E. resinifera*.

In primer OP-L13, 2 positive markers (235, 290) were observed in *E. citriodora*. 3 negative markers (260, 405) were observed in *E. citriodora* and a marker (200) was observed in *E. resinifera*. Meanwhile, no unique band was recorded in *E. camaldulensis*.

Genetic similarity matrix and cluster analysis

According to the similarity matrix of the 4 genotypes combinations (Table 9), the highest similarity (0.948%) was found between *E. gomphocephala* and *E. camaldulensis* while the lowest similarity (0.659%) was between *E. resinifera* and *E. citriodora*. Figure 6 reveals the dendrogram tree of the four species of *Eucalyptus* resulting from the UPGMA of values presented in Table

Table 9. Unique DNA fragments (markers) of different genotypes and their molecular weights detected by the different employed primers.

Primer	Genotype	Positive marker (amplified fragment)	Negative marker (non-amplified fragment)
OP-B12	*E. citriodora*	850	-
		640	-
		590	-
		500	-
OP-C15	*E. citriodora*	945	-
		885	-
		800	-
		725	-
		560	-
		-	630
		-	525
		-	360
		-	270
		-	250
		-	175
OP-D01	*E. gomphocephala*	690	-
	E. resinifera	435	-
		395	-
	E. citriodora	-	175
OP-I15	*E. citriodora*	-	530
		480	-
		-	445
		420	-
	E. resinifera	-	335
OP-L13	*E. citriodora*	-	405
		290	-
		-	260
		235	-
	E. resinifera	-	200

9. Cluster analysis by UPGAM suggests the existence of groups with higher similarities. In the dendrogram, the species of *Eucalyptus* formed two main groups. Three species were clustered together in the first main group, one of which (*E. resinifera*) was clustered in the first subgroup and two (*E. gomphocephala, E. camaldulensis*) were in the second subgroup. In the second main group, the *E. citriodora* were clustered.

Besides the use of a particular type of molecular marker, molecular characterization also depends on successful isolation of quality DNA. Problems are reported for the isolation of plant DNA. DNA preparations may contain colored substances, polysaccharides and phenolic compounds (Aras et al., 2003; Temiesak et al., 1993; Vanijajiva et al., 2005). The use of DNeasy Plant Mini Kit allowed the isolation of DNA from the plants studied, which we found suitable for RAPD-PCR amplification.

The classification between various subgenera, species and subspecies is based primarily on morphological attributes. However, these morphological characters may be unstable and influenced by environmental conditions (Goodrich et al., 1985). Over the years, the methods for detecting and assessing genetic diversity have extended from analysis of discrete morphological traits to biochemical and molecular trait. However, the advents of the protein electrophoresis as an analytical tool provides indirect methods for genome probing (Cooke, 1984; Gilliand, 1989).

This is the first report describing the genetic diversity in four species *of Eucalyptus* using RAPD markers. The use of RAPD markers to identify genetic variations was

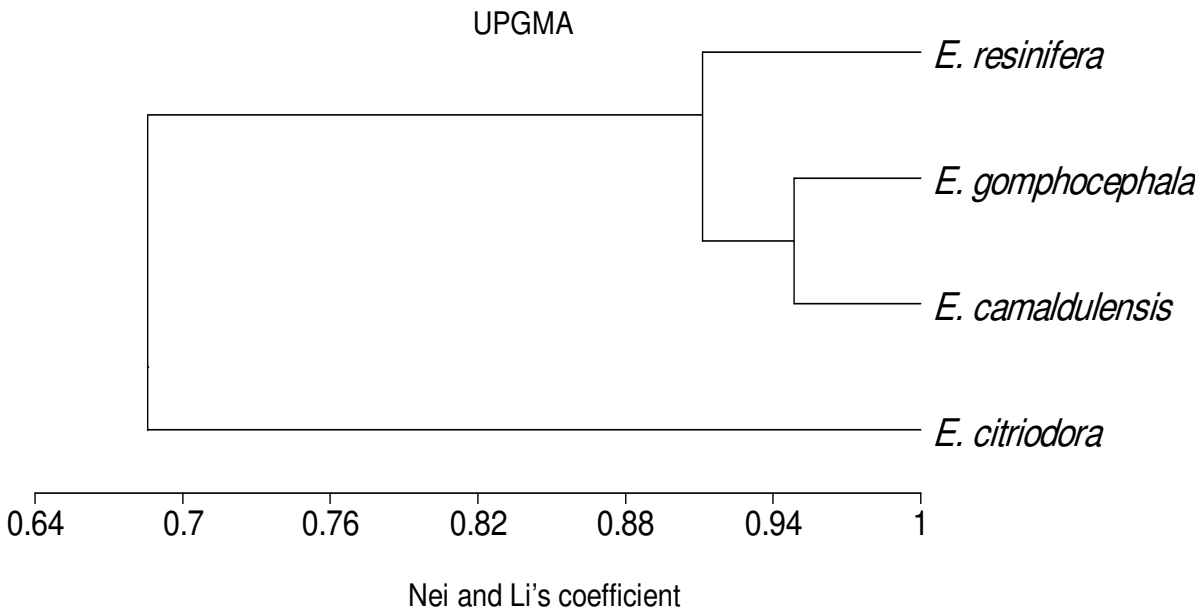

Figure 6. Dendrogram demonstrating the relationship among four species of *Eucalyptus* based on data recorded from polymorphism of RAPD markers.

preferred over conventional morphological and biochemical markers since these are completely devoid of any interference from environmental effect and growth stages of experimental material; thus, making them highly reliable.

Conclusion

In this study, the RAPD technique was used to identify *Eucalyptus* species genome. This strategy is rapid, simple, and produces reliable results, since it was possible to demonstrate that a standard set of primers can be used to distinguish *Eucalyptus* species. Phenotypic traits, whether morphological or chemotypic, are results of the genotype expression, while DNA markers are independent of environment, age and tissue and are expected to reveal the genetic variation more conclusively in assessing such variations.

Traditional classification of plant species is based mainly on the morphological and anatomic characters, however, these features are changeable and sometimes difficult to observe, so it is necessary to be supported by molecular techniques. This study represents a first approach in using molecular markers as a tool to study molecular systematics in *Eucalyptus*. In future studies, the analysis of additional population and species, additional primers for RAPD-PCR studies and the use of different types of molecular markers such as AFLP (amplified fragment length polymorphism), SSR (self sustained sequence replication), and ITS (international transcribed spacers) will improve the accuracy of resolution of accurate *Eucalyptus* classification.

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