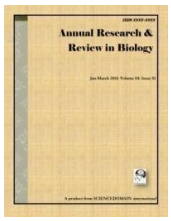

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Genetic Diversity Analysis of Date Palm (*Phoenix dactylifera L.*) Genotypes using RAPD Markers

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Authors' contributions

This work was carried out in collaboration between both authors. Author AB designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author GMN managed the analyses of the study. Both authors read and approved the final manuscript.

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ABSTRACT

Date palm (*Phoenix dactylifera L.*) is one of the most important fruit crops in the Middle East. In the present research, RAPD DNA marker was used to characterize six date palm genotypes grown in the Jiroft region of Iran. The pre-screening of 18 primers on genotypes allowed selection of 16 primers which revealed polymorphism and gave reproducible results. All analyzed genotypes were distinguishable by their band patterns. Cluster analysis by UPGMA showed two main clusters for palm genotypes based on difference in fruit color with variation in sexuality and early or mid-season maturities. Mozafati, Kabkab and Mordarsang genotypes were located on the first cluster, while the second cluster consisted of Kaloteh, Male211 and Male504 genotypes, which were most closely related with the highest value in conformity with Nei and Li's similarity value of 0.58. Dendrogram was confirmed as valuable cluster Principal Coordinate Analysis (PCoA) using the first principal component from the molecular data. The results of molecular analysis were adapted with genetic variation. RAPD technology therefore appears very effective for identifying genotypes, polymorphism and genetic distance of date palm.

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Keywords: Date palm; diversity; molecular markers; RAPD.

1. INTRODUCTION

The date palm (*Phoenix dactylifera* L.), 2n=2x=36, is a dioecious long-lived monocotyledonous plant, belonging to the Arecaceae family. It is one of the excellent candidate crops in arid and semiarid regions of the world with high tolerance to environmental stresses. In addition to its valuable fruit, the tree is cultivated for fuel, fiber and as shelter for ground crops [1]. The annual world production of dates has reached 6-8 million mt (metric tons), representing a market exchange value of over 1 billion USD. The fruit is nutritionally rich and can be processed into several products, thus generating employment and socioeconomic benefits to most local people [1]. Morphological characterization of cultivars requires a large set of phenotypic data that are normally difficult to collect, and statistically variable due to environmental effects [2]. 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 [3,4]. 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, although the exhibited polymorphism was low [2,5]. The RAPD markers have been used to detect somaclonal variation in TC (Tissue Culture) derived plant from four date palm varieties. RAPD markers were used by [6] to analyze the genetic stability of somatic embryogenesis-derived regenerates and mother plant in the Iranian-grown date palm cultivar Khinaizi. Al-Khalifah and Askari [7] has already used 19 date palm cultivars from Saudi Arabia for micro-propagation studies and subjected the regenerates to RAPD for genetic variation analysis. Other RAPD analyses were used for the identification and assessment of genetic diversity for the conservation of date palm germplasm in Saudi Arabia. Most of these findings indicated that the RAPD technique is reliable for the identification and construction of genetic linkage maps, but other reports have suggested that RAPD markers have significant difficulties for cultivar characterization because of low polymorphism, irreproducibility and the construction of weak grouping associations [8,9,10]. Therefore, the present investigation was conducted to define the genetic diversity among female and male genotypes grown in the Jiroft, Iran region using RAPD markers.

2. MATERIALS AND METHODS

2.1 Plant Materials

The planting material consists of fresh juvenile leaves from adults trees. A set of six genotypes, comprising four female and two male were used for the study (Table 1). The date palms considered here were collected from Jiroft, Iran.

