The Application of Random Amplified Polymorphic DNA (RAPD) in the Classification of Egyptian Date Palm (*Phoenix dactylifera* L) Cultivars

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Abstract: This study aims to classify date palm **(*Phoenix dactylifera* L)** cultivars using Randomly Amplified Polymorphic DNA (RAPD). Eight date palm genotypes; Amhat, Barhy, Bint Aisha, Zaghloul, Hayany, Amry, Kuboshy and Samany cultivars and one male genotype were analysed. Twenty random primers were used to screen the genotypes but five produced reproducible bands. The five primers yielded 179 bands (with an average of 36 bands per primer), including eleven polymorphic bands. The primer OPK4 in particular, produced a large number of strongly amplified and individual fragments (47 fragments), whereas, primer OPK1 produced the lowest number (21 fragments). Primer OPK1 gave the highest percentage of polymorphism (9.52), while OPK4 gave the lowest percentage of polymorphism (4.25). Two primers OPK4 and OPK5 produced amplification products that were monomorphic across all the female cultivars. Three primers OPK1, OPK3 and OPK7 revealed low polymorphisms (6.14%). Analysis of all the bands recorded showed 93.36% similarity (11 polymorphic fragments out of a total of 179). The results showed distinct variation in the profiles within the female cultivars and male genotype especially with primers OPK1, OPK3 and OPK. RAPD-PCR showed that the male genotype is different from the female cultivars and that there is a high degree of similarity among the female cultivars of the date palm.

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1. Introduction

Molecular biology techniques based on the polymerase chain reaction (PCR) offer a new tool for genetic analysis and construction of linkage maps. The random amplified polymorphic DNA (RAPD) technique utilizes arbitrary primers for the amplification of genomic DNA, thus enabling identification of individual cultivars and differentiation of species (**Welsh and McClelland, 1990, Liu *et al.*, 2005., Gaafar and Saker, 2006**). The use of arbitrary primers for evolution studies and linkage analysis has been found effective in several plant species (**Halward *et al.*, 1992, Ahmed and AL-Qaradawi, 2009., Abdulla and Gamal, 2010**).

RAPD has been effectively utilized in the analysis of the date palm genetic variations. **Sedra *et al.* (1998a)** studied genetic variation among 43 date palm accessions from Morocco, Iraq and Tunisia using RAPD markers. Selection of 19 primers revealed polymorphism among the 43 accessions tested in the study. Each accession revealed distinguishable RAPD patterns, enabling the identification of each accession. Thus, RAPD technology appears very effective for identifying accessions of date palm. However, cluster analysis of the accessions showed a weak relationship between the accessions although morphologically similar varieties were clustered together.

Hela *et al.* (2000) built a random genomic library of Tunisian date palm varieties from total cellular DNA previously amplified according to an RAPD procedure. The resultant recombinant DNA, ranging from 200 to 1600 bp inserts, constitutes a large number of potential markers for molecular characterization of date palm varieties. **Trifi *et al.* (2000)** performed DNA amplification fingerprinting analysis on a collection of Tunisian date palm varieties using a set of universal decamer primers. The findings showed that good fruit quality varieties can be clustered. For example, the Deglet Nour and Kentichi varieties, characterized by their contrasting fruit qualities, seem to be dissimilarly related to the others. These data provide evidence of RAPDs as a powerful technique which may be used to get phylogenetic information within Tunisian date palm varieties but does not identify them as monophyletic groups.

On the other hand, **Soliman *et al.* (2003)** utilized RAPD-PCR to determine the genetic similarity between male and female Egyptian date cultivars and identify unknown males of Egyptian date palm through known female cultivars using five

primers. Although genetic similarity was estimated between male and female Egyptian cultivars, the results obtained were not sufficient to identify unknown males among the cultivars analyzed. It seemed that more advanced techniques are required for the identification of male Egyptian date palm varieties.

Since RAPD-PCR seemed to be useful in genetic analysis of date palm varieties, it is utilized in this project aimed at classifying several date palm genotypes.

