Kuwait J. Sci. **45 (1)** pp 108-114, 2018

Genetic variability among seven cultivars of date palm (*Phoenix dactylifera* L.) based on embryonic DNA of old fruit

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Abstract

One of the important factors in molecular biology analysis is DNA extraction process to obtain high quality DNA. Previously, all attempts to extract DNA from date palm involved either the use of fresh leaves or germinated seeds. The aim of this study was to extract genomic DNA from various parts of three years old date palm fruit and to evaluate the genetic diversity among seven cultivars of date palm. The fruit of each cultivar was dissected into perianth, seed without embryo, zygotic embryo, and endocarp-mescocarp-epicarp to extract the DNA. The results showed that genomic DNA could be easily extracted in sufficient quantity from zygotic embryo. Both RAPD and ISSR markers could effectively determine the genetic relationship among date palm cultivars with polymorphism percentage ranging between 32.09% and 36.49% respectively. RAPD markers showed that cultivars with highest similarity value were Lbanah and Nabtat Ali (80.0%), followed by Khalas and Sukkary (73.81%), while the least were Ajwa and Safawi (59.56%). ISSR revealed that the highest similarity values were for Anbar and Khalas (88.0%), followed by Lbanah and Nabtat Ali (86.18%), while the least were Khalas and Safawi (73. 81%).

Keywords: Date palm; Genetic variability; DNA extraction.

1. Introduction

The date palm tree (Phoenix dactylifera L.) (Family, Arecaceae-Palmae) is a common traditional tree in Middle Eastern countries and highly liked by people all over the world. The date palm fruit was considered as the first most widely consumed fruit in the Kingdom of Saudi Arabia and in all Muslim countries, especially Ajwa cultivar. It is considered as a symbol of life in dry areas, as it shows high tolerance to temperatures and salinity compared to many other crop plant species. All parts of date palm trees provide for a lot of necessities in life.

Taxonomically, the number of identified date palm varieties all over the world is about 5000 varieties, out of which nearly 450 varieties were found in the Kingdom of Saudi Arabia (Bashah, 1996). There are many variations in morphological characters of seed among date palm varieties, although each seed has a single, oblong, one-seeded berry with a terminal stigma, fleshy pericarp and a membranous endocarp (Mansour, 2005). The morphological markers have been used widely to describe various varieties of date palm, but it has many limitations such as long vegetative phase.

Biochemical markers such as RAPD, ISSR, proteins, etc., have been used intensively to identify plant varieties (Al-Salameen *et al*., 2013; Bendiab *et al*., 1993; Bennaceur *et al*., 1991). The random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) are potentially simple, rapid, reliable and effective methods, which do not need prior knowledge of DNA sequence (Wolfe & Liston, 1998; Mcgregor *et al*., 2000).

Current protocols for genomic DNA extraction of date palm have some difficulties, as it is necessary to get fresh leaves or germinated seed, and the parent tree itself should have started fruiting for the correct identification. Therefore, our goal is to extract genomic DNA from different parts of three-year-old date palm fruit as a first attempt, and to evaluate the genetic diversity among seven date palm cultivars including Ajwa, Anbar, Lbanah, Safawi, Khalas, Sukkary and Nabtat Ali.

2. Materials and methods

2.1 Plant materials

During January 2014, three year old fruit of seven date palm cultivars (Phoenix dactylifera L.) were collected from three different locations in Kingdom of Saudi Arabia. Cultivars of Ajwa, Anbar, Lbanah and Safawi were collected from Al-Madīnah Al-Munawwarah region (24° 28' 7" North, 39° 36' 51" East – Alt.- 619 meters), Sukkary and Nabtat Ali were collected from Qassim region (27° 4' 60" North, 43° 28' 0" East- Alt.- 753 meters), while Khalas was collected from Wadi Addawasir region (20°27' 52" North, 44°47'14" East, Alt.- 624 m). Each fruit was dissected into perianth, seed without embryo, embryo, and endocarp-mescocarp-epicarp.

