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RAPD marker analysis for delineating of genetic diversity among date palm (*Phoenix dactylifera* L.) cultivars

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Abstract
Date palm (*Phoenix dactylifera* L.) is belongs to Arecaceae family. It is a dioecious and long lived monocotyledonous fruit tree on the Globe. The fruit of date palm has great socio-economic importance with high nutritional value for arid and semi arid regions of world. Date palm can survive at very high adverse environmental conditions. In the present study, twenty Random Amplified Polymorphic DNA (RAPD) markers have been profiled in eight cultivars of date palm collected from date palm farm of ICAR-CIAH, Bikaner. On the basis of reproducibility and reliable fingerprints, ten RAPD primers were selected for genetic diversity analysis. RAPD has generated a total of 99 fragments. Out of those, sixty four fragments showed high polymorphism among date palm cultivars which delineate 63.57% genetic diversity. Jaccard similarity coefficients were used for the evaluation of pair wise genetic divergence; cluster analysis of the similarity matrices was performed to estimate inter-specific diversity.

Keywords: Date palm, random amplified polymorphic DNA (RAPD), genetic diversity, similarity coefficient, PCR

Introduction
Date palm (*Phoenix dactylifera* L.), 2n=2x=36, of the family Arecaceae is the dioecious long lived monocotyledonous ancient domesticated fruit tree with a great socioeconomic importance and nutritional value for arid and semi arid regions of world with high tolerance to environmental stresses. The tree plays an important role in the development of sustainable agriculture in many drought and saline affected regions of the world (El-Hadrami and Al-Khayri, 2012) [7]. Date palms have always been clonally propagated to ensure the identity and uniformity of the cultivars. However, the existence of intra-cultivar variation could potentially cause confusion in cultivar nomenclature, preservation and utilization. Discrimination among closely related cultivars and clones is often extremely difficult. Morphological characterization of cultivars requires a large set of phenotypic data that are normally difficult to collect, and statistically variable due to environmental effects (Sedra *et al*., 1998) [13]. Morphological markers such as fruit characteristics have been used to describe the varieties, but these markers are significantly affected by environmental factors. In general, identification and evaluation of genetic diversity between cultivars based on morphological markers are very difficult and time consuming (William *et al*., 1990; Clark *et al*., 1993) [17, 5]. Random amplified polymorphic DNAs (RAPD) are DNA fragments amplified by the polymerase chain reaction using short (usually 10 bp) synthetic primers of random sequence. RAPD markers have been used for identification and DNA fingerprinting of date palm varieties. The RAPD technique has been used for cultivar genotyping (Ben-Abdallah *et al*., 2000; Trifi *et al*., 2000) [4, 16] and for analyses of phylogenetic relationships and genetic diversity (Al-Khalifah and Askari, 2003; Al-Moshileh *et al*., 2004; El-Tarras *et al*., 2007) [2, 3, 8]. Estimating the genetic distance assists in studying genetic diversity, a trait that is important for parent selection associated with genetic mapping and for marker-assisted selection in breeding programs (Lapitan *et al*., 2007; Trethowan and Kazi, 2008) [10, 15]. Therefore, the present investigation was conducted to define the genetic diversity and phylogenetic relationship among eight date palm genotypes grown in the Bikaner region using RAPD markers.

Materials and Methods

Collection of plant material and isolation of genomic DNA

The experimental material for the investigation consisted of young leaf samples of eight date palm (*Phoenix dactylifera* L.) cultivars (Khadrawy, Shamran, Zahidi, Braim, Chipchap, Sabiah, Seddami and Medjool) collected from date palm farm, Indian Council for Agricultuture Research (ICAR)-Central Institute for Arid Horticulture (CIAH), Bikaner. The genomic DNA was isolated from date palm leaf sample using DNeasy Plant Mini Kit (Qiagen, Ltd., Crawley, UK). All the chemicals and biochemicals used in analysis were of fine analytical grade and obtained from standard manufacturer and all reagents, solutions, Ependroff tubes, microcentrifuge tubes, PCR tubes and tips were sterilized at 15 psi, 121ºC for 20 minutes in autoclave before use. Ten primers (5 OPE and 5OPF series, operon technologies Inc. Alameda, California) were used for analysis.

