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Investigation on molecular phylogeny of some date palm (*Phoenix dactylifra* L.) cultivars by protein, RAPD and ISSR markers in Saudi Arabia

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Abstract

Date palm (*Phoenix dactylifra* L.) is one of the most important fruit crops in the Middle East. Kingdom of Saudi Arabia lies among the largest date palm fruit producer worldwide. However, little knowledge is currently available about the molecular characterization of date palm cultivars in this country. Determination of genetic variability and cultivar identification in date palm are two major importance in breeding programs, characterization of germplasm, and conservation purposes. Three types of markers such as protein, RAPD-PCR, and ISSR were applied on four important cultivars in Saudi Arabia (Med300l b1, Sugay1 b1, Khalas b1 and Sukkarib1). Intervarietal variations were investigated using five RAPD and five ISSR markers. Most of the studied markers showed intervarietal polymorphism. Protein markers were also applied to assess the genetic polymorphism. Cluster analysis by UPGMA showed two main clusters. Cultivar Sukkarib1 was located on the cluster A, with Nei and Li's coefficient equal to 0.55. Cluster B consisted of 3 other cultivars, Med300lb1, Sugayb1and Khalasb1 with 0.66-0.85 Nei and Li's similarity range. Sugay b1 and Khalas b1 were the two most closely related cultivars among the four cultivars with the highest value in the similarity for Nei and Li's coefficient 0.85. Med 300l b1 was also closely related to Sugay b1 and Khalas b1 with second highest value in the similarity matrix 0.66. The average similarity among the four cultivars was a bit more than 55%. Most of cultivars had the narrow genetic diversity as already expected. The result of the analysis can be used for the selection of possible parents to generate mapping populations.

Keywords: Date palm, RAPD-PCR, protein, ISSR, SDS-PAGE, diversity, conservation

Introduction

The green revolution was mainly based on use of high yielding varieties. Date palm has been domesticated for at least 5000 years in the Middle East region as a most important fruit crop and represents a big source of income to oases and creates the favorable condition for improving secondary crop cultures like barely, Alfa Alfa and cloves as forage. The numbers of known date palm varieties that are distributed all over the world are approximately 5000 out of which about 450 are found only in Saudi Arabia (Bashah 1996). The average number of date palm trees in Arab countries is estimated to be about 62 million trees. In 1996 Saudi Arabia produced 570.000 tons of date worth 203 million US$ (FAO 1996). There are three main types of date based on fruit moisture, content, etc. such as soft, semidry and dry cultivars. Dates (*Phoenix dactylifra* L.) are dioecious perennial, monocotyledon fruit trees that belong to the family of Arecaceae and its heterogeneous genetic form makes its progeny strongly heterogeneous and variable (Fakir and Munier 1992). Multiplication of date palm is mainly done vegetatively. Therefore, individuals with economically important traits are easily cloned. Clonally propagation of elite cultivars with known high performance is highly diseried in Saudi Arabia. The main limitation for this type of propagation is the minimal production of offshoots some of which will die when separated from the mother plants. Over years many varieties have been transplanted to the areas other than original regions, and they may have been adapted and cultivated with different names. As a result, a variety may have a different name in different plantation areas or even two genetically varieties may have the same name (Torres and Tisserat 1980). This matter also reduces the genetic diversity of the cultivars, making them vulnerable to biotic and abiotic stresses. The morphological markers such as fruit characteristics have been used to describe the varieties, but these markers are significantly affected by the environment. In general, identification and evaluation of genetic diversity between cultivars based on morphological markers are very difficult and time

Table 1. Showing the sequence of the ten decamer arbitrary RAPD-PCR primers (Operon model) assayed in RAPD-PCR

No	Primer name	Sequence
1	OP C-02	5'-GTGAGGCGTC-3'
2	OPC-06	5'-GAACGGACTC-3'
3	OP C-10	5'-TGTCTGGGTG-3'
4	OPC-11	5'-AAAGCTGCGG-3'
5	OP C-15	5'-GACGGATCAG-3'

