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PHYLOGENETIC DIVERSITY AND RELATIONSHIPS AMONG SIX *Aloe* SPECIES GROWN IN EGYPT USING RAPD AND MORPHOLOGICAL CHARACTERS

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ABSTRACT

This study was performed on six species belongs to genus *Aloe* grown in Egypt; namely; *Aloe barbadensis, A. camperi, A. ciliaris, A. grandidentata, A. nobilis* and *A. vera*. The aim of this study is to explore the taxonomic relationships between these species using randomly amplified polymorphic DNA (RAPD) markers to elucidate the pattern of genetic diversity among these individuals of different species of *Aloe*. In addition to the morphological characters. A total of 9 RAPD primers were screened of six spices of *Aloe.* The percentage of polymorphic bands was 91.33% with molecular size ranged from 0.100 kb to 2.5 kb. Only 3 of 29 bands were commonly detected in all samples which reflected certain homology of sample. From the obtained results the six *Aloe* species were divided into three clusters, cluster (I) containing *Aloe barbadensis* and *Aloe vera* at homology 20% related with cluster (II) at homology 7% which containing *Aloe grandidentata and Aloe nobilis*. The last cluster (III) containing *Aloe camperi* and *Aloe ciliaris* at homology 12%.

Key words: *Aloe*, RAPD and morphological characters.

INTRODUCTION

Aloe, a genus belonging to a family Liliaceae or Aloaceae. It is to be continued for a family Asphodelaceae, subfamily Asphodeloideae (Stevens, 2001). In the past, it has been assigned to the family Aloaceae (now included in the Asphodelaceae) or to a broadly circumscribed family Liliaceae (the lily family). The plant *Agave americana*, which is sometimes called "American Aloe", belongs to the Asparagaceae, a different family.

According to International Rules of Botanical Nomenclature, *Aloe vera* (L.) is the legitimate name for this species (Newton, 1979; Tucker *et al.,* 1989 ; Bradley, 1992). The genus *Aloe* has also been placed taxonomically in a family called Aloeaceae. It is an important medicinal plant with many cosmetic properties. This plant is cultivated throughout the world for its thick flesh leaves from which many medicinal and cosmetic products are prepared. It has been shown that the products of this plant are anti-bacterial Ferro *et al.* (2003), anti-viral Kahlon *et al.* (1991), anti-fungal Kawai *et al.* (1998) and have properties like anti-septic, anti-tumoral, anti-inflammatory, anti-oxidant and immune-stimulant Weiner and Weiner (1994). The juice of this plant is considered useful in stomach disorders Foster *et al.* (1999). It is one of herbaceous or woody plants, and characterized by stemless, large, thick and fleshy leaves, a sharp apex and a spiny margin. *Aloe* is widely distributed in the world Steenkamp and Stewart (2007). Most *Aloe* species have a rosette of large, thick, fleshy leaves. *Aloe* flowers are tubular, frequently yellow, orange, pink, or red, and are borne, densely clustered and pendant, at the apex of simple or branched, leafless stems. Many species of *Aloe* appear to be stemless, with the

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rosette growing directly at ground level; other varieties may have a branched or unbranched stem from which the fleshy leaves spring. They vary in colour from grey to bright-green and are sometimes striped or mottled. Some Aloes native to South Africa are tree-like arborescent (Rodd and Stackhouse, 2008). A number of different *Aloe* species are marketed as *Aloe* products. The important species contributing to the great market of *Aloe* products throughout the world are *Aloe andongensis, A. arborescens* (Kidachi *Aloe*), *A. vera* (=*A. barbadensis*), *A. ferox (*Cape *Aloe)*. Out of these species, *Aloe vera* scores in quantity and quality over the others.

Molecular markers could be reflect the differences amang species directly without affecting the environment (Wang *et al.,* 1996). Several molecular markers particularly the Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP) and Variable Number of Tandem Repeats (VNTR) have been proven useful in detecting genetic diversity. RAPD technique has several advantages such as speed, low cost and the usage of small amounts of plant materials (Becerra and Gepts, 1993; Heun *et al.,* 1994; Jain *et al.,* 1994).