2. Material and Methods

Date palm samples were collected from the Experimental Station at Al-Kassassin, Egyptian Ministry of Agriculture, Ismailia Governorate and private orchards, respectively, during the 2003 and 2004 seasons. The samples are eight female cultivars (Amhat, Barhi, Bint Aisha, Zaghloul, Hayany, Amry, Kuboshy and Samany) and one male genotype grown from seed in full production stage. The ages of Amhat, Barhy, Zaghloul, Samany and Amry cultivars and the male cultivars ranged from 16-20 years while the Bint Aisha, Hayany and Kuboshy cultivars were 25-30 years old. All the female cultivars are soft date except Amry which is a semi-dry date.

DNA extraction

DNA was extracted from the leaf of the different date palm female cultivars and male genotype using the cetyl trimethyl ammonium bromide (CTAB) method of **Doyle and Doyle (1990)**. Briefly, 300 mg frozen date palm leaf was finely grounded and homogenized in 300 uL extraction buffer before incubating for 15 min at 65 °C. 700 uL chloroform-isoamyl alcohol (24:1 v/v) was added to the homogenate and mixed carefully. The extract was centrifuged at 8000g for 4 min in a bench-top centrifuge. The pellet was removed and one volume of chloroform-isoamyl alcohol (24:1 v/v) was added to the supernatant. The mixture was then centrifuged for 4 min at 8000g and the resulting pellet removed. This procedure was repeated until the final supernatant seemed clear. Finally, 3 volumes of ice cold absolute alcohol was added to the final supernatant and centrifuged at 8000g for 10 min. The supernatant was decanted and the resulting pellet was washed twice with 70% ethanol before being suspended in minimal volume of distilled deionized water.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) amplification was performed in 25 µl reaction mix containing 40 ng genomic DNA, 0.5 unit Taq polymerase (Appliegene, Germany), 200 µM each of dATP, dCTP, dGTP, dTTP, 10 p mole random primers K1 (5′-TGCCGAGCTG -3), K3 (5′-CCCTACCGAC -3), K4 (5′-TCGTTCCGCA-3′), K5 (5′-CACCTTTCCC-3′) and K7 (5′-CCACAGCAGT-3′) and appropriate amplification buffer. Following an initial denaturation step at 92°C for 2 min, the amplification programme was 44 cycles of 30s at 92°C (denaturing step), 30s at 36°C (annealing step), and 2 min at 72°C (extension step) in Perkin Elmer thermocycler (USA). Reactions were finally incubated at 72°C for 10 min. All primers used were 10-mer random oligonucleotide sequences obtained from Operon Technologies Inc. (Alameda, CA, USA). These primers were selected after preliminary experiments.

Amplification was performed and reactions repeated on different days with different DNA template preparations and reproducibility of bands verified. The amplification products were separated on 2% in TAE (Tris-acetate EDTA) buffer, (pH 8.0) stained with ethidium bromide (0.2 µg /ml) and photographed under UV light using red filter. The RAPD bands were scored for their presence (+) or absence (-) and the genetic similarity was calculated for each primer separately and average for all primers was carried out with each comparison.

3. Results and Discussion

DNA of eight date palm cultivars and one male genotype was isolated from the leaf and amplified by PCR using random oligonucleotide primers. Amplification products were separated by agarose gel electrophoresis to reveal band polymorphism.

Table 1 shows the results of RAPD analysis of different date palm cultivars using five random primers. Out of 20 random primers screened, only five primers produced clear reproducible bands. The five primers yielded 179 scorable bands (with an average of 36 bands per primer), including eleven polymorphic bands. The number of bands from each primer varied from 21 to 47 bands. However, primer OPK4 in particular, produced a large number of strongly amplified and individual fragments (47), whereas, primer OPK1 produced the lowest number (21) of amplicons. On the other hand, primer OPK1 gave the highest percentage of polymorphism (9.52) while, the lowest percentage of polymorphism (4.25) was obtained using the primer OPK4.

RAPD variation has also been reported in many studies. For example, **Sedra *et al.* (1998b)** reported that analysis of the 19 selected primers among the 43 date palm (*Phoenix dactylifera* L.) accessions generated 56 bands, 37 of which were polymorphic. There were 1.9 polymorphic bands per primer on average. The RAPD technology was applied to four

genotypes (ADM, AIB, BSTN and ZAHD) representing different geographical origins. The number of amplification bands per primer varied between 0 and 13, with a mean of 5 major bands per primer (**Sedra *et al.*, 1998a**).