2.2 DNA extraction protocol

The genomic DNA from each dissected part was extracted using DNeasy Plant Mini Kit (supplied by QIAGEN) with slight modification as follows: 0. 049 g from each part was ground using mortar and pestle in the presence or absence of liquid nitrogen and 400 μl of buffer AP1 and 4 μl of RNase were added. Resulting mixtures were incubated for 10 min at 65 °C, then 130 μl of buffer AP2 was added to the lysate. The lysate was centrifuged (20,000 g, 5 min.), then the supernatant was transferred to QIA shredder spin column. The column was centrifuged (20.000 g, 2 min), then the flow-through was transferred into a new tube without disturbing the pellet. Buffer AW1 (900 μl) was added and mixed carefully, then 650 μl of the mixture was transferred to a "DNeasy" mini spin column and placed in a 2ml collection tube. The column was centrifuged (7,000 g, 1 min.), then the spin column was placed into a new 2ml collection tube. Buffer AW2 (500 μl) was added and then centrifuged (7,000 g, 1 min.). This step was repeated twice. The spin column was transferred to a new 1.5 ml collection tube and added with 100 μl of buffer AE. Each sample was incubated at 23 °C for 5 min and centrifuged (20,000 g, 1 min.). A Thermo Scientific™ BioMate 3S UV-Visible at 260nm was used to measure the absorbance of the DNA. The quality of DNA was evaluated by electrophoresis on 1.0 % agarose gel for 60 minutes in 0.5 × TBE buffer stained with ethidium bromide, then visualized by UV-transilluminator and documented in gel documentation system (Biorad Laboratories, USA) (Figure 1).

2.3. PCR amplification and visualization

Eight RAPD and eight ISSR primers were used for PCR amplification that produced clear and reproducible banding patterns for diversity analysis (Table 1). Each 25 µl of reaction volume of DNA amplification contains 50 ng of genomic DNA, 5 pM primer, 200 μM each dNTPs, 0.5 unit Taq DNA polymerase, 1.5 mM MgCl2 and 1× PCR buffer (El Zalabani *et al*., 2012). The PCR condition was adjusted as follows: an initial denaturation of 94°C for 1 min, followed by 49 cycles each consisting of 1 min denaturation (94°C), 1 min annealing (38°C) and 1 min extension (72°C), then amplification was terminated by a final extension for 10 min at 72°C. The amplification products were electrophoresed at 90 V for 60 minutes in 1.5% agarose gel in 0.5 × TBE buffer as described above (section DNA extraction protocol). To determine the genetic diversity among date palm cultivars, the amplified bands were automatically scored through image analysis using gel documentation system for presence or absence. Jaccard's similarity coefficient, squared euclidean distance and agglomerative cluster analysis method were applied by using the Community Analysis Package Software Program (CAP).

Table 1. Sequence of RAPD and ISSR biomarkers

RAPD primers (ID)	**Sequence (5' – 3')**
Oligo 203	CACGGCGAT
Oligo 342	GAGATCCCTC
Oligo 345	GCGTGACCCG
Oligo 42	TTAACCCGGC
OPK 8	GAACACTGGG
OPJ 1	CCCGGCATAA
Oligo 349	GGA GCC CCC T
OPE-18	GGACTGCAGA
ISSR primers (ID)	Sequence (5' – 3')
Primer 3	TGGATGGATGGATGGA
Primer 4	CACACACACACACAAG
UPC 888	CACCACACACACACACA
UBC 823	TCTCTCTCTCTCTCTCC
UBC 824	TCTCTCTCTCTCTCTCG
UBC 826	ACACACACACACACACC
UBC 842	GAGAGAGAGAGAGAGACG
Primer 1	AGAGAGAGAGAGAGAGC

