Assessment of Genetic Distance Among Some Iraqi Date Palm Cultivares (*Phoenix Dactylifera* L.) Using Randomly Amplified Polymorphic DNA

Mohammed S. Alansari*[1], Abdul Kareem A. Al-kazaz[1], Hussam S. Khierallah[2]
[1]Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq.
[2]Date Palm Research Unit, College of Agriculture, University of Baghdad, Iraq.

Abstract

The aim of this study to determine the genetic distance and relationship among some Iraqi date palm cultivars by using the Random Amplified Polymorphic DNA (RAPD) technique. Molecular analysis was performed by using 10 random primers. These primers produced 176 fragment lines across 14 cultivars, Of these, 166 or 94.3% were polymorphic. The size of the amplified bands ranged between 200-2250 bp. The genetic polymorphism value of each primer was determined and ranged between 7.5-16.9%. In terms of unique banding patterns, the most characteristic banding pattern was for the Barhee cultivar with primer OP-M06 and for the Khadhrawy Mandily cultivar with primer OP-C02. Genetic distance values ranged from 0.868 to 0.125 among studied date palm cultivars. Analysis of genetic relationship showed there were two main groups (A and B), each main group had two subgroups, The first one divided into two subgroups, A1 and A2. A2 subgroup contains the cultivar 'Maktoom', while A1 divided into two subgroups A1a and A1b that contains (9) cultivars (Helaly, Barhee, Khadhrawy.Baghdad, Eusta-Omran, Barbun, Zahdi, Gamal Al-Deen, Mtawag and Khadrawy.Mandily). The main group B divided into two subgroups, B1 subgroup included 'Khastawy' and 'Sultany' cultivars, while B2 subgroup contains 'Ashrasi' and 'Bream' cultivars. According to above results, RAPD markers can be consedered the useful tool to study the genetic relationship among Iraqi date palm cultivars.

Keywords: *Phoenix dactylifera*, Randomly amplified polymorphic DNA, Genetic distance, Phylogenetic Tree

تقدير البعد الوراثي لبعض اصناف نخيل التمر العراقية بأستخدام مؤشرات التضاعف العشوائي المتعدد الاشكال لسلسلة الدنا

محمد سعد الانصاري*[1]، عبد الكريم عبد الرزاق القزاز[1]، حسام سعد الدين خير الله[2]
[1]قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق.
[2]وحدة ابحاث النخيل، كلية الزراعة، جامعة بغداد، العراق .

الخلاصة:

الهدف من هذه الدراسة هو تحديد العلاقة والبعد الوراثي لبعض اصناف نخيل التمر العراقية بأستخدام تقانة مؤشرات التضاعف العشوائي المتعدد الأشكال لسلسلة DNA (RAPD). أجري التحليل الجزيئي على مجين 14 صنف بأستخدام عشرة بادئات عشوائية وانتجت هذه البادئات 176 حزمة رئيسية، من ضمنها، 166

*Email:mohammedsma@yahoo.com

حزمة متباينة وبنسبة 94.3%. تراوحت احجام الحزم المضاعفة ما بين 200−2250 زوج قاعدي. تم تحديد قيمة التباين الوراثي لكل باديء وقد تراوحت هذه القيم بين 7.5−16.9%. من حيث الانماط الحزمية الفريدة ، كان النمط الحزمي الاكثر تميزا للصنف 'برحي' مع الباديء OP−M06 وصنف 'خضراوي مندلي' مع الباديء OP−C02. تراوحت قيم الابعاد الوراثية بين 0.125− 0.868 مابين اصناف نخيل التمر المدروسة، بينما اظهر تحليل العلاقة الوراثية ان هناك مجموعتين رئيسيتين وهي: Aو B ،وكل مجموعة ضمت مجموعتين ثانويتين، المجموعة الأولى قسمت إلى مجموعتين ثانويتين(A1 و A2). احتوت المجموعة A2 صنف 'مكتوم' ، بينما قسمت المجموعة A1 بدورها إلى مجموعتين (A1a و A1b) واللتان احتوتا على 9 اصناف (هلالي، برحي ، خضراوي بغداد، اسطة عمران، بربن، زهدي، جمال الدين، مطوك وخضراوي مندلي). اما المجموعة الرئيسية B فقد قسمت بدورها الى مجموعتين ثانويتين (B1 و B2) اذ تضمنت المجموعة B1 صنفي 'خستاوي' و'سلطاني' بينما ضمت المجموعة B2 صنفي 'اشرسي' و'بريم' . اعتمادا على النتائج اعلاه، يمكن اعتبار مؤشرات RAPD اداة مفيدة لدراسة العلاقة الوراثية بين أصناف النخيل العراقية.