Various molecular markers have been developed as powerful tools for genetic diversity analysis and establishing relationships between species and cultivars. The assessment of genotypic identity among individuals of a species is central to making valid biological interpretations for population structure, breeding systems, reproductive biology and micro evolutionary processes within and among the species. Among various molecular markers, the Random Amplified Polymorphic DNA (RAPD) is most widely used because it allows a rapid and inexpensive assay with different primers (Williams *et al.,* 1990). Due to the technical simplicity and speed of RAPD methodology, it has been successfully used for the assessment of genetic structure and phylogenetic analysis (Gepts, 1993).

Therefore, the present study was carried out characterize the local *Aloe* germplasm which collected from different geographical regions of Egypt that cultivated in the garden of Applied Research Center for Medicinal Plants, National Organization for Drug Control and Research "NODCAR" Giza, Egypt. So the main objective of this study was to assess the genetic variation among local *Aloe* germplasm using RAPD markers.

MATERIALS AND METHODS

Plant Material

The available local *Aloe* germplasm at the garden of Applied Research Center for Medicinal Plants, National Organization for Drug Control and Research "NODCAR" Giza, Egypt were used. Six spices of *Aloe* representing different geographical regions of Egypt were used for the study. *Aloe barbadensis, Aloe camperi, Aloe ciliaris, Aloe grandidentata, Aloe nobilis,* and *Aloe vera.* All the plants used in the present study were about five years of age.

Morphological Characteristics

Characters such as plant height, leaf length, leaf width and leaf texture, colour, leaf apex and leaf margin were recorded in all *Aloe* germplasm for comparative studies.

Isolation of DNA

PCR based analytical methods are highly sensitive to the purity of DNA templates (Yau and Nagan, 2002). DNA isolation from plant materials is not always simple or routine (Smith *et al*., 1991). Unlike the non plant DNA isolation protocols, the methods need to be adjusted to each plant specie and even for each tissue due to the pleothora of primary and secondary metabolites in plants (Sangwan *et al.,* 1998). DNA was isolated from 3 g of the leaf tissue of all species (excluding the gel like mesodermal region) according to the protocol described by (Khanuja *et al.,* 1999) and dissolved in 200 µl of high salt TE buffer (pH 8.0). For the removal of RNA, 15 µg of RNAse was added to the DNA solution and incubated for 10 minutes at 37°C. The plant tissues were ground using both a mortar and pestle after -80°C treatment for 1 week. Modified CTAB DNA extraction protocol (Doyle and Doyle, 1987; Shah *et al.,* 2000) was applied using ground tissue. The DNA was then purified by phenol: chloroform extraction and ethanol precipitation. The DNA obtained dissolved in

TE buffer. The DNA obtained was run on 0.8% agarose gels (in 1 × TBE) containing ethidium bromide (1.5 µl ethidium bromide/100 ml gel). Initially, 2 µl DNA with 0.5 µl loading dye was used. All the specimens were run at once at 100 V (70 mA) for an appropriate time. The gel was visualized under a UV source and the amount of DNA per µl was estimated by relative comparisons of the DNA bands with each other and the λ-DNA ladder digested with restriction enzymes. An RNAse A treatment (0.2 µl per 50 µl) for each sample for 1 hr., at 37°C was performed in a shaking water bath.

The quantity and purity of the DNA were assessed, checked for quality and purity by electrophoresis in a agarose gel in 1 × TAE buffer *(*Sambrook *et al.*, 1989). Quantification of DNA was done by UV spectrophotometer (Hewlett Packard 8453, USA) by measuring optical densities at 260 nm and the ratio of OD 260/280 nm., respectively. Finally all DNA samples were diluted to get 50 ng /µl then solutions were stored at -20°C for use in RAPD assay.