3. Results and discussion

3.1. DNA extraction method

The quality and purity of genomic DNA obtained from embryo with this extraction method was high, either it was grinded in the presence or absence of liquid nitrogen (Figure 1). The ratio value of A260/A280 of DNA from dissected embryos was 1.8, indicating the purity of DNA in regards to protein contamination. The result of DNA amplification also ensures this purity, since these techniques cover the entire genome. The most recent method to extract DNA from the date palm is to grind the fresh leaves with sterile sand particles and sodium chloride (Ibrahim *et al*., 2010). There was no DNA extracted from perianth, seed without embryo, and endocarp-mescocarp-epicarp. It is probably due to the presence of polyphenolic compounds, polysaccharides, or phenolic chemicals like quercetin, isorhamnetin heterosides, (-)-epicatechin, (+)-catechin, 5-caffeoylshikimic acid (Ziouti *et al*., 1996). Therefore, embryo of old date palm fruit could be used for isolation of genomic DNA. It could yield pure and sufficient amount of DNA, suitable for molecular research and very precisely represent the cultivar of date palm, which is a cross pollinated one.

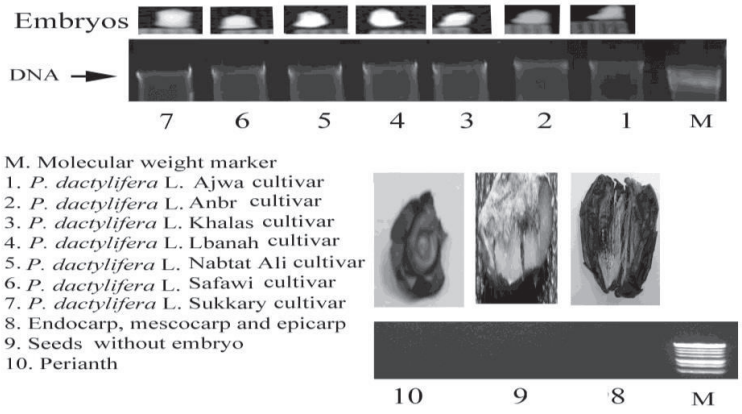

Fig. 1. DNA extracted from various parts of three years old date palm fruit. (17-) DNA fragment extracted from embryos; (8,9,10) no DNA fragment extracted from perianth seed without embryo, and endocarp-mescocarp-epicarp; (M) molecular weight marker.

3.2. Genetic diversity of date palm cultivars

The results showed that DNA amplification using RAPD and ISSR markers clearly could characterize, differentiate and estimate the genetic diversity of P. dactylifera cultivars (Figure 2). Amplification of total genomic DNA using eight RAPD primers resulted 133 DNA bands in total of which 36.49 % were polymorphic. Oligo 345 had the highest number of bands (25.0) followed by Oligo 42 (24.0) and OPJ 1 (22.0), while OPK 8 had the least number (4.00). OPJ 1 had the highest rate of polymorphism (54.5%) followed by Oligo 203 (43.8%), Oligo 349 (42.1%) and Oligo 42 (41.7%), while Oligo 345 had the lowest rate (24.0%) (Table 2).

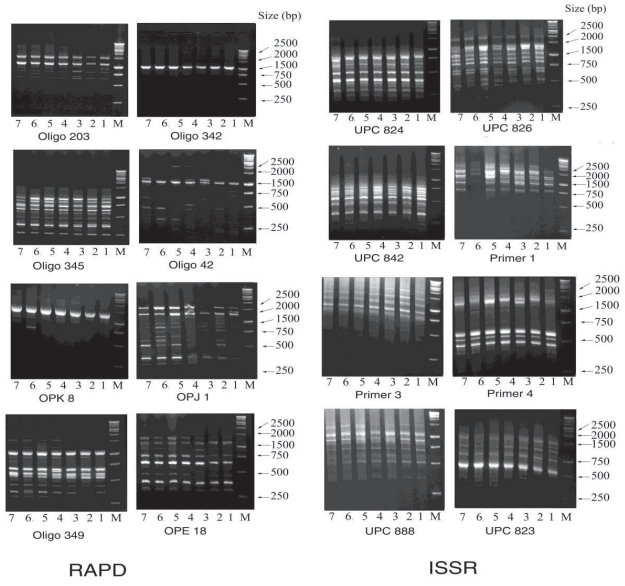

Fig. 2. RAPD and ISSR profiles of seven cultivars of date palm cultivars. Lane 1, Ajwa; Lane 2, Anbr; Lane 3, Khalas; Lane 4, Lbanah; Lane 5, Nabtat Ali; Lane 6, Safawi; Lane 7; Sukkary; M, Molecular weight marker.