Introduction:

Date palm (*Phoenix dactylifera* L., 2*n*= 2*x* = 36) is one of earliest domesticated fruit tree[1] It is a dioecious,perennial, monocotyledonous fruit tree that belongs to the family Arecaceae, and is of major socio-economic importance in west Asia and Africa not only for its fruit as staple food but also as an ornamental plant[2]. Date palm has been cultivated for at least 7,000 years and is believed to have originated in Mesopotamia (southern part of Iraq) or western India [3]. Because of its high nutritional value, great yields and its long life the date palm has been mentioned as the "tree of life" [4]. Originates from its fruit '*Phoenix*' presumably derived from the Greek word for purple or red fruit, and "*dactylifera*" from the Greek word "daktulos" meaning finger like appearance of the fruit's form [5]. Dates are a main income source and staple food for local populations in many countries in which they are cultivated, and have played significant roles in the economy, society, and environment of those countries[6]. The number of known cultivars distributed all over the world was reported to be approximately 5000 [7], out of which 650 are cultivated in Iraq [8], 340 in Saudi Arabia [9] and 135 in United Arab Emirates [10]. During the Gulf and Iran-Iraq wars, many palm trees were destroyed and more died when the southern marshes were drained. Genetic diversity of this crop was therefore, negatively affected by these ecological stresses [11].

There are several techniques available to investigate different genotypes of crop species and determine the purity of the variety to help in plant breeding program. Prior to the availability of DNA-based marker, most genetic diversity studies in various crops were carried out using morphological and biochemical markers. These markers can be affected by environmental factors, growth practice and they are taking a long time to access. DNA-based markers provide useful information on genetic diversity of plant cultivars, as they remain unaffected by environmental factors and the developmental stage of plants. Such markers also provide a nearly unlimited potential to uncover differences at the molecular level [12], and have been revealed a powerful tools [13] to provide information on the relatedness of cultivars that are difficult to distinguish morphologically such as those of date palm. The DNA-based markers chosen for this study are Random Amplified Polymorphic DNA (RAPD). RAPD can generate many useful genetic markers for the analysis of genetic diversity and phylogenetic relationships in closely related groups [14]. It is possibly the simplest test technically [15] and the fastest of all recently applied DNA-based markers for the detection of genetic variation within date palm [16].

Advantages include rapid analysis, highly informative results, low cost and simplicity. The technique relies on using short primers (≈10 bases) with random sequences and less stringent demands for pairing that allows amplification of DNA polymorphisms randomly distributed all over the genome and giving varied patterns of banding on gels that can be used for distinction between species or linkage mapping [14]. several studies implied RAPD for the molecular characterization of date palm of Tunisia [13], Morroco [17], Saudi Arabia [18,19, 20], and Egypt [16, 21, 22]. The aims of the current study are (i) identification and differentiation of various Iraqi date palm varieties by generating a DNA fingerprint for each variety, (ii) estimation of the genetic diversity and determination of the genetic relationship among studied varieties by using RAPD markers.

Materials and Methods:

Plant material

A total of 14 well-defined reference Iraqi date palm cultivars used in this study. These cultivars were collected from Al-Zaafarania date palm Statio belonging to the Ministry of Agriculture in Baghdad table-1.

Genomic DNA Isolation

The DNA extracted from leaf tissue according to the CTAB method described by [23].

Purity and concentration of DNA was measured by spectrophotometer according to [24]. Genomic DNA integrity was detected by running on 0.8% agarose gel electrophoresis followed by staining with ethidium bromide and visualized under UV light [25]. DNA samples were diluted to a working concentration of 50 ng/µl in order to be use in the RAPD PCR experiment.