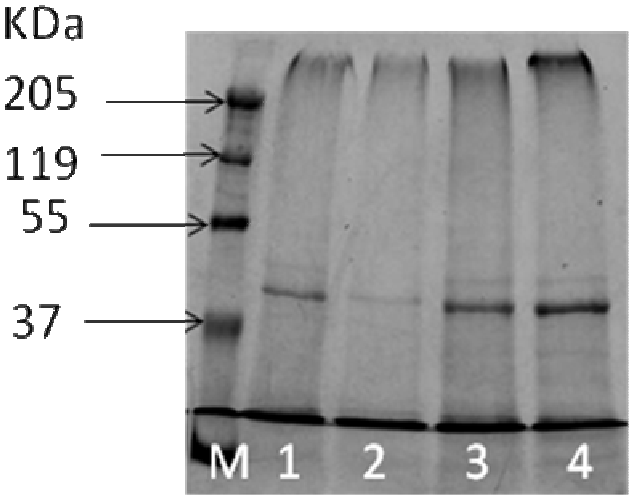

Fig 1. Polyacrylamide gel electrophoresis SDS-PAGE stained with coomassie blue R-250 brilliant blue for total protein of date palm from leaves lane (M) High range protein marker from Bio-Rad lane 1- Med300lb1, lane 2- Sugay1 b1, lane 3- Khalas b1 and lane 4-Sukkar1 b1.

consuming because the onset of fruiting takes approximately between 3 to 5 years. Furthermore, characterization of varieties requires a large set of phenotypic data that are normally difficult to collect and statistically variable, due to environmental effects (Sedra et al., 1993, 1996 and 1998). Biochemical markers (isozymes and proteins) have proven to be effective in varietal identification as well. (Bendiab et al., 1993; Bennaceur et al., 1991; Fakir and Munier. 1992). However, they give limited information and are usually indirect approach for detecting genomic variation. The molecular markers are useful tools of identification and phylogenetic analysis of different plant species and cultivars. Genetic fingerprinting using molecular markers have many important applications, including germplasm conservation, assessment of seed purity and verification of labeling and identity of plants in production and marketing (Henry 1998)

Random amplified polymorphic DNA (RAPD-PCR) is a powerful technique, which can be used to identify and determine plant genomes or to estimate the phylogenetic relationship among the individual genomes of date palm. RAPD is based on the polymerase chain reaction (Williams et al., 1990; Clark and Lanigan 1993). ISSR (inter simple sequence repeat) markers have also been proven to encompass many advantages over the other molecular techniques as it requires less time, low cost, small quantity of DNA for the analysis and co-dominant nature (Powel et al., 1996). The objectives of the present study were 1) screening the suitable primers for development of diversity markers in date palm and 2) to analyze the genetic diversity among the four different cultivars using these developed markers.

Materials and methods

Plant materials

The plant materials were collected from various plantation areas of the Kingdom of Saudi Arabia for four commercial cultivars Med300l b1, Sugay1 b1, Khalas b1 and Sukkar1 b1. The cultivars were provided by SAPAD Company (Saudi American Plant Development)

Protein analysis by SDS-PAGE

Total proteins of fresh leaves were analyzed by SDS-PAGE. Leaves were grinded on liquid nitrogen in 0.2 M Tris pH 8, 2% w/v SDS, 10% sucrose and 1% BME. Proteins were separated by SDS-PAGE according to Laemmli, 1970. Gel slab was scanned using gel pro-analyzer ver. 3.3 (Media Cypermetics 93-97). Data were statistically analyzed by multiple comparison procedure at $p \leq 0.05$ using t-test and mean separation by least significant difference (LSD) (Steel and Torrie, 1980).

Total Genomic DNA extraction

Total genomic DNA was extracted from young leaves. The leaves were first grinded into a fine powder in liquid nitrogen using a pestle and morter following the steps of CTAB protocol (Porebski et al., 1997; Hussien et al., 2003).