Table (1): RAPD-PCR amplification products of DNA extracted from leaves of date palm cultivars using five random primers.

Primer	Sequence 5′ ----------3′	Total number of scorable bands	Number of polymorphic bands	% of polymorphism
OPK1	TGCCGAGCTG	21	2	9.52
OPK3	CCCTACCGAC	37	2	5.40
OPK4	TCGTTCCGCA	47	2	4.25
OPK5	CACCTTTCCC	42	3	7.14
OPK7	CCACAGCAGT	32	2	6.25
Overall totals		179	11	6.14

% of polymorphism = No. of polymorphic bands ÷ Total No. of scorable bands × 100

The distribution and size of polymorphic bands from eight date palm cultivars and one male genotype using five primers are shown in Table 2, Figs. 1 (A,B), 2 (A,B) and 3. It could be observed that the primers generated a set of amplification products ranging from size 100 bp in OPK3 (Fig. 1-B) to 1000 bp in OPK4 (Fig. 2-A).

Results in Table (2) also reveal that using primer OPK1, two polymorphic bands of 800 bp and 700 bp were absent in Barhy, Bint-Aisha cultivars and the male genotype but are present in the other date palm cultivars (Fig. 1-A, black arrows). With primer OPK3, one polymorphic band of 1000 bp was absent in the Amhat, Barhy and Hayany cultivars and the male genotype. Also, one polymorphic band of 500 bp was absent in the Barhy, Hayany and the male genotype (Fig. 1-B, Table 2).

The fragment patterns visualized after gel electrophoresis revealed that with primer OPK4, two polymorphic bands of 1000 bp and 500 bp were absent in the male genotype but present in all female cultivars (Fig. 2 A). A similar observation was seen with primer OPK5 where, three polymorphic bands of 600, 300 and 200 bp were detected in all females cultivars (Fig. 2 B).

Using primer OPK7, one polymorphic band of 1000 bp was present in the male genotype but is absent in all female cultivars (Fig. 3). However, one polymorphic band of 800 bp was absent in the Samany, Amry and Kuboshy cultivars but was detected in the other cultivars and the male genotype. In this respect, **Adawy *et al*. (2002)** stated that in the RAPD analysis of five date palm cultivars using 10 primers, the fragment sizes ranged from 310 to 2800 bp. In the present study, the fragments ranged from 100 to 1000 bp. These discrepancies could be attributed to the use of different primers and different reaction conditions.

Table (2): Distribution and size of polymorphic bands from date palm cultivars using five random primers

Primer	Size of polymorphic band (bp)	Distribution of polymorphic bands								
		1	2	3	4	5	6	7	8	9
OPK1	800	+	---	---	+	+	+	+	+	---
	700	+	---	---	+	+	+	+	+	---
OPK3	1000	---	---	+	+	---	+	+	+	---
	500	+	---	+	+	---	+	+	+	---
OPK4	1000	+	+	+	+	+	+	+	+	---
	500	+	+	+	+	+	+	+	+	---
OPK5	600	+	+	+	+	+	+	+	+	---
	300	+	+	+	+	+	+	+	+	---
	200	+	+	+	+	+	+	+	+	---
OPK7	1000	---	---	---	---	---	---	---	---	+
	800	+	+	+	+	+	---	---	---	+

+, refers to the presence and ---, absence of a RAPD marker (polymorphic band)

Numbers from 1 to 9 represent genotypes as follows: 1, Amhat cv. 2, Barhy cv. 3, Bint-Aisha cv. 4, Zaghloul cv. 5, Hayany cv. 6, Samany cv. 7, Amry cv. 8, Kuboshy cv. 9, Male genotype.