2.2 Total Genomic DNA Extraction

Total genomic DNA was extracted from young leaves. The leaves were first ground into a fine powder in liquid nitrogen using pestle and mortar. Then, CTAB protocol [11] was used to extract the genomic DNA. Adding 0.6 g CTAB, 0.03 g PVP and 0.015 g activated charcoal, leaves were softly homogenized in 3 ml extraction buffer contain 100 mM Tris-HCl (pH 8), 2.0 M NaCl, 20mM EDTA (pH 8). The homogenate was then transferred into a microcentrifuge tube and incubated at 55ºC for 30 min with frequent agitation, avoiding the suspension to settle. It was then cooled down to room temperature and centrifuged at 16,000 g for 10 min at room temperature. After the transfer of supernatant to a new tube, 1 volume of chloroform-isoamylalcohol (4% (v/v)) was added to the supernatant and vortexed thoroughly. Then, Centrifuged at 16,000 g for 10 min at room temperature and the aqueous (upper) phase was transferred to a new tube. If cloudiness in the solution persists, the chloroform-isoamylalcohol extraction should be repeated again. The supernatant was then transferred to a new tube and 0.45 volume of isopropanol was added and mixed by inversion. The mixture was incubated at 25ºC for 1 hour and centrifuged at 700 g for 10 min at room temperature. The supernatant was discarded and the remaining pellet was washed by adding 1 mL of wash buffer followed by vortexing. After centrifugation at 900 g for 10 min at room temperature and discarding the supernatant the pellet was air dried and re-suspended in 50µL of distilled water.

2.3 RAPD-PCR

Amplification of genomic DNA was made on a Perkin Elmer DNA Cycler (BIOMETRA, Germany), sixteen arbitrary decamers (Sina Colon Co.) in 25-µL reaction volumes containing 1 unit of Taq polymerase, 2.5µl Tris-HCl (pH 9.0), 4.2 mM MgCl2, 0.2 mM of each dNTP, 0.5mM each of random primer and 50 ng of template DNA. The cycle program included an initial 5 min denaturation at 95ºC, followed by 30 cycles of 1 min at 95ºC, 30 sec at 35ºC and 2 min at 72ºC, with a final extension at 72ºC for 8 min. RAPD fragments were separated on 3% agarose gels in 1X TBE buffer, stained with ethidium bromide and photographed on a UV transilluminator using a digital camera. DNA from each plant was amplified using primer and finally their banding patterns were compared (Fig. 1).

2.4 Data Analysis of RAPD

The gel profile was visually scored by assigning a number to each distinctive band. The PCR reactions for polymorphic primers were repeated to verify reproducibility of results. From the total of 18 primers, only 16 primers which exhibited polymorphism were selected for the analysis (Table 2). Fragment sizes were designated as amplified bands. The presence or absence of bands was scored as 1 or 0, respectively. Estimation of genetic similarity (GS) was calculated for all pairs of varieties using Nei [12] coefficient. UPGMA was performed with matrix of GS estimates to measure the quality of each marker. Distance matrix was performed through the Jaccard's similarity coefficient [13] using NTSYS-pc Ver. 2.1 [14].

2.5 Principal Coordinates Analysis (PCoA)

Principal coordinate analysis (PCOA) was performed and consequently two-dimensional plot was drawn based on the first two main components. The program Power Marker version 3.25, Liu and Muse [15] was used to calculate allele frequencies and alleles per locus. Polymorphism information content (PIC) which indicated the ability to distinguish between genotypes for each primer combination also was calculated.