RAPD-PCR

RAPD was performed as described by Williams et al. (1990) with slight modification. PCR reactions were carried out in 25 µl volumes containing 25 ng of total genomic DNA, 10 pmol primer, 200µM dNTP, 2mM MgCl2, 1X PCR buffer and 2 units ampli Taq polymerase (RTS *Taq* DNA polymerase). Five random oligonuclotide primers OPC2, OPC6, OPC10, OPC11 and OPC15 were used in the experiment (Operon technologies, Alameda, USA) (Table 1). Amplification was performed in Perkin Elmer 9600 thermal cycler (Foster City, USA) with the following temperature profile: 94°C for 5 min followed by, 40 cycles of 94°C for 1 min, 36°C for 1 min, and extension at 72°C for 90s. The final extension step was carried out by 72°C for 5 min. The list of oligonucleotide is given in Table 1.

Table 2. The sequence of the 5 pairs of primers used in ISSR

Primer	Forward	Reverse	Repeat unit
DPssr 118	5-CAAAAAGAAAGAGGGGAAAAA-3	5-TGGCATAATGGATCAGGTCTT-3	$(TC)_{19}$
DPssr 7	5-GGTAGACGCAAATCGAAAGAACG-3	5-CAGTAAACAGGAAGAGTACCAGTAG-3	$(TGG)_5$
DPssr8	5-CATCTGCCATTTCTTCTGACCA-3	5-GACCGGCATTCCTATTTCTCTGTT-3	?
DPssr11	5-TGCTTTCCCCTCTTCTTCTTCTCC-3	5-CTACCCGCAGCTCACCTCTTCTAT-3	?
DPssr12	5-TTGTCGTAATACCAGCAGGAA-3	5-GGTGTGGAGTAATCATGTAGTAGA-3	?

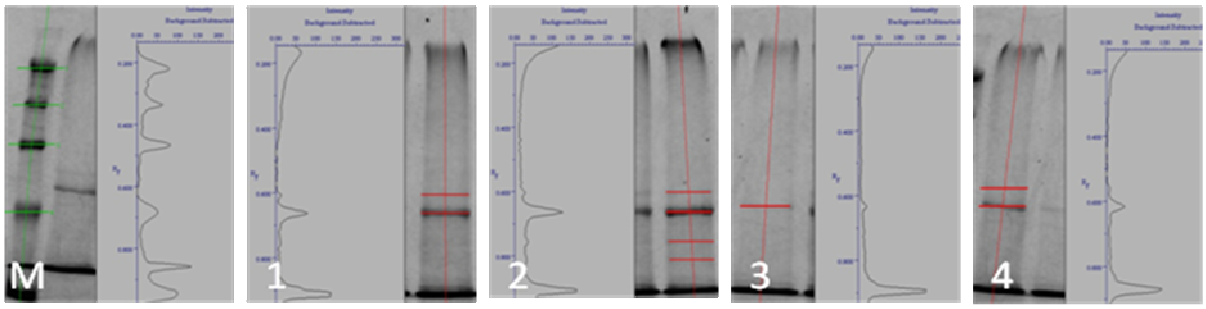

Fig 2. Scanning of SDS-PAGE gel of the total protein of date palm leaves. Lane (M) High range protein marker from Bio-Rad , lane 1- Med300lb1, lane 2- Sugay1 b1, lane 3- Khalas b1 and lane 4-Sukkar1 b1

Table 3. comparative analysis of optical density (O.D), molecular weight (M.wt) and relative front (RF) of SDS-PAGE protein profile of date palm (*Phoenix dactylifra* L.) leaves.