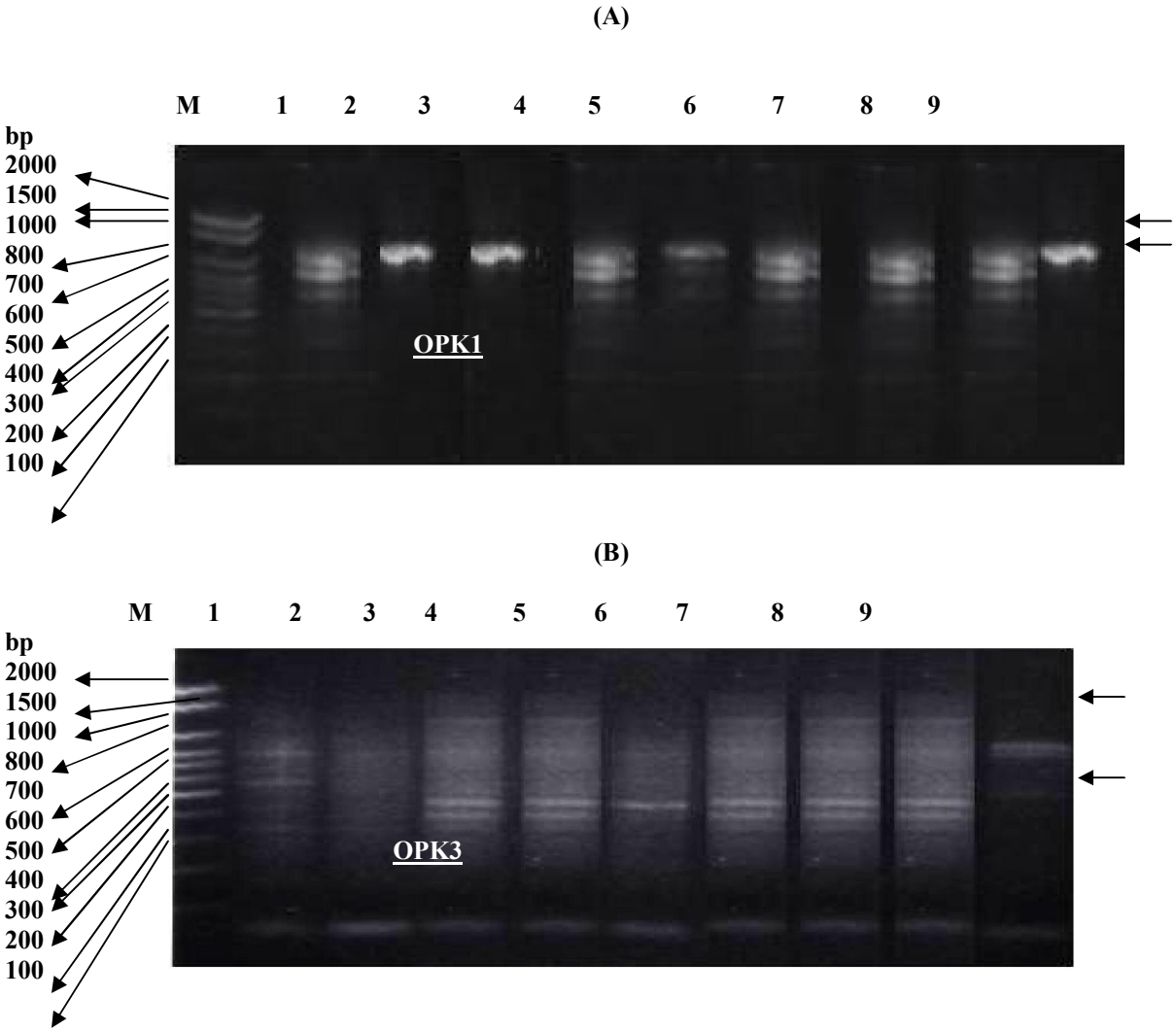


Figure (1). Agarose gel electrophoresis of RAPD fragments generated by primers OPK1 (A), OPK3 (B) of different date palm female cultivars.
Amhat cv. (lane 1); Barhy cv. (lane 2); Bint-Aisha cv. (lane 3); Zaghloul cv. (lane 4); Hayany cv. (lane 5); Samany cv. (lane 6); Amry cv. (lane 7); Kuboshy cv. (lane 8) and Male genotype (lane 9). Molecular marker (bp) (lane M). Primers used are designated *below* the gels. Polymorphic DNA fragments are indicated by arrows