Table 1- Date palm cultivars used in this study

Number	Name
1	Barhee
2	Maktoom
3	Ashrasi
4	Gamal Al-Deen
5	Barbun
6	Bream
7	Khastawy
8	Khadhrawy Mandily
9	Khadhrawy Baghdad
10	Zahdi
11	Sultany
12	Helaly
13	Eusta omran
14	Mtawag

Primer selection and RAPD assay

To identify primers promising detectable polymorphisms among date palm cultivars, 13 decamers of oligonucleotides (Operon Technologies Inc., Alameda, California, USA) were tested. After an initial screening, the primers classified into two groups according to results obtained. The first group gave no amplified products and this group included (OP-R18, O-A07 and OP-K18). The second group gave results in term of amplification and polymorphism, including (OP-F10, OP-M06, OP-X04,OP-C11, OP-C13, OP-E07, OP-P07, OP-A13, OP-C02 and OP-D20) table-2 [26,27]. Only primers that had been earlier found to be polymorphic among date palm varieties were used in this study.

Amplification reaction were performed in a volume of 25 µl containing 12.5 µl of Go Taq®Green Master Mix (Promega-USA), with concentration (1X) containing (10mM Tris-HCl (PH8), 50mM KCL, 1.5mM $MgCl_2$, 200µM each deoxynucleotide triphosphate (dNTP) and 1 unit DNA polymerase), Amplification was carried out using thermocycler (Labnet international . Inc - USA), using the following program:-

1 cycle of 5 min at 94°C for initial separation, followed by 45 cycles of 1 min at 94°C for denaturation, 1 min at 36°C for annealing and 2 min at 72°C for primer extension. Finally, 1 cycle of 10 min at 72°C was used for the final extension, followed by a hold at 4°C [28]. Each PCR reaction was repeated twice to ensure reproducibility.

Twenty microliter of PCR products were analyzed by electrophoresis in 1.5% agarose gel at 5 volt/cm for 2 hour in 0.5X TBE (10mM Tris-Borate,1 mM EDTA) buffer, agarose gels were stained with ethidium bromide 0.5 µg/ml for 20-30 minutes. The 100 bp DNA ladder (100-1,500) bp (promega-USA) was used as a molecular marker. After electrophoresis, images of gels were captured using Gel Documentation System (ATTA-Japan).

Table 2-The names of the random primers used in the study and their sequences.

No.	*Primer's name*	**Sequence 5'------ 3'*
1	OP-A13	CAGCACCCAC
2	OP-C02	GTGAGGCGTC
3	OP-C11	AAAGCTGCGG
4	OP-C13	AAGCCTCGTC
5	OP-D20	ACCCGGTCAC
6	OP-E07	AGATGCAGCC
7	OP-F10	GGAAGCTTGG
8	OP-M06	CTGGGCAACT
9	OP-P07	GTCCATGCCA
10	OP-X04	CCGCTACCGA

Data analysis:

Molecular weight estimation

Molecular weight was calculated by using a computer software M.W. Detection program, Photo-Capture M.w. program from Consort version 1.0 [29], based on comparing the PCR products with the known size of DNA fragments of a PCR markers (which consist of 11 bands from 100 to 1,500 bp).

Primers parameters

Total bands number were scored visually. Polymorphic fragments and fragment lines were also scored. Polymorphism percentage was calculated by dividing number of polymorphic fragments amplified by the total number of bands amplified by the same primer X100. Discrimination power for each primer was calculated by dividing the number of polymorphic fragments amplified by primer by the total number of polymorphic fragments obtained X100. Efficiency for each primer was calculated by dividing the total number of bands amplified by primer by the total number of bands obtained X100. All bands were visually scored as present (1) or absent (0) to create the binary data set [11].

Estimation of Genetic Distances

Only data generated from the detection of polymorphic fragments were analyzed. The amplification profile of all the used cultivars for any given primer were compared with each other, the presence of band scored as "1" and the absence of the same band of the same size in other cultivars scored as "0". The intensity of the bands was not taken into account. Only clear and reproducible amplified fragments were considered for genetic relationship analysis. genetic distance between the 14 cultivars were estimated. A Cluster analysis was performed to construct genetic relationship tree diagrams among studied date palm cultivars using an Unweighted Pair-Group Method with Arithmetic Average (UPGMA) [11]. All computations were carried out using Palaeontological Statistics (PAST) software version 1.62 [30].