Lane Number	Band Number	Relative Front	Mol.wt. KDa	Peak OD	Average OD
1	1	0.22	205	176.502	138.024
1	2	0.339	119	162.759	135.976
1	3	0.467	99	185.638	146.415
1	4	0.684	52	160.44	139.911
2	1	0.563	74.495	114.638	112.699
2	2	0.618	63.223	154.058	133.511
3	1	0.634	64.34	113.241	108.33
4	1	0.608	74.091	122.241	117.93
4	2	0.664	63.223	188.739	147.228
5	1	0.604	74.927	116.86	113.087
5	2	0.666	62.812	217.981	155.872
5	3	0.757	47.153	124.72	120.767
5	4	0.814	39.174	121.058	119.026

ISSR

ISSR scorable primers were designed and screened for PCR amplification (Table 2). The PCR reactions were prepared by using 50 ng of genomic DNA, 1x PCR buffer, 200µM dNTP, 2mM $MgCl_2$, and 2 units of ampli *Taq* polymerase (RTS-*Taq* DNA polymerase) and 15 ng ISSR primers. The following temperature profile was used for amplification: 94°C for 5 min followed by 45 PCR cycles of 94 °C for 1 min, 49°C for 45 sec and 72°C for 2 min. A final extension step of 7 min at 72°C was also carried out. The PCR products were separated on 1.4% (RAPD) and 1.6 % (ISSR) Agarose gel in 1x TAE buffer containing 0.1 μgml^{-1} of Ethiduim bromide for about 2 hrs at 80 V. Gel was photographed under UV light with Tracktel GDS-2 gel documentation system.

Data analysis

The gel profile were visually scored by assigning a number to each distinctive band. The PCR reactions for polymorphic primers were repeated to verify reproducibility of results. 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 and Li (1979) coefficient. UPGMA was performed with matrix of GS estimates to measure the informativeness of each marker.

Results and discussion

The aim of the present study is to produce the molecular markers for identification of four very common cultivars of date palm in Saudi Arabia. The SDS-PAGE analysis of the total protein profile from leaves revealed no significant differences in protein content of cultivars, qualitative (Fig 1&2) and quantitatively (Table 1). No major differences recognized in banding pattern of the protein of the studied cultivars. Although the scanning analysis showed that cultivar Sukkarib1 has a slightly different protein profile than protein profile of the other three cultivars.

Five RAPD-PCR primers were used and results showed that the sizes of the fragments ranged from 300 to 3000bp.

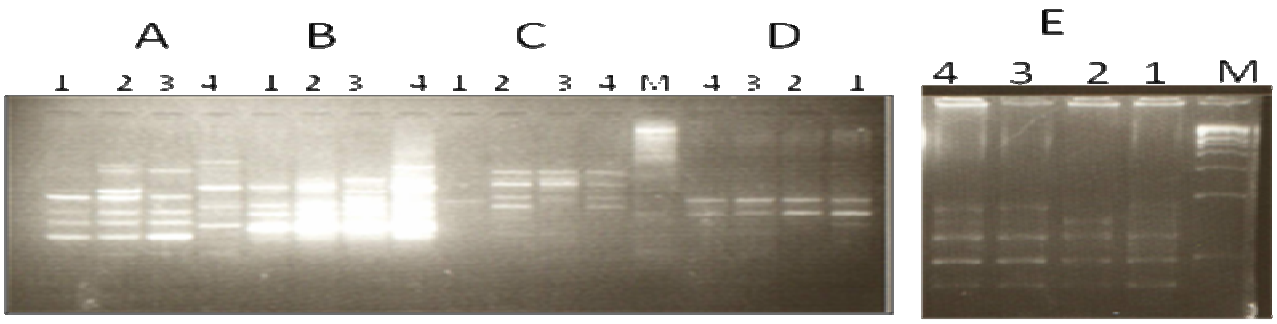

Fig 3. RAPD –PCR profile of four date palm cultivars group A using OPC2 primer: lane 1- Med300lb1, lane 2- Sugay1 b1, lane 3- Khalas b1 and lane 4-Sukkar1 b1, group B using OPC6 Primer lane 1- Med300lb1, lane 2- Sugay1 b1, lane 3- Khalas b1 and lane 4-Sukkar1 b1, group C using OPC10 Primer: lane 1- Med300lb1, lane 2- Sugay1 b1, lane 3- Khalas b1 and lane 4-Sukkar1 b1, group D using OPC11 Primer: lane : 1- Med300lb1, lane 2- Sugay1 b1, lane 3- Khalas b1 and lane 4-Sukkar1 b1and group E using OPC15 Primer: lane : 1: Med300lb1, lane 2- Sugay1 b1, lane 3- Khalas b1 and lane 4-Sukkar1 b and lane M :1 Kb Marker.