Preparation of Reaction Mixture and programming of PCR

DNA amplification reaction was performed in 25 µl reaction mix made of: 12.5 µl 2 × Taq PCR Master Mix Kit (0.625 U/µl Tag DNA polymerase; 2 mM MgCl2; 0.2 mM each dNTP) (Qiagen, India), 1 µl primer (concentration 5 pmol/µl), 7.5 µl twice distilled water and 4 µl template DNA (50 ng/ µl). PCR amplifications were performed on a Gradient PCR Thermal Cycler DW-K960 (Shanghai Drawell Scientific Instrument Co., Ltd., China) following amplification profile: initial denaturation at 95 °C for 5 min., 35 cycles of denaturation at 94 °C for 0.5 min., annealing at 36 °C for 1 min., extension at 72 °C for 2.5 min. and a final extension at 72 °C for 5 min. PCR products were loaded into a 1.4% agarose gel for electrophoresis which was prepared in 1X TAE buffer containing 0.5 µg/ml of the ethidium bromide. The amplified products were electrophoresed for 2.5 – 3.5 hrs at 100 V. and were photographed by Syngene eel documentation system and stored as JPEG files.

Data Analysis

The DNA bands generated by each primer were counted and their molecular sizes were compared with those of the DNA markers. The bands scored from DNA profiles generated by each primer were pooled together. Then the presence or absence of each DNA band was treated as a binary character in a data matrix (coded 1 and 0, respectively) on Gel Documentation System. By applying the unweighted pair-group arithmetic average (UPGMA) method (Sneath S& okal 1973) using the SHAN subroutine by using the Numerical Taxonomy and Multivariate Analysis System NTSYS-pc version 2.10e (Rohlf, 1988), dendrograms representing the genetic relationship among eight date palm cultivars. Percentage of polymorphic bands was calculated as the percentage of polymorphic bands amplified by the primer to the total number of bands was produced by the same primer.

Results and Discussion

RAPD markers were used to determine genetic variation within available germplasm. PCR analysis showed that the selected ten RAPD primers produced polymorphic bands which could be scored unambiguously (Fig. 1). The results from our work proved that RAPD primers are useful tools for genetic variation study in date palm. A set of 5 primers of OPE series and set of 5 primer of OPF series were used to characterize 8 date palm cultivars to ascertain the phylogenetic relationship among the cultivars. The number of generated bands varied from 7 to 13 with size 300-3200 bps. Primer OPE 5, OPF 6 and OPF 7 produced maximum number of bands i.e. 13 bands; however, primer OPE 3 and OPF 2 produced only 7 bands. Degree of polymorphism varied according to the primer. The maximum polymorphism per cent was found in primer OPE 2 and OPF 9 (77.78%) followed by OPE 5 (76.92%), OPF 6 (69.23%), OPF 8 (62.50%), OPF7 (61.54%), OPE 1 (60%), OPE 3 (57.14%) OPE 4 (50%) and minimum per cent of polymorphism was found in OPF 2 (42.86%) with average polymorphism was 63.57% (Table 1). Primers produced typical band combination, which could be used for the varietal identification Table 2.

The pair-wise genetic distance were estimated by using Jaccard similarity co-efficient for the RAPD data ranged from ranged from 0.558 (Khadrawy and Seddami) to 0.835 (Braim and Chipchap) [Table 3]. Cultivars Braim and Chipchap were closely related with a similarity index of 0.835, followed by cultivars; Sabiah with Chipchap (0.798), Sabiah with Medjool (0.747), Seddami with Sabiah (0.741). Cultivars Khadrawy and Seddami, Seddami and Braim showed least similarity (0.558) and (0.578) respectively showing poor relationship between the cultivars. It is evident that the dendrogram is grouped into two major clusters A and B (Fig. 2), out of 8 genotypes that got clustered into two groups, the first group consists of monophytic branch of Khadraway and second major group B includes seven cultivar (Shamran, Zahidi, Braim, Chipchap, Sabiah, Medjool and Seddami). The second group B can further be divided into two sub-groups viz., 'a' and 'b'. The sub-group 'a' consist of two sub group a1 (Shamran and Zahidi) which shows closer affinity among each other and showed close relationship. Sub group a2 consists of the remaining four cultivars viz., Braim, Chipchap, Sabiah and Medjool which are distinctly related. Braim and Chipchap showed the close relation each other. Sub group 'b' consist of distinct cultivar Seddami having monophytic branch. In the light of above mentioned results of RAPD high level of polymorphism was observed in eight date palm cultivars which indicates high level of genetic diversity among the cultivars.