Results and Discussion:

DNA amplification and cultivar identification

The genetic diversity and the relationship among date palm cultivars were evaluated using RAPD markers amplified from 13 universal primers. Among the 13 decamer primers, there were three primers (R18, A07 and K18) that gave no PCR products; hence, they were eliminated from the analysis. Each of the remaining ten primers varied in their ability to resolve variability among cultivars.

Primers parameters

A total of 1243 scorable bands were generated from those primers with average of 124 bands per primer table-3. The number of amplified bands per cultivar varied from 78 bands for the primer OP-D20 showing the lowest primer efficiency (6.27%) to 174 bands for the primer OP-E07 showing the highest primer efficiency (13.9%). In term of size, the amplified bands ranged from 200 bp (OP-C11) to 2250 bp (OP-C13).

RAPD primers amplified 176 fragments across all cultivars genomes with average of 18 fragments per primers. Among these fragments scored, 166 fragment (94.3%) were polymorphic with average of 17 polymorphic fragments per primers across the 14 date palm cultivars. This average was higher than observed in other date palm studies using RAPD markers, with an average of 3.1, 3.5 and 8.1polymorphic band per primer [31, 32, 33]. This discrepancy may related to cultivars and the selection of RAPD primers with scorable bands or the use of more diverse cultivars.

Table 3- Number of amplified bands, polymorphic fragments, fragment lines, primer efficiency and discrimination power of the 10 primers used for RAPD analysis

No. of fragment lines	% discrimination power	% polymorphsim	Polymorphic fragment(no.)	% primer efficiency	Total bands (no.)	Primer name
12	6.62	14.1	11	6.27	78	OP-D20
17	10.2	16.5	17	8.28	103	OP-C11
20	9.63	9.19	16	13.9	174	OP-E07
12	5.42	7.5	9	9.65	120	OP-A13
21	12.6	15.2	21	11.1	138	OP-M06
22	12.0	13.7	20	11.6	145	OP - C02
23	13.8	16.9	23	10.9	136	OP-X04
20	12.0	14.7	20	10.9	136	OP-C13
15	9.03	12	15	10.0	125	OP-P07
14	8.43	15.9	14	7.07	88	OP-F10
176			166		1243	Total
18			17		124	Average

The primer OP-X04 amplified 23 polymorphic fragments (16.9% polymorphism) showing the highest discrimination power (13.8%). While the primers OP-A13 amplified 9 polymorphic fragments (7.5 % polymorphism) showing the lowest discrimination power (5.42 %), this variation is related to primers in which they differ from each other in their number of polymorphic fragments produced. A total of 176 useful fragment lines were scored from the amplification products with the ten random primers of DNA from 14 date palm cultivars, of these 176 PCR products generated, 5.7% (10 fragments) were monomorphic across all cultivars. The remaining 166 fragments (94.3% of the total products scored) were polymorphic among the studied cultivars, this was a relatively high level of the percentage of polymorphic fragments obtained by random primers compared to reports of other RAPD studies in date palm which were 66.0% [34] and 78.6% [35], while this percentage was comparatively similar to other date palm studies at 92.4% [36] . The arbitrary primers OP-F10, OP-D20, OP-C02 and OP-M06 were useful for discrimination cultivars of distinct characteristics figure-1 and figure-2. Some cultivars could be distinguished from all other cultivars with selection of these primers. For example, Gamal Al-Deen and Eusta Omran gave specific banding pattern with primers OP-D20, OP-F10, OP-E07 and OP-X04, and Khadhrawy Mandily gave unique banding pattern with primers OP-C02 and OP-M06; while Barhee gave unique banding pattern with primers OP-E07 and OP-M06.

Genetic distance

The genetic distances (G.D) index revelead from Jaccard similarity index was calculated for the 166 RAPD polymorphic fragments according to formula (G.D = 1 - similarity) [37], of the 14 cultivars table-4. The cultivar Khadhrawy baghdad' was highly divergent from 'Sultany' with distances of 0.868. The cultivar 'Gamal Al-Deen' was very closely related to 'Mtawag' with distances of 0.125. In this study, the highest value of genetic distance is relatively similar when compared to the report of other RAPD study that obtained 0.908 genetic distance among a subset of Tunisian date-palm elite cultivars [13], while the lowest value of genetic distance is high when compared with results obtained 0.007 genetic distance among cultivars of Iranian date palm [36].