Table 2. Polymorphism of eight RAPD primers.

Primer ID	Total numbers of bands	No. of polymorphic bands	No. of monomorphic bands	No. of unique bands	Polymorphism %
Oligo 203	16.0	7.00	5.00	4.00	43.8
Oligo 342	10.0	3.00	7.00	0.00	30.0
Oligo 345	25.0	6.00	17.0	2.00	24.0
Oligo 42	24.0	10.0	4.00	10.0	41.7
Oligo 349	19.0	8.00	11.0	0.00	42.1
OPK 8	4.00	1.00	1.00	2.00	25.0
OPJ 1	22.0	12.0	3.00	7.00	54.5
OPE-18	13.0	4.00	7.00	2.00	30.8
Total	133	51.0	55.0	27.0	36.49

Similarity coefficient between seven date palm cultivars based on RAPD biomarkers revealed that the highest similarity value was between Lbanah cv. and Nabtat Ali cv. (80.0%), followed by Khalas cv. and Sukkary cv. (73.81%), while the lowest was between Ajwa cv. and Safawi cv. (59.56%) (Table 3). The dendrogram separated seven date palm cultivars into two main clusters at 24.2 of linkage distance. The first cluster was consisted of Ajwa cv., Anbr cv. and Khalas, while the rest were included in the second cluster (Figure 3).

Table 3. Genetic similarity % among seven date palm cultivars based on RAPD markers

Cultivar	Ajwa	Anbar	Khalas	Lbanah	Nabtat Ali	Safawi	Sukkary
Ajwa	100.0						
Anbar	62.90	100.0					
Khalas	64.17	72.73	100.0				
Lbanah	63.78	65.41	73.39	100.0			
Nabtat Ali	61.19	66.42	71.54	80.00	100.0		
Safawi	59.56	68.38	68.42	69.06	73.57	100.0	
Sukkary	63.08	67.16	73.81	72.93	73.72	74.45	100.0

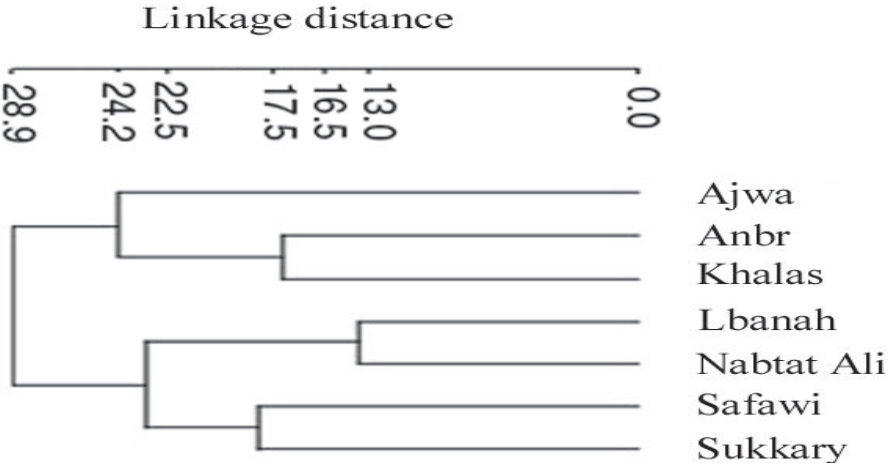

Fig 3. Dendrogram of seven date palm cultivars based on RAPD markers.