RAPD Amplification

PCR amplications were carried out in a 20 µl reaction mix containing 50 ng template DNA, 1× Buffer (provided by the manufacturer of the Taq enzyme), 200 µM each dNTP (QIAGEN), 0.6 µM primer (Operon Primer Technology), 2, 5-4 mM MgCl2 (Table 1), 0, 75 U Taq DNA polymerase (2.5 units/reaction) (QIAGEN). PCR reactions were run in a T-GRADIENT Thermocycler, cat. ≠ 050-801 (Biometera, Inc. Germany) according to the following program: one cycle of 95°C for 15 min, 40-45 cycles (Table 1) of 95°C for 60 sec, annealing temperature of each primer (Table 1) for 60 sec, 72°C for 90 sec., and final elongation cycle of 10 min at 72°C. Reproducibility of amplification profiles was tested for each primer. Only those bands consistently reproduced in different analyses were considered. Poor amplifications occurred systematically with individuals from different populations; these were excluded from the analysis and they mainly account for the different sample sizes of this study.

Amplified fragments were scored as presence (1) or absence (0) of individuals. The data was used for similarity-based analysis using the software program (Biogene Software).RAPD analyses were analyzed using the Nei genetic similarity index (Nei and Li, 1979). On the basis of the equation, SI= 2Nij / (Ni + Nj)

Where Nij is the number of common bands shared between samples i and j, Ni and Nj are the total number of DNA bands for geno types i and j, respectively. A dendrogram was constructed on the basis of the similarity matrix data by unweighted pair group method arithmetic average (UPGMA) cluster analysis using Biogen software VILBER LOURMAT. Inc., France.

RESULTS AND DISCUSSION

Morphological Characteristics

Data presented in Table 2 and Fig. 1 indicate that all studied species have striping leaf shape. *A. barbadensis* showed the maximum leaf length and width (103-110 × 12-13.2 cm) over all studied species, whereas, *A. vera* showed the minimum values (3-4 × 0.4-0.5 cm). *A. camperi* and *A. grandidentata* recorded intermediate values (39-48 × 3.2-4.4) and (36-41 × 3.8-5.4), respectively. Both, *A. ciliaris* and *A. nobilis* were nearly the same for length and width (11.5-15 × 2.8 × 3.5) and (8.5-10 × 3.2 × 4.5), respectively.

A. camperi and *A. vera* have grayish green leaves, *A. barbadensis* has dull grayish green, *A. ciliaris* has dark green leaves. *A. grandidentata* has pale green leaves with irregular white blotches. *A. nobilis* has lustrous green leaves with white lines.

It is clear from data that leaf texture of *A. ciliars*, *A. nobilis* and *A. vera* have fleshy thick, while *A. barbadensis* and *A. grandidentata* have leathery texture. *A. camperi* has fleshy texture. All studied species have apex and margine spine.

RAPD Analysis

The RAPD technique provides a useful approach for evaluating genetic differentiation, particularly in those species that are poorly known genetically and are propagated vegetatively such as *Musa* (Bhat and Jarret, 1995) and *Lilium*

Table 1. 9-mer primers used for RAPD analysis, primer sequences, number of PCR cycles, optimal annealing temperatures and optimal MgCl2 concentration

Primer	Sequence 5` - 3`	N. cycles	T. annealing	MgCl2
OPB1	5` -GTTTCGCTCC-3`	40	44°C	2.5mM
OPB5	5`- TGCGCCCTTC- 3`	40	45°C	2.5mM
OPB6	5`- TGCTCTGCCC-3`	40	45°C	2.5mM
OPB7	5`-GGTGACGCAG-3`	45	47°C	2.5mM
OPB8	5`-GTCCACACGG– 3`	45	47°C	2.5mM
OPB10	5`-CTGCTGGGAC- 3`	40	38°C	2.5mM
OPB11	5`-GTAGACCCGT- 3`	40	44°C	2.5mM
OPB16	5`-CCAAGCTGCC- 3`	40	44°C	2.5mM
OPB17	5`-TGACCCGCCT- 3`	45	47°C	4.0mM

15 µl aliquots of amplification products were separated on 1.6 (*W/V*) agarose gels containing ethidium bromide (0.5 µg/ml) in 1X TAE buffer and gels were photographed using VILBER LOURMAT. Inc., France. Gel Documentation System.