RAPD markers have been successively used for phylogenetic studies in many plant species (Xuemei *et al*., 2012) [18] and RAPD analysis could be used for an effective identification and DNA fingerprinting of date palm (Abdulla & Gamal, 2010; Jan *et al*., 2011) [1, 9]. The genetic similarity matrices were estimated for the date palm cultivars and used to develop dendrogram revealing the genetic relationships. Moreover, the polymorphism detected and its reproducibility suggests that RAPD markers are reliable for identification of date palm cultivars (Eissa *et al*., 2009) [6]. Our results are supported by Sonboli *et al*., 2011 and Xuemei *et al*., 2012 that RAPD marker system reveal high levels of polymorphism among species indicating its effectiveness for evaluating intra- and inter-specific genetic diversity in the genus. On the basis of above observations on DNA level, it can be concluded that there is high genetic diversity and stable difference between date palm cultivars. These results are supported by Markhand *et al*., 2010, with previous studies conducted on fruit characterization of Pakistani date palm that the fruits are significantly diverse from one cultivar to another.

The current study implies that molecular markers as RAPD are effective tools to discriminate various date palm genotypes. Molecular markers are indispensible for modern breeding to achieve date palm genetic variation improvement considering the lengthy and dioecious nature of date palm.

These results suggest that Random amplified polymorphic DNAs may differentiate genotype based on morphological characters and also gave some unique markers in some genotypes.

Table 1 Sequence of primers and bands produced including polymorphic bands and per cent polymorphism in RAPD analysis

S. No.	Oligo Name	Sequence (5' to 3')	Band sizes (bp)	Total no. of band	No. of polymorphic bands	% Polymorphism
1	OPE1	CCCAAGGTCC	400-1700	10	6	60.00
2	OPE2	GGTGCGGGAA	380-1500	9	7	77.78
3	OPE3	CCAGATGCAC	550-1400	7	4	57.14
4	OPE4	GTGACATGCC	480-1600	10	5	50.00
5	OPE5	TCAGGGAGGT	500-2500	13	10	76.92
6	OPF2	GAGGATCCCT	300-1500	7	3	42.86
7	OPF6	GGGAATTCGG	500-3000	13	9	69.23
8	OPF7	CCGATATCCC	350-2200	13	8	61.54
9	OPF8	GGGATATCGG	700-3200	8	5	62.50
10	OPF9	CCAAGCTTCC	400-2800	9	7	77.78
Total				99	64	Avg. (63.57%)

Table 2: Typical band combinations for varietal identification

Cultivars	Typical band combination
Khadrawy	OPE 1 (550 bp) and OPE 5 (1200 bp)
Shamran	OPE 1(550), OPE 2(450 bp,1000 bp), OPE 4 (1450bp), OPE 5(700 bp), OPF 6 (2000 bp) and OPF9 (450 bp)
Zahidi	OPE1 (400 bp,500bp), OPE 2 (450 bp), OPE 4 (1450bp), OPE 5(700 bp) and OPF9 (450 bp)
Braim	OPE1 (400 bp,500bp) and OPF 8(1400 bp , 3000 bp)
Chipchap	OPE 5 (2100bp), OPF 6(2000bp) and OPF 8(1400 bp , 3000 bp)
Sabiah	OPE 5 (1500bp, 2100bp), OPF 6 (580bp, 2000bp)and OPF 8 (1400bp)
Seddami	OPE 2 (1000bp), OPE 5(700 bp,1500 bp,2100bp),OPF 2 (1500bp)OPF 6 (580bp)OPF 7 (1200bp) and OPF 9 (500bp)
Medjool	OPE 3(550 bp,1200bp)OPF 6 (580bp),OPF 8 (3000bp) and OPF 9 (500bp)

Table 3: Jacard's similarity indices (%) among the eight Date palm cultivars based on RAPD analysis

	Khadrawy	Shamran	Zahidi	Braim	Chipchap	Sabiah	Seddami	Medjool
Khadrawy	1.000							
Shamran	0.697	1.000						
Zahidi	0.583	0.704	1.000					
Braim	0.650	0.593	0.699	1.000				
Chipchap	0.651	0.671	0.698	0.835	1.000			
Sabiah	0.605	0.663	0.690	0.698	0.798	1.000		
Seddami	0.558	0.598	0.607	0.578	0.633	0.741	1.000	
Medjool	0.617	0.619	0.609	0.655	0.674	0.747	0.659	1.000