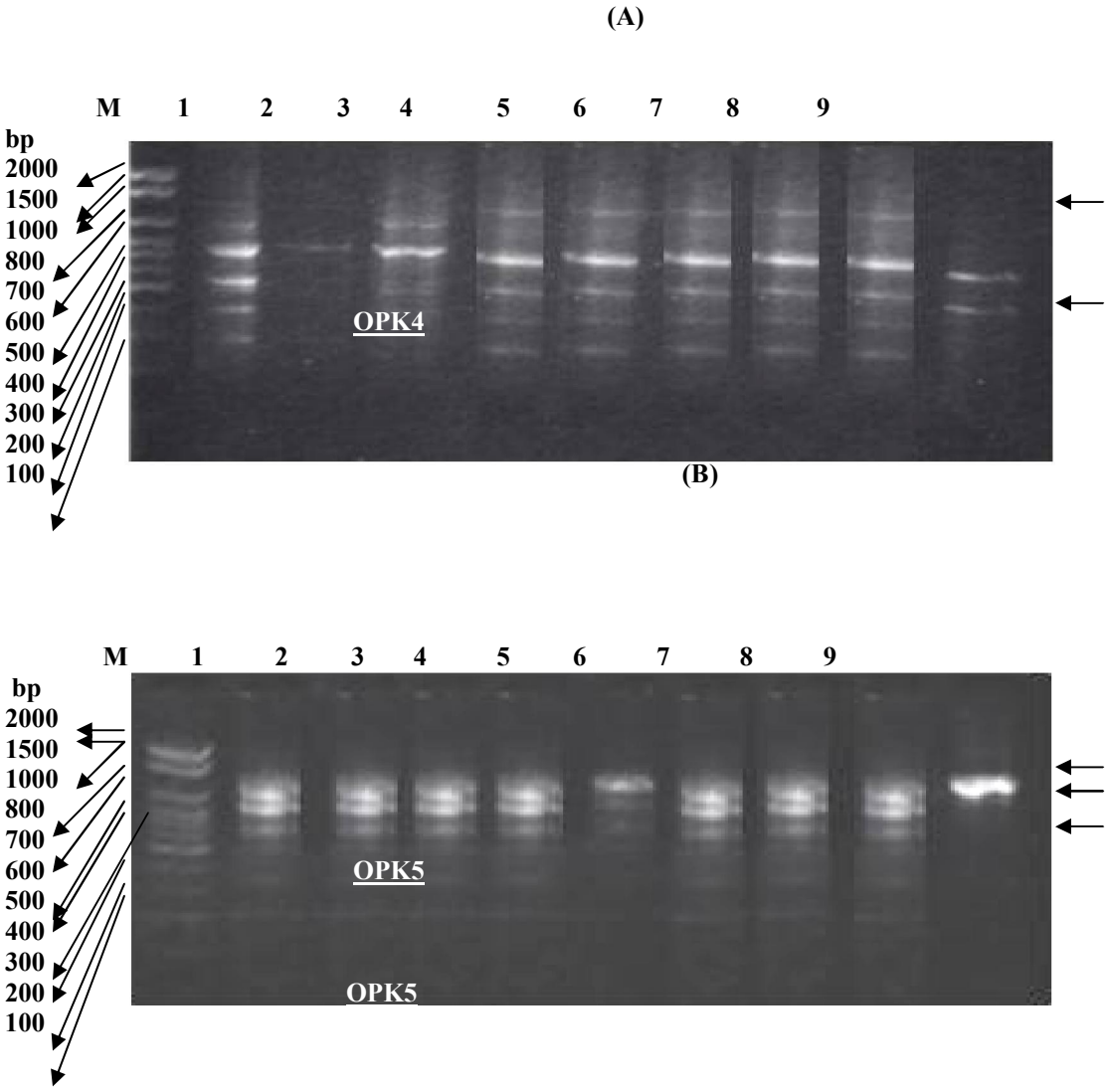
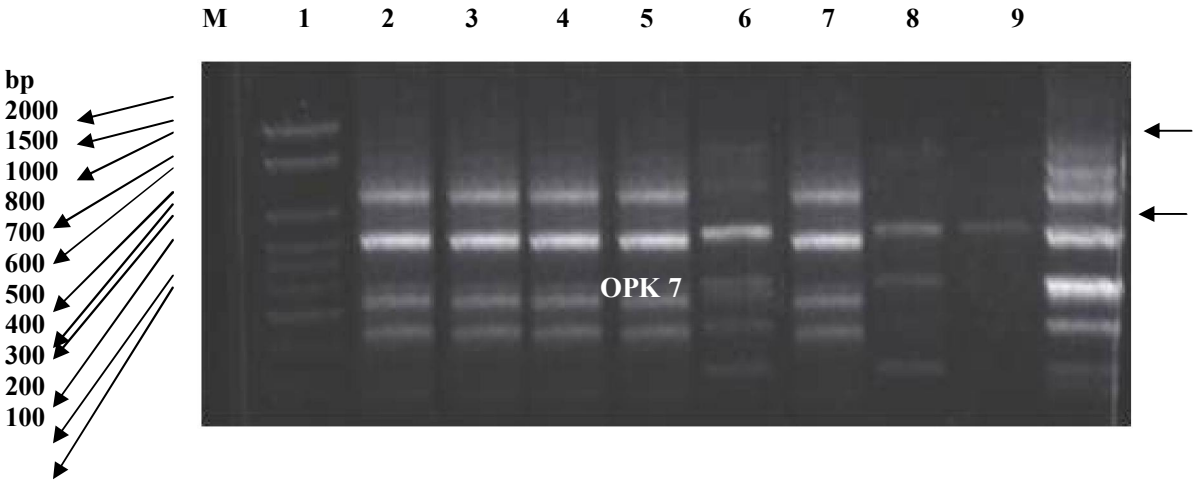