Table 2. Morphological information of the leaves of the six species of *Aloe*

Character	*Aloe barbadensis*	*A. camperi*	*A. ciliaris*	*A. grandidentata*	*A. nobilis*	*A. vera*
Shape	striping	striping	striping	striping	striping	striping
Length cm	103-110	39-48	11.5-15	36-41	8.5-10	3-4
Width cm	12-13.2	3.2-4.4	2.8+3.5	3.8-5.4	3.2-4.5	0.4-0.5
Colour	Dull grayish green	Grayish green	Dark green	Pale green with irregular white blotches	Iustrous green with white lines	Grayish green
Texture	Leathery	Fleshy	Fleshy thick	Leathery	Fleshy thick	Fleshy thick
Apex	Spine	Spine	Spine	Spine	Spine	Spine
Margin	Spine	Spine	Spine	Spine	Spine	Spine

(1)

Aloe ciliaris

(2)

Aloe vera

(3)

Aloe grandidentata

(4)

Aloe nobilis

(5)

Aloe camperi

(6)

Aloe barbadensis

Fig. 1. Photographs showing leaves of the six studied *Aloe* species

(Haruki *et al.*, 1998). Not only the extent of variation but also RAPD provides markers even for cultivar identification (Torres *et al.*, 1993) and germplasm evaluation (Shioda *et al.*, 2003).

A total of 9 RAPD primers were screened of six spices of *Aloe*. Out of these, only 4 of the primers (Table 3 and Fig. 2) that showed reproducible results were chosen to amplify the whole six spices. A total of 29 bands were amplified among 6 species using 4 primers and the polymorphic bands 27. Monomorphic bands are those which present in all individuals polymorphic are present in one or more but not all individuals and unique ones are present in at least one individual not any other.

The mean percentage of polymorphic bands was 91.33% with molecular size ranged from 0.100 kb to 2.5 kb. Only 3 bands of 29 bands were commonly detected in all samples which reflected certain homology of sample. Sources of polymorphism in RAPD assay may be due to deletion, addition or substitution of base within the priming site sequence (Willams *et al.*, 1990). High diversity is the reflection of adaptation to environment, which is beneficial to its propagation, resources conservation, the domestication of wild species and the screen of specified locus.

Significant inter and intra specific variations could be visualized as evident from the similarity coefficients (Table 4) developed on the basis of relative indices among all possible pairs. Similarity values varied from 0.05 to 0.20 Maximum similarity of 20% was observed between *Aloe barbadensis* and *Aloe camperi* while lowest similarity of 5.0% was observed between *Aloe barbadensis* and *Aloe vera*. DNA-based markers such as RAPD, are practically unlimited in number, remain unaffected by environment and growth conditions and are simply inherited (Karihaloo *et al.*, 2003). Thus, this RAPD analysis provided phylogenetic relationships as well as some unique DNA bands to quantify the genetic variation.

A phylogenetic tree (Fig. 3) was constructed from four primers using unweighted pair-group method using arithmetic averages (UPGMA method) of (Nei and Li, 1979) (Biogene Software). The genetic distances among six *Aloe* species were shown diagrammatically by linkage dendrogram which resulting from those primers. In this case the linkage dendrogram provide a good visual idea about clustering and variabitity present in populations. The six *Aloe* species were divided into three clusters, cluster (I) containing *Aloe barbadensis* and *Aloe vera* at homology 20% related with cluster (II) at homology 7% which containing *Aloe grandidentata* and *Aloe nobilis*. The last cluster (III) containing *Aloe camperi* and *Aloe ciliaris* at homology12%. The same results were obiained in (Fig. 3 A, B). These results indicated that *Aloe barbadensis* and *Aloe vera* were closely related. Also *Aloe camperi* and *Aloe ciliaris* were closely related.

Although in (Fig. 3 C, D) The six *Aloe* species were divided into two clusters, cluster (I) containing *Aloe grandidentata* and *Aloe nobilis* at homology 20%, 28%, respectively related with cluster (II) at homology 10% which containing *Aloe camperi* and *Aloe vera*. Cluster analysis clearly branched out *Aloe barbadensis* and *Aloe ciliaris* from rest of *Aloe* species suggesting that these two are more divergent from other species. Hence, phenotypic characters combined with RAPD analysis provided a better relationship to identify these species.