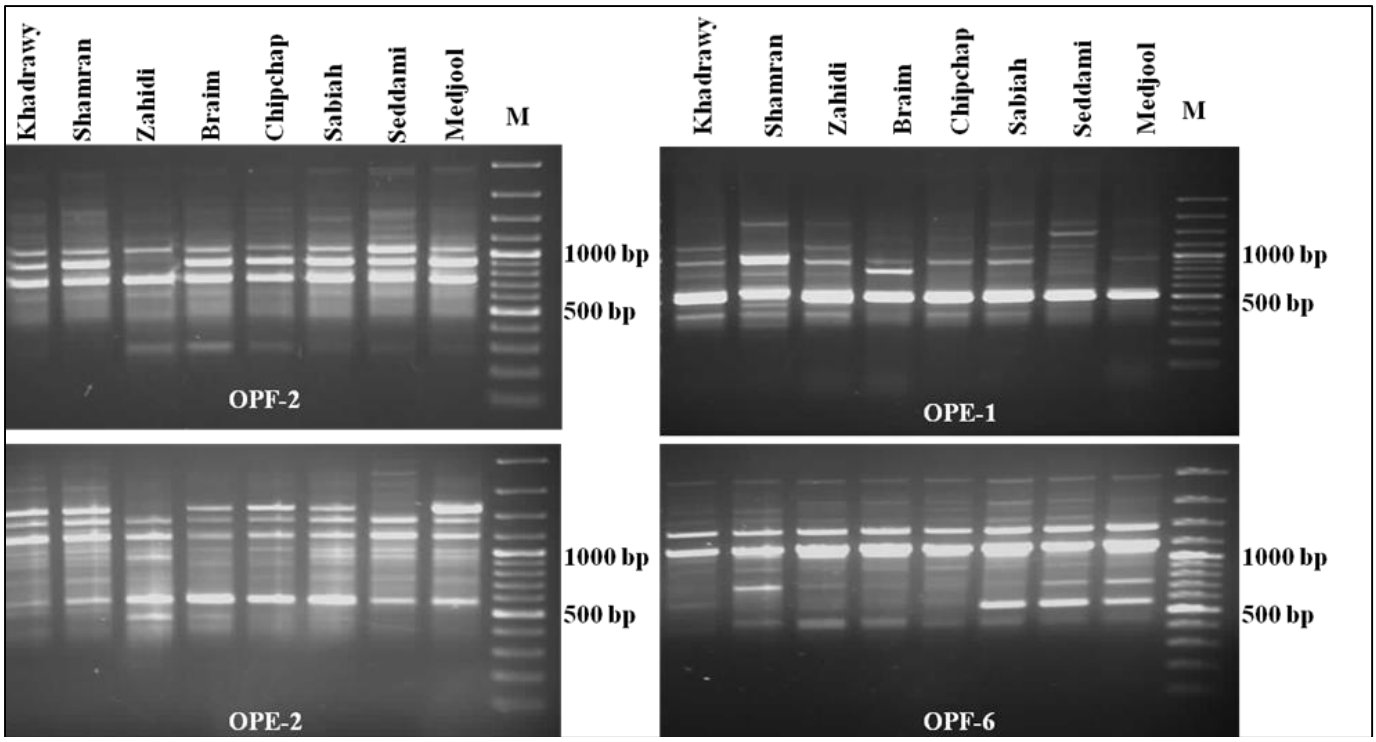

Fig 1: RAPD banding patterns of eight date palm cultivars generated by primers OPF-2, OPE-1, OPE-2, OPF-6, and M-Marker 100 bp DNA

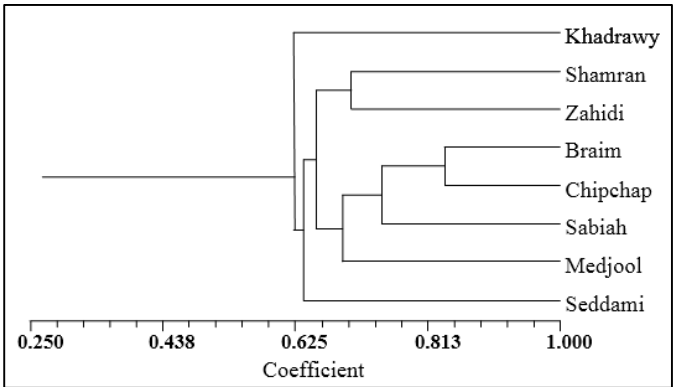

Fig 2: Dendrogram showing the phylogenetic realtionship among eight date palm cultivars

Conclusions

It was concluded that the primer OPE 5, OPF 6 and OPF 7 produced maximum number of bands which shows their ability to assess the phylogenetic relations among cultivars. Based on the study it was concluded that Khadraway and Saddami are distantly related to all other cultivars under study. However, closer relationships were observed between Shamran and Zahidi, and also between Braim and Chipchap.

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