3. RESULTS AND DISCUSSION

3.1 Genetic Diversity Analysis

In total, 107 amplified DNA fragments were obtained from the RAPD primers. Two primers were monomorphic among the genotypes and the rest of the 16 RAPD primers were used for genetic diversity assessment. Polymorphism information content (PIC) value of the primer OPQ6 showed the highest polymorphism (0.81) with lowest allele frequency for major allele (0.5) with sequence 5´ GAG CGC CTT G 3´. Marker OPK14, OPF5, OPJ20, OPA9, OPU12, and OPD7 has highest allele frequency for major allele (0.83), which showed minimum PIC (0.23). PIC values varied from 0.23 to 0.81 with an average of 0.32, in which the highest value belonged to OPQ6 (PIC = 0.81) (Fig. 1) (Table 2). Similarly, three female specific markers were identified in date palm [16]. Nei's gene diversity (h) and Shannon's Information index (0.19), (0.28) was obtained respectively, which showed genotypes diversity. Similarly, Trifi et al. [10] has already observed 66.07% polymorphism among 43 date palm accessions using 19 RAPD primers. The polymorphism was much higher than our study which might be due to a diversity of date palm accessions. A dendrogram (Fig. 2) based on UPGMA analysis grouped the six genotypes into two main clusters, with Jaccard's similarity coefficient at the level of 0.58. The dendrogram showed two main clusters (A and B) with four and two genotypes respectively. Cluster A has two sub-clusters (A1 and A2). Subcluster A1 consists of Mozafati and Kabkab genotypes which are late maturing (female) with black fruits and highly resistant to rain water, while subcluster A2 comprises Mordar-sang genotype, which is seasonal, late maturing with yellow fruit and show medium resistance to rain water. Cluster B was further divided into two subclusters. Subcluster B1 consists of Male211, an early maturing and seasonal male and Male504, a good quality male genotype which were most similar; B2 includes Kaloteh, an early maturing and seasonal female. Al-Moshileh et al. [17] observed similarity with coefficient ranges from 0.70 to 0.84 in six cultivars of date palm using RAPD profiles. This higher similarity reflects less diversity of germplasm due to collections coming from the same geographic region. In another study done [2], RAPD markers have been used for identification and DNA fingerprinting of date palm varieties, although the exhibited polymorphism was low and also other reports have suggested [10], that RAPD markers have significant difficulties for cultivar characterization because of low polymorphism, irreproducibility and the construction of weak grouping associations. In conclusion, our study revealed that RAPD markers have high polymorphism and appears to be a powerful

technique for analysis of genetic diversity of date palm germplasm against previous researches. These results suggest that RAPD may differentiate genotype based on morphological characters and also gave some unique markers in some genotypes.

3.2 Principle Coordinate Analysis

The validity of obtained dendrogram was evaluated by cophentic coefficient (r=0.91), then the dendrogram was confirmed as valuable cluster. PCoA was done using the first principal component from the molecular data and these data were scattered on biplot to simplify the reaction of genotypes according to the biplot and 3-dimentional biplot. Based on the biplot and 3-dimentional biplot analysis, genotypes were divided into two groups: First group showed a number of genotypes including Mozafati, Kabkab and Mordar-sang (female/ seasonal) as classified in the same group. Male211, Male504 and Kaloteh showed different reactions based on first 2 and first 3 principle components. The genotypes were grouped together as good males, same age and the same location (Figs. 3,4). Hamza et al. [16] has already found grouping of date palm cultivars according to their fruit characteristics. PCoA results showed the grouping based on difference in fruits color with variation in sexuality and early or mid-season maturities. These results may further be confirmed with a large genotypes and primers. The results of this study showed clear and high yields of amplified DNA fragments. RAPD method appears to be a powerful technique for analysis of genetic diversity of date palm germplasm. Many oligonucleotide primers are now known for future genetic diversity analysis of date palm and for identification of variable varieties.

Table 1. Morphological characters of six date palm genotypes samples from Iran

No.	Genotype	Female trees	Fruit color	Resistance to rain water	Fruit taste
1	Mozafati	Late maturity	Black	High	Much sweet
2	Kabkab	Late maturity	Black	High	Much sweet
3	Mordar- Sang	Seasonal	Yellow	Medium	Sweet
4	Kaloteh	Early maturity	Brown	Susceptible	Medium sweet
No.	**Genotype**	**Male trees**	**Quality**	**Source**	**Plant age**
5	Male211	Early maturity	Medium	Main plant	15
6	Male504	Early maturity	Good	Main plant	15