Cluster analysis

Dendogram was constructed based on [37] genetic distance using UPGMA cluster analysis and depicted genetic relationships among 14 date palm cultivars, showing two main clusters A and B figure-3. The first one consisted of two subclusters, A1 and A2. A2 subcluster contains the cultivar 'Maktoom', while A1 divided into two subclusters A1a and A1b. A1b subcluster included 'Helaly' cultivar, while A1a subcluster included two subclusters A1a1 and A1a2. A1a2 subcluster contains 'Barhee' cultivar, on the other hand, A1a1 subcluster contains two subclusters, A1a1c that include 'Khadhrawy Mandily', 'Mtawag' and 'Gamal Al-Deen' cultivars, and A1a1d subcluster that contains 'Zahdi', 'Barbun', 'Eusta-Omran' and 'Khadhrawy Baghdad' cultivar. The second main cluster B divided into two subclusters, B1 subcluster included 'Khastawy' and 'Sultany' cultivars, while B2 subcluster included 'Ashrasi' and 'Bream' cultivars.

In conclusion, although Iraqi date palm trees have been grown in Iraq for thousands years, high genetic diversity is clear among them as shown in our results. Genetic identification of date palm

cultivars using morphological markers is usually not possible until fruits are produced and frequently requires a large set of phenotypic data that is often difficult to assess and some-times variable due to environmental influences, in contrast to DNA markers which reflect precisely the polymorphisms occurred among date palm cultivars. According to results, most of RAPDs universal primers used were efficient for discrimination Iraqi date palm cultivars.