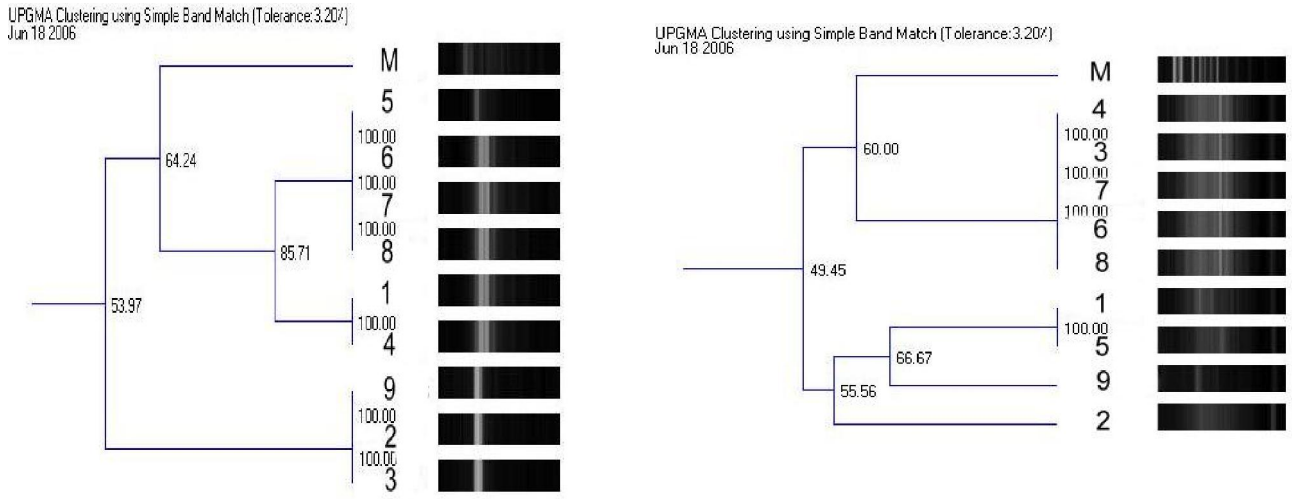