Eight ISSR primers resulted 128 bands with 32.09% of polymorphism. UPC 842 generated maximum number of bands (25.0), followed by Primer 1 (18), UPC 888 and UPC 826 (17), while UBC 823 and UPC 824 had the least number of bands (11.00) (Figure 2; Table 4). Primer 1 had the highest rate of polymorphism (72.2%) followed by Primer 4 (43.8), while primer UBC 823 had the lowest rate (9.1%). Primer UPC 842 had the highest numbers of monomorphic bands (14.0), while Primer 1 had the lowest numbers (3). The highest number of unique bands (4.0) was recorded from Primer 4, followed by Primer 1, UPC 888 and UPC 842 (2.00), while no unique band was recorded from primer 3, UBC 823 and UPC 824. Similarity coefficient based on ISSR markers, showed that the highest similarity value was between Anbar cv. and Khalas cv. (88.0%), followed by Lbanah cv. and Nabtat Ali cv. (86.18%), and between Ajwa cv. and Anbar cv. (85.71%). The least similarity value was between Khalas cv. and Safawi cv. (73. 81%) (Table 5). The dendrogram separated the genotypes of seven date palm cultivars into three clusters at 13.5 of linkage distance. The first cluster was Anbr cv., Khalas cv. and Ajwa cv. The second cluster was Lbanah cv., Nabtat Ali cv. and Sukkary cultivar. While the third cluster was Safawi cv. only (Figure 4).

Table 4. Polymorphism of eight ISSR primers

Primer ID	Total numbers of bands	No. of polymorphic bands	No. of monomorphic bands	No. of unique bands	Polymorphism %
Primer 3	13.0	2.00	11.0	0.00	15.4
Primer 4	16.0	7.00	5.00	4.00	43.8
UPC 888	17.0	3.00	12.0	2.00	17.6
UBC 823	11.0	1.00	10.0	0.00	9.1
UPC 824	11.0	3.00	8.00	0.00	27.3
UPC 826	17.0	6.00	10.0	1.00	35.3
UPC 842	25.0	9.00	14.0	2.00	36
Primer 1	18.0	13.0	3.00	2.00	72.2
Total	128	44.0	73.0	11.0	32.09

Table 5. Genetic similarity % among seven date palm cultivars based on ISSR markers

Cultivar	Ajwa	Anbar	Khalas	Lbanah	Nabtat Ali	Safawi	Sukkary
Ajwa	100.0						
Anbar	85.71	100.0					
Khalas	79.55	88.00	100.0				
Lbanah	74.81	83.06	82.54	100.0			
Nabtat Ali	77.44	80.00	83.72	86.18	100.0		
Safawi	74.40	75.61	73.81	75.83	78.69	100.0	
Sukkary	76.92	80.95	80.47	79.84	81.10	67 .76	100.0