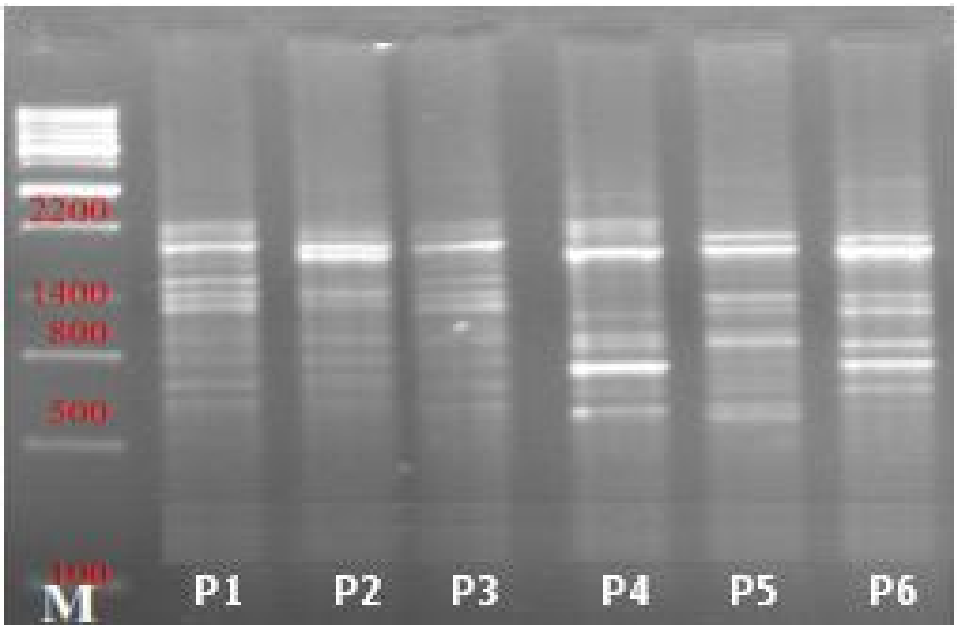

Fig. 1. RAPD markers of six date palm accessions amplified with primer OPQ6 (M= 1Kb ladder, lane numbers represent the accessions numbers while arrows indicate polymorphic markers)

Table 2. Sequences of the random nucleotide primers used to evaluate six palm genotypes from Iran

Sequence	Primer	No.	Sequence	Primer	No.
5′ GAG CGC CTT G 3′	OPQ6	9	5′ GAC GGA TCA G 3′	OPK14	1
5′ TGG ACC GGT G 3 ′	OPC8	10	5′ GGG TAA CGC C 3′	OPA9	2
5′ GGA CAC CAC T 3′	OPJ19	11	5′ TCA CCA GCC A 3′	OPU12	3
5′ GTG GGC TGA C 3′	OPP6	12	5′ TGT CTG GGT G 3 ′	OPD7	4
5′ TGG TCG CAG A 3′	OPJ18	13	5′ ACG AGG GAC T 3′	OPJ21	5
5′ ACC TTT GCG G 3′	OPU6	14	5′ AAG CGG CCT C 3′	OPJ20	6
5′ CCC GGC ATA A 3′	OPJ1	15	5′ CCG AAT TCC C 3′	OPF5	7
5′ ACA GCC CCC A 3′	OPU20	16	5′AGA TGC AGC C 3′	OPE7	8

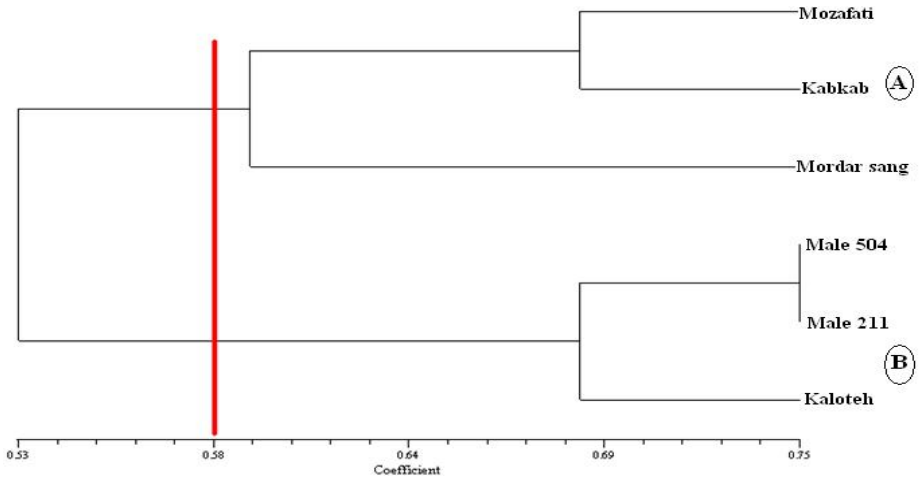

Fig. 2. Dendrogram of six date palm accessions based on RAPD markers according to the unweighted pair group mean algorithm (UPGMA) method based on a similarity matrix by NTYSYs 2.1 software