Figure (2). Agarose gel electrophoresis of RAPD fragments generated by primers OPK4 (A), OPK5 (B) of different date palm female cultivars.
Amhat cv. (lane 1); Barhy cv. (lane 2); Bint-Aisha cv. (lane 3); Zaghloul cv. (lane 4); Hayany cv. (lane 5); Samany cv. (lane 6); Amry cv. (lane 7); Kuboshy cv. (lane 8) and Male genotype (lane 9). Molecular marker (bp) (lane M). Primers used are designated *below* the gels. Polymorphic DNA fragments are indicated by arrows



Figure(3). Agarose gel electrophoresis of RAPD fragments generated by primers OPK7 of different date palm female cultivars.
Amhat cv. (lane 1); Barhy cv. (lane 2); Bint-Aisha cv. (lane 3); Zaghloul cv. (lane 4); Hayany cv. (lane 5); Samany cv. (lane 6); Amry cv. (lane 7); Kuboshy cv. (lane 8) and Male genotype (lane 9). Molecular marker (bp) (lane M). Primers used are designated *below* the gels. Polymorphic DNA fragments are indicated by arrows.

(A) **(B)**



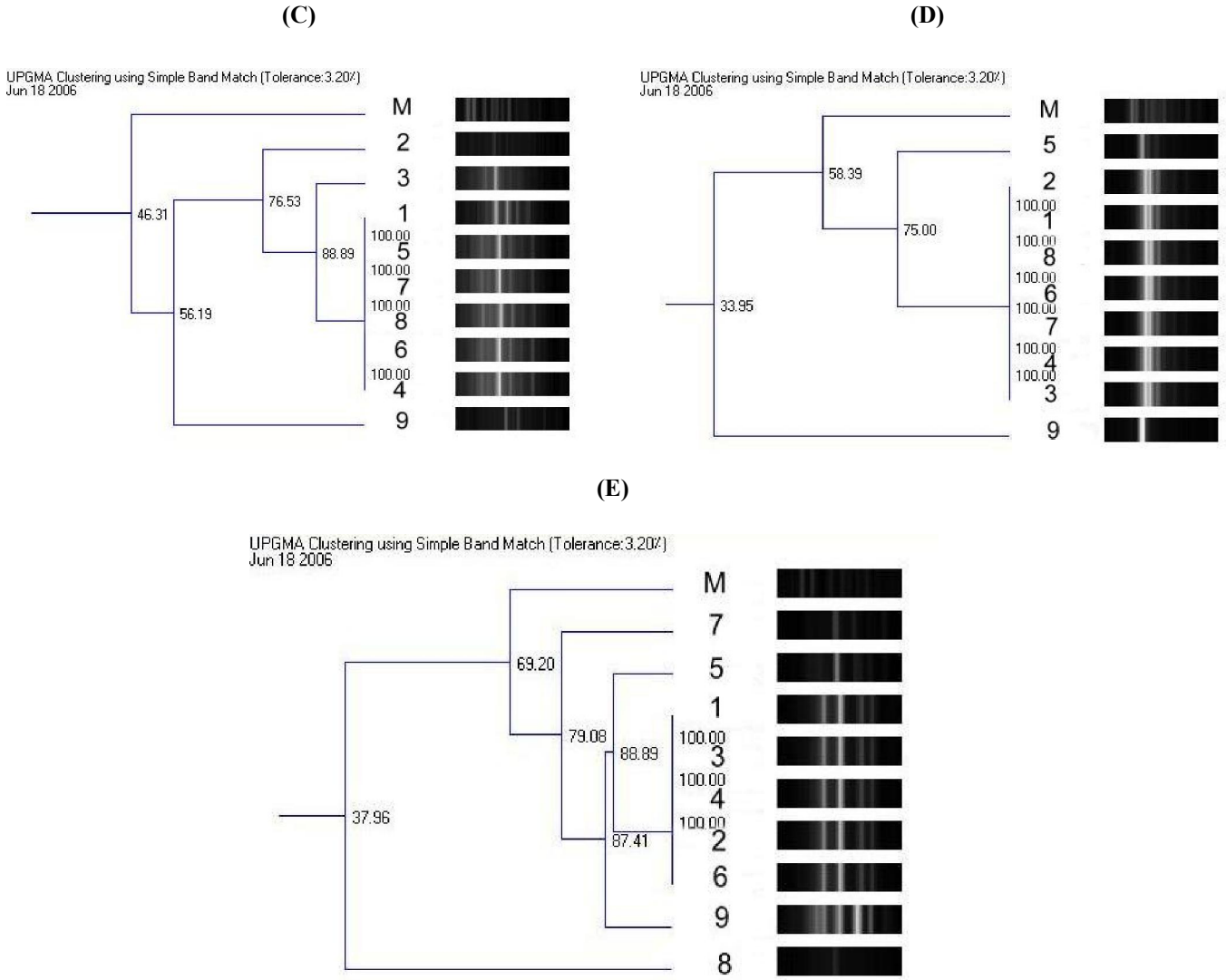


Figure (4). Dendrogram resulted from UPGMA clustering analysis based on the similarity polymorphism of DNA of the studied date palm genotypes isolates by PCR-based RAPD with 5 primers using agarose gel electrophoresis. (A) Primer OPK1; (B) Primer OPK3; (C) Primer OPK4;(D) Primer OPK5; (E) Primer OPK7.

Molecular marker (lane M); Amhat cv. (lane 1); Barhy cv. (lane 2); Bint-Aisha cv. (lane 3); Zaghloul cv. (lane 4); Hayany cv. (lane 5); Samany cv. (lane 6); Amry cv. (lane 7); Kuboshy cv. (lane 8) and Male genotype (lane 9).

In the present study using 5 primers, a total of 179 bands including eleven polymorphic bands were obtained, of which 6.14% showed polymorphism. The female cultivars showed monomorphic RAPD profiles in comparison with the male genotype, especially with primers OPK4 and OPK5. **Soliman *et al.* (2003)** compared genetic material from four female date palm and four unknown male trees of the Egyptian date palm. They found that the genetic similarity between the four female date palm (Zaghloul, Amhat, Samany and Siwi) ranged from 87.5 to 98.9%. The banding profiles obtained suggest that two of the male cultivars (male 3 and 4) are genetically related to the four female cultivars. In this study, of the 5 primers tested, primers OPK4 and OPK5 each produced amplification products that are monomorphic across all the female