The high levels of genetic diversity of RAPD markers observed in this study probably were associated with the extensive range of genetic diversity represented in *Aloe* species. We took into account not only differences in geographical range but also results of morphological variation. These results are in agreement with previous observations in soybeans, barley and corn (Powell *et al*., 1996; Russell *et al*., 1997; Pejic *et al*., 1998).

Conclusion

The results of this study have demonstrated that RAPD technique can be applied to measure the degree of variability between *Aloe* species. RAPD values for estimation of between species variation and a very good tool to investigate genetic variability of *Aloe* species.

Table 3. RAPD primers data and the percentage of polymorphic bands

Primer code	Nucleotide sequence	Size of frigment (bP)	Polymorphic band	Monomorphism (%)	Polymorphism (%)
OPB2	5-GTTTCGCTCE-3	250-2000	6	17.18	89.06
OPB5	5-GTCCACACGG-3	500-1000	5	-	100
OPB7	5-GTAGACCCGT-3	400-1500	8	-	100
OPB8	5-CCAAGCTGCC-3	500-1500	3	21.04	83

Table 4. Genetic similarity index from RAPD data of different special of *Aloe* species Nei's similarity coefficient

Sample	*Aloe barbdensis*	*A. camperi*	*A. cliaris*	*A. grandidentate*	*A. nobilis*	*A. vera*
A. barbdensis	1.00					
A. camperi	0.20	1.00				
A. ciliaris	0.06	0.06	1.00			
A. grandidentata	0.11	0.08	0.09	1.00		
A. nobilis	0.11	0.10	0.07	0.19	1.00	
A. vera	0.05	0.19	0.07	0.04	0.11	1.00