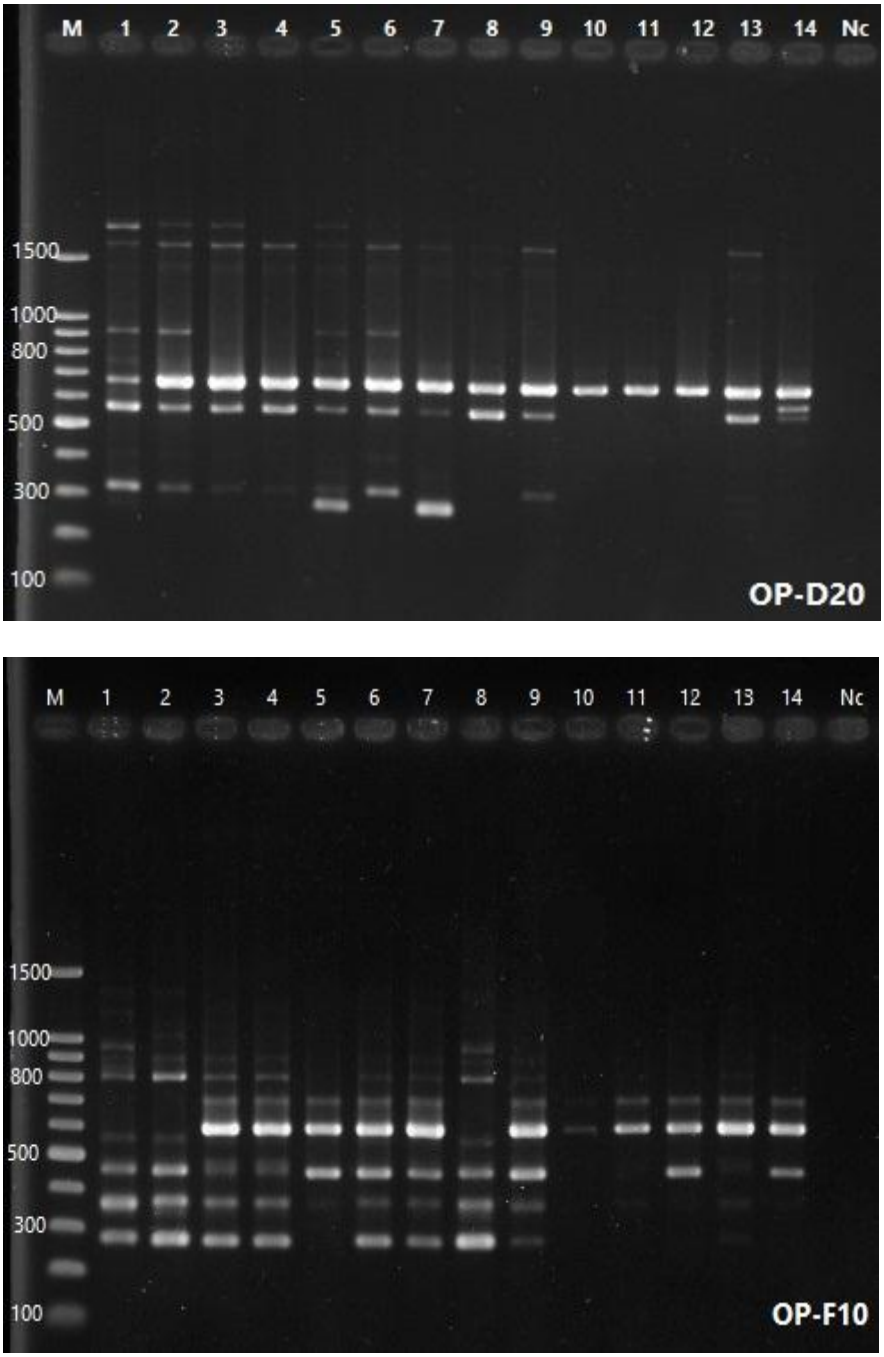

Figure 1- Agarose gel electrophoresis of primers OP-F10 and OP-D20 for DNA samples of the date palm plants. Bands were fractionated by electrophoresis on 1.5% agarose gel (2hr, 5V/cm, 0.5X Tris-borate buffer) and visualized by ethidium bromide staining. M: 100 bp ladder. Lanes: 1. Helaly, 2. khadhrawy Baghdad, 3. Gamal Al-Deen, 4. Mtawag, 5. Barhee, 6. Eusta Omran, 7. Barbun, 8. Zahdi, 9. Khadhrawy Mandily, 10. Sultany, 11. Khastawy, 12. Ashrasi, 13. Maktoom and 14. Bream. NC: Negative control.