cultivars. The size of the monomorphic bands produced by these primers ranged from 200 bp in OPK5 (Fig. 2-B) to 1000 bp in OPK4 (Fig. 2-A and Table 2). The other three primers (OPK1, OPK3 and OPK7) revealed scoreable polymorphisms.

Generally, RAPD profiles of female cultivars of date palm exhibited similar banding patterns (monomorphic RAPD profiles) especially primers OPK4 and OPK5 which suggest homology among these cultivars. The results of molecular analysis also showed distinct variation in the profiles within the female cultivars and the male genotype especially with primers OPK1, OPK3 and OPK7.

The present study provides valuable information on the molecular basis of polymorphism detected as RAPD markers in some date palm cultivars. RAPD analysis demonstrated that the polymorphism observed (6.14%) is very low. The similarity obtained from the analysis of all the bands recorded showed 93.86% similarity (11 polymorphic fragments out of a total of 179). The reliability of RAPD as a marker system to certify genetic stability or variability of date palm needs to be confirmed by phenotypic characteristics and other molecular marker techniques.

Cluster analysis using simple band match (Tolerance: 3.2%) as shown in Figure (4) shows that OPK4 and OPK5 primers differentiated between the male genotype and all female cultivars with similarity levels of 56.19 and 33.95%, respectively. OPK1 primer differentiated Barhy and Bint-Aisha cultivars and the male genotype from other cultivars with similarity levels of 53.97%. OPK3 primer differentiated Amhat, Barhy and Hayany cultivars and the male cultivars with similarity level of 49.45%. OPK7 primer differentiated Kuboshy cultivar from the other genotypes with similarity level of 37.96%.

Thus, it is possible to classify the date palm cultivars using RAPD-PCR technique. The results obtained are reproducible and genetic analysis can be correlated to phenotypic evaluation of the date fruit. Further attempts are being conducted to obtain detailed information on these cultivars.

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