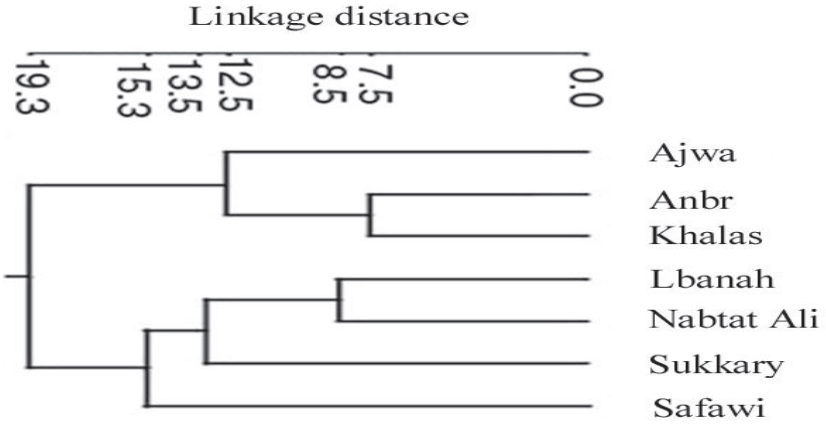

Fig 4. Dendrogram of seven date palm cultivars based on ISSR markers

In this study, there are variations between the number of resulted bands from RAPD and ISSR markers, which is probably due to differences in the number of alleles per locus in the genome of date palm cultivars. Previous study showed that many conditions affected the bands produced by a biomarker such as primer structure, amount of genomic DNA and the number of annealing sites (Kernodle *et al*., 1993). In another study, there were variations in polymorphism rate, average number of bands and the hierarchical clustering resulted from RAPD markers and ISSR markers in barley plants (Guasmi *et al*., 2012). RAPD markers generated more specific bands in date palm genome than ISSR markers, supporting earlier study that RAPD marker could determine genetic diversity with higher resolution than ISSR marker in 42 sugar beet accessions (Izzatullayeva *et al*., 2014). This study also confirms the existence of shared DNA fragments among studied date palm cultivars as revealed by monomorphic bands generated from RAPD and ISSR markers. Based on ISSR markers, Jaccard's similarity coefficient among seven date palm cultivars ranged between 88.0% and 73.81%, while based on RAPD markers ranged between 80.00% and 59.56 %. To some extent, these values are in agreement with the principle provided by Weier *et al*. (1982) that operational taxonomic units (OUT) should be above 85% for the same species and above 65% for the same genus. Therefore, both ISSR and RAPD markers could be effectively used in characterization and determination of the genetic relationships among date palm cultivars.

4. Conclusion

The genomic DNA of date palm could be extracted using zygotic embryo of three-years-old date palm fruit. Both ISSR and RAPD markers could be used to evaluate the genetic diversity of date palm.

5. Acknowledgement

The researchers greatly acknowledge King Abdulaziz City for Science and Technology (KACST) for the financial support under grant number (A-I-35-398).

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Submitted: 02/02/2016
Revised : 06/05/2017
Accepted : 07/05/2017

التباين الجيني بين سبعة أصناف مستنبة من نخيل التمر (Phoenix dactylifera L.) بناءً على الحمض النووي المجيني genomic DNA المستخرج من الثمار العتيقة

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خـلاصـة

إن عملية استخراج الحمض النووي للحصول على حمض نووي عالي الجودة هي واحدة من العوامل الهامة في تحليل البيولوجيا الجزيئية. ففي السابق، كانت جميع محاولات استخراج الحمض النووي من نخيل التمر تنطوي إما على استخدام أوراق فتية أو بذور مستنبتة. وكان الهدف من هذه الدراسة هو استخراج الحمض النووي المجيني من أجزاء مختلفة من ثمار شجرة نخيل التمر عمرها ثلاث سنوات وتقييم التنوع الجيني بين سبعة أصناف مستنبتة من نخيل التمر. تم تشريح ثمرة كل صنف مُستنبت إلى غلاف الزهرة، بذور بدون جنين، الجنين الزيغوتي، و(الغلاف الثمري الداخلي – الغلاف الثمري الوسطي – الغلاف الثمري الخارجي) endocarp-mescocarp-epicarp لاستخراج الحمض النووي. وأظهرت النتائج أنه يمكن استخراج الحمض النووي المجيني بسهولة وبكمية كافية من الجنين الزيغوتي. يمكن لكل من مؤشرات تقنيتي الدنا متعدد الشكل المُضخم عشوائياً RAPD وتكرارات التسلسل البسيط البيني ISSR أن تُحدد بشكل فعال درجة القرابة الوراثية بين أصناف نخيل التمر المستنبة مع نسبة تعدد الأشكال تتراوح بين ٪32.09 و ٪36.49 على التوالي. وأظهرت مؤشرات RAPD أن الأصناف المستنبتة ذات أعلى قيم في التشابه هي اللبانة Lbanah ونبتت علي (٪80.0) Nabtat Ali، يليها الخلاص Khalas والسكري (٪73.81) Sukkary، في حين كانت أقلها العجوة Ajwa والصفوي (٪59.56) Safawi. وأظهرت مؤشرات ISSR أن أعلى قيم في التشابه كانت بالنسبة للأنبار Anbar والخلاص (٪88.0) Khalas، يليها اللبانة Lbanah ونبتت علي (٪86.18) Nabtat Ali، في حين كانت أقلها الخلاص Khalas والصفوي (73.81%) Safawi.