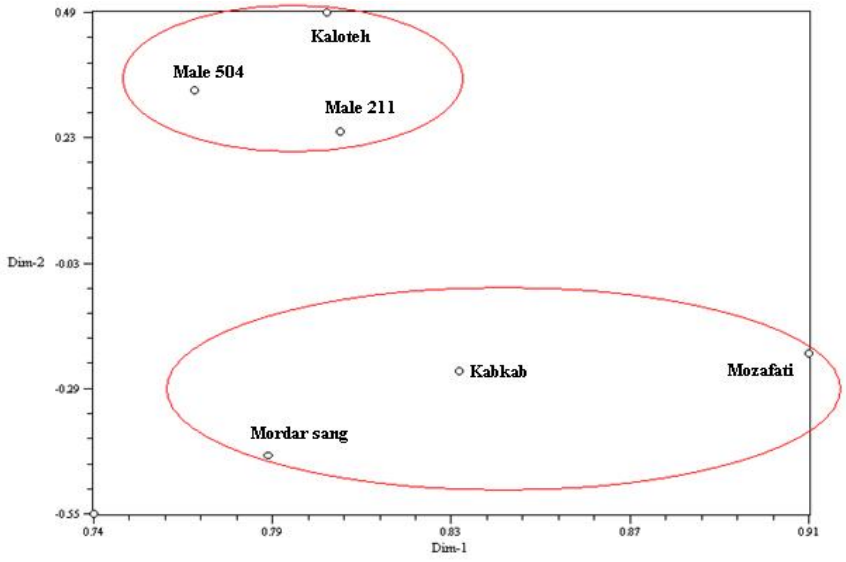

Fig. 3. Biplot of six date palm first two principal components

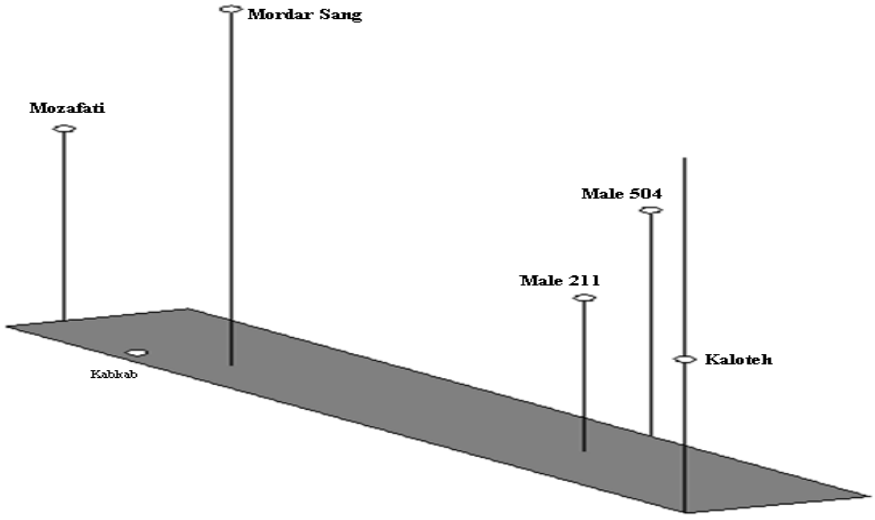

Fig. 4. 3D-Biplot of 6 date palm first three principal components

4. CONCLUSION

The date palm is known in the Jiroft, Iran region, due to its great potential to generate income in extreme environmental conditions, unproductive and semiarid lands. Characterization of germplasm is a prerequisite for crop improvement and systematic study. 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

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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