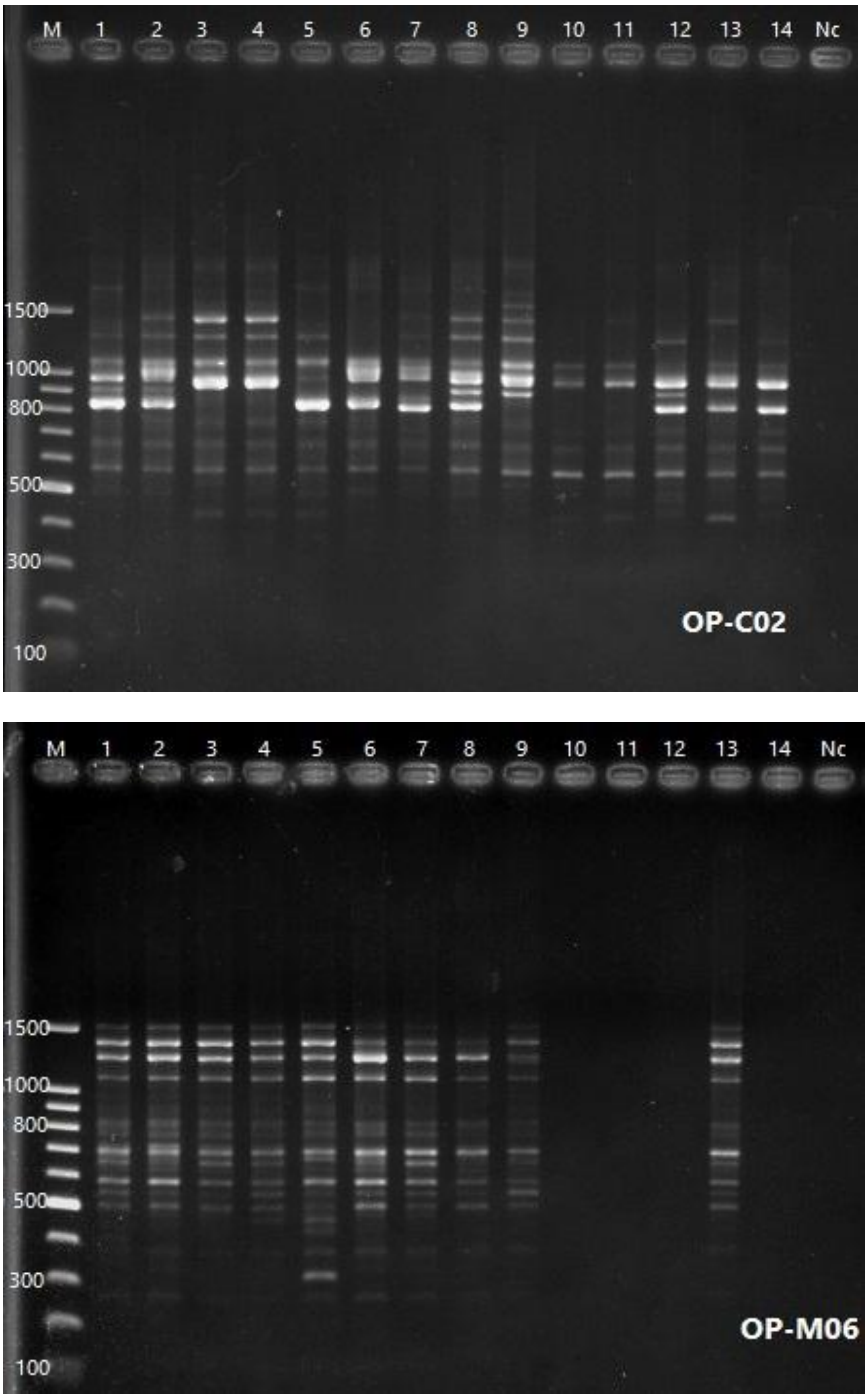

Figure 2- Agarose gel electrophoresis of primers OP-M06 and OP-C02for DNA samples of the date palm plants. Bands were fractionated by electrophoresis on 1.5% agarose gel (2hr, 5V/cm, 0.5X Tris-borate buffer) and visualized by ethidium bromide staining. M: 100 bp ladder. Lanes:1. Helaly, 2. khadhrawy Baghdad, 3. Gamal Al-Deen, 4. Mtawag, 5. Barhee, 6. Eusta Omran, 7. Barbun, 8. Zahdi, 9. Khadhrawy Mandily, 10. Sultany, 11.Khastawy, 12. Ashrasi, 13. Maktoom and 14. Bream. NC: Negative control.

	Bream	Maktoom	Ashrasi	Khastawy	Sultany	Khadhrawy_M.	Zahdi	Barbun	Eusta_Omran	Barhee	Mtawag	Gamal_Al-Deen	Khadhrawy_B.	Helaly
Bream	0.000													
Maktoom	0.547	0.000												
Ashrasi	0.370	0.562	0.000											
Khastawy	0.536	0.643	0.559	0.000										
Sultany	0.619	0.760	0.600	0.390	0.000									
Khadhrawy_M.	0.593	0.390	0.630	0.670	0.787	0.000								
Zahdi	0.650	0.460	0.664	0.730	0.845	0.378	0.000							
Barbun	0.630	0.415	0.655	0.709	0.804	0.344	0.265	0.000						
Eusta_Omran	0.629	0.434	0.653	0.748	0.816	0.315	0.372	0.285	0.000					
Barhee	0.621	0.450	0.656	0.760	0.828	0.442	0.437	0.331	0.328	0.000				
Mtawag	0.636	0.358	0.650	0.667	0.785	0.301	0.386	0.311	0.362	0.403	0.000			
Gamal_Al-Deen	0.653	0.382	0.677	0.685	0.800	0.285	0.370	0.323	0.307	0.388	0.125	0.000		
Khadhrawy_B.	0.698	0.489	0.701	0.788	0.868	0.427	0.375	0.343	0.265	0.369	0.377	0.350	0.000	
Helaly	0.629	0.460	0.655	0.757	0.830	0.426	0.395	0.400	0.370	0.424	0.434	0.406	0.336	0.000

Table 3-Genetic distance's values among the 14 date palm cultivars of Iraq as revealed by RAPD analysis.