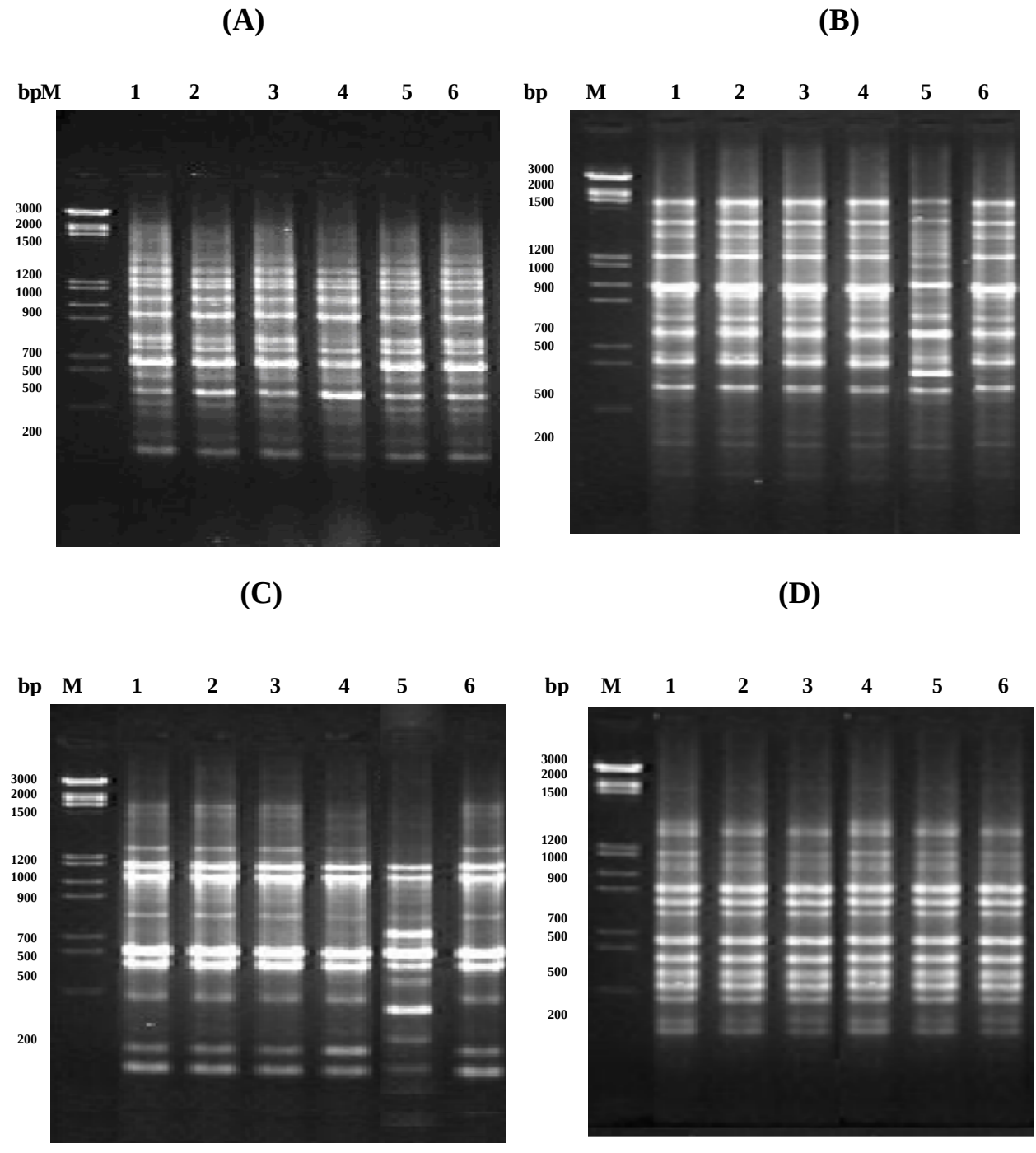

Fig. 2. Polymerase Chain Reaction-Amplified among six *Aloe* species showed random amplification of polymorphic DNA assay fingerprints, separated by gel electrophoresis and detected by ethidium bromide staining. Based on RAPDs. (A)=OPB2, (B)= OPB5, (C)= OPB7 and (D)= OPB8.(1)= *A. barbdensis*, (2)= *A. camperi* ,(3)= *A. ciliaris* ,(4)= *A.grandidentata*,(5)= *A. nobilis* and (6)= *A. vera*. M=GeneRuler™ DNA Ladder cat, No # SM0247/2/3 MBI Fermentas, Lithuania