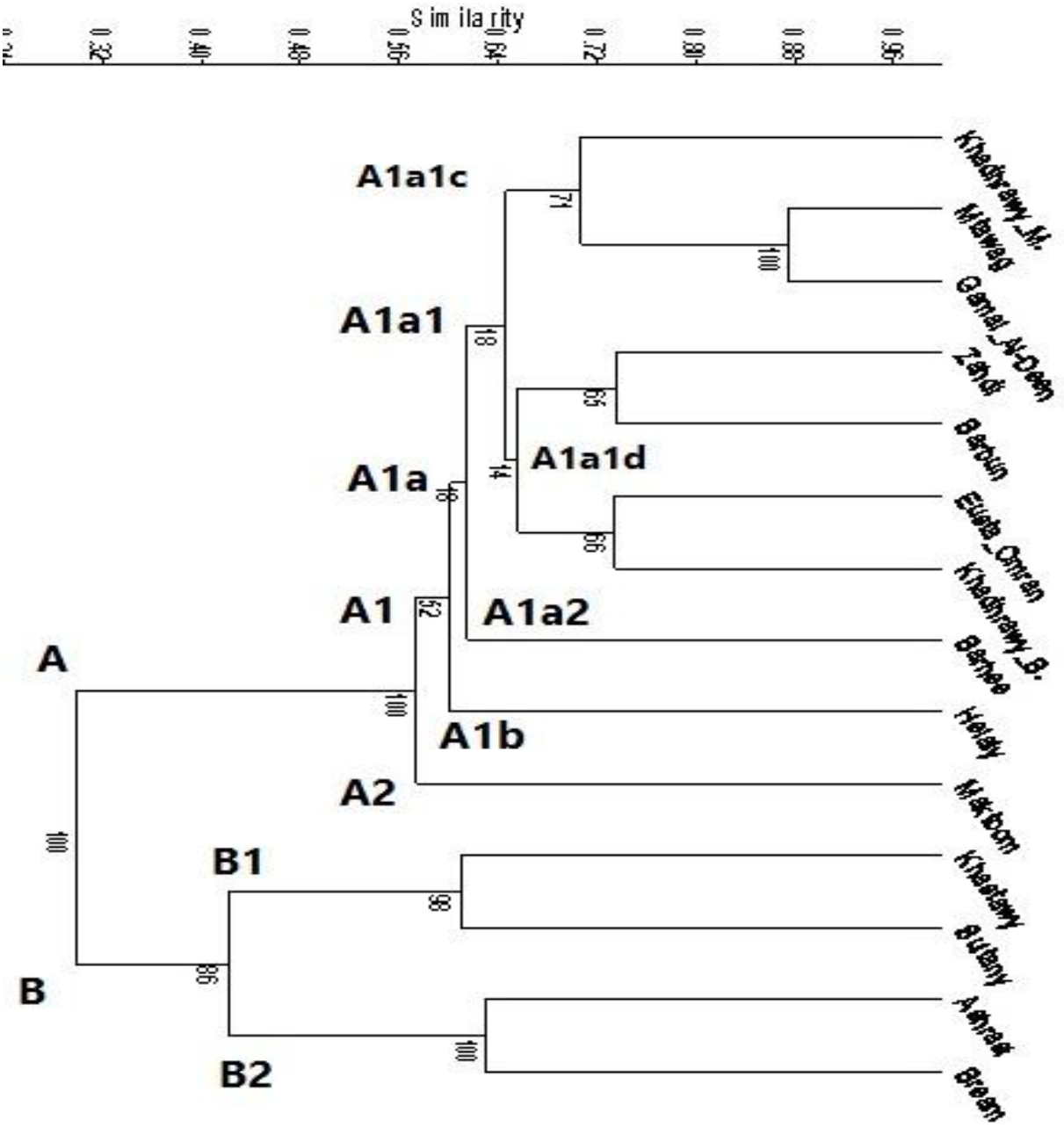

Figure 4- Genetic relationships among the 14 date palm cultivars estimated by RAPD analysis.

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