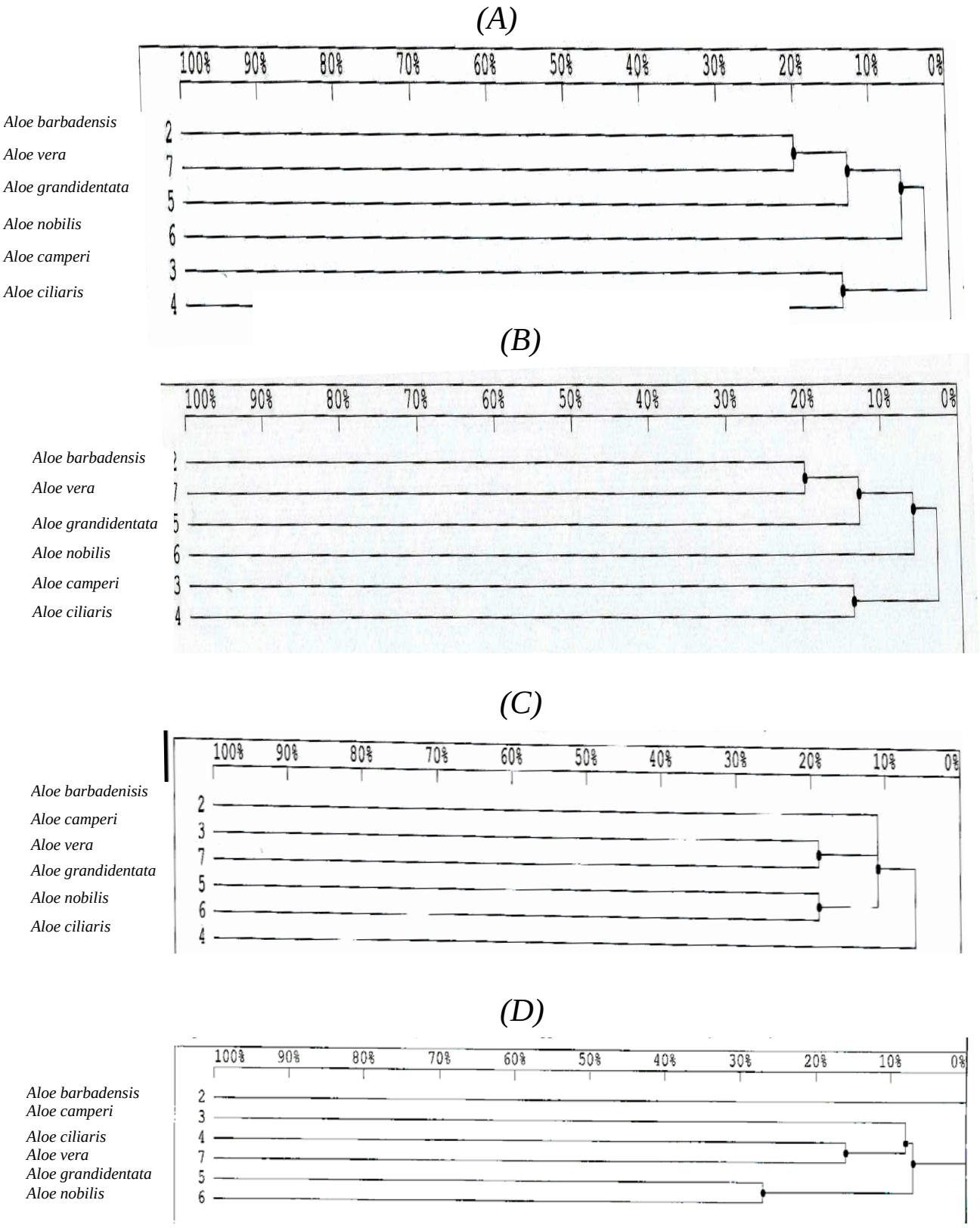

Fig. 3. Genetic similarity among six *Aloe* species revealed by UPGMA cluster analysis based on RAPDs. A=OPB2, B= OPB5, C= OPB7 and D= OPB8

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التباعد والعلاقات الوراثية بين ستة أنواع من الصبار النامى في مصر باستخدام الأشكال العشوائية للحمض النووى والصفات المورفولوجية

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١- معمل البيوتكنولوجى مركز الدراسات التطبيقية لبحوث النباتات الطبية- الهيئة القومية للرقابة والبحوث الدوائية القاهرة- مصر

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تم إجـــراء هـــذه الدراســة على ستة أنواع تتبـع جنس الصبار (*Aloe*) والمنزرعة في مصر وهذه الأنواع هي كالآتى: *Aloe barbadensis, A. camperi, A. ciliaris, A. grandidentata, A. nobilis* and *A. vera*، وكان الهدف من هـذه الدراسة هو أستكشاف العلاقة التقسيمية بين هذه الأنواع باستخدام الأشكال العشوائية للحمض النووى وذلك لتوضيح نمط التباعد الوراثى لهذه الأنواع المختلفة من *Aloe*، بالإضافة إلى الصفات المورفولوجيه، باستخدام عدد تسعه بادئات للأشكال العشوائية للحمض النووى تم عمل مسح للستة أنواع من *Aloe*، نسبة متوسط الاشكال العشوائية من الحمض النووى كانت ٩١.٣٣% عند الأوزان الجزيئية التى تتراوح بين ٠.١ كليو قاعدة نيوكليوتيدية و٢.٥ كليو قاعدة نيوكلويتيدية، فقـط ثـــلاث حزم من ٢٩ حزمه الموجودة كانت شائعة فى كل عينات *Aloe* مما أنعكس على التجانس بين العينات، النتـــائج أوضحـت أن الســـتة أنـــواع مـــن *Aloe* تم تقســـيمها إلى ثلاث مجموعات: المجموعة الأولى وتشمل *A. barbadensis* و *A. vera* عند مستوى تجانس٢٠% والذي يرتبـط مع المجموعة الثانية عند مستوى تجانس ٧% وهذه المجموعة تشمل *A. grandidentata* و*A. nobilis* أما المجموعة الثالثة والتي تحتوى على *A. camperi, A. ciliaris* مستوى تجانسها ١٢%.

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