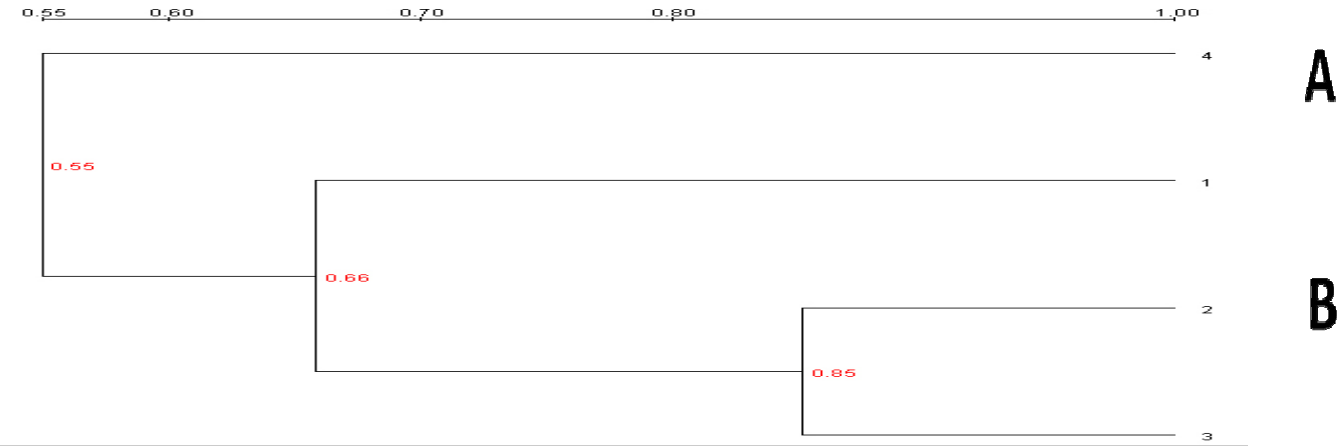

Fig 4. Dendogram of phylogenetic relationships among four cultivars of date palm based on Nei and Li's similarity coefficient obtained from 5 RAPD primers.

The average number of amplified band was five. All genotypes revealed a unique profile with the primers and results were reproducible and thus can be used for DNA fingerprinting. Different primers produced a different level of polymorphism among the four cultivars Fig (3). The pair-wise genetic distance and cluster analysis were estimated by unweighted paired group method of arithemetic mean (UPGMA) and showed two main clusters. Cluster A consisted of one cultivar Sukkarib1 which did not belong to the other 3 cultivars, with 0.55 Nei and Li's coefficient in the similarity matrix. Cluster B consisted of 3 cultivars Med300lb1, Sugayb1and Khalasb1 with 0.66-0.85 Nei and Li's similarity range. Sucayb1 and Khalas b1 were shown the most closely related cultivars among the four cultivars with the highest value in the similarity matrix for Nei and Li's coefficient of 0.85. Med 300lb1 was closely related to Sucayb1 and Khalas b1 with second highest value in the similarity matrix of 0.66. The average similarity among the four cultivars was more than 55%. As expected most of the cultivars have a narrow genetic base. The result suggests that RAPD analysis could be used for an efficient identification and DNA fingerprinting for the date palm varieties in Saudi Arabia. This will help in the collection and cataloguing of the germplasm in the form of a germplasm bank (Fig 4 and Table 4).

Table 4. Similarity matrix of date palm genotypes obtained from RAPD marker.

	4	**3**	**2**	**1**
4	**100**	64.2	58.2	42.6
3	64.2	**100**	85.2	60.9
2	58.2	85.2	**100**	70.8
1	42.6	60.9	70.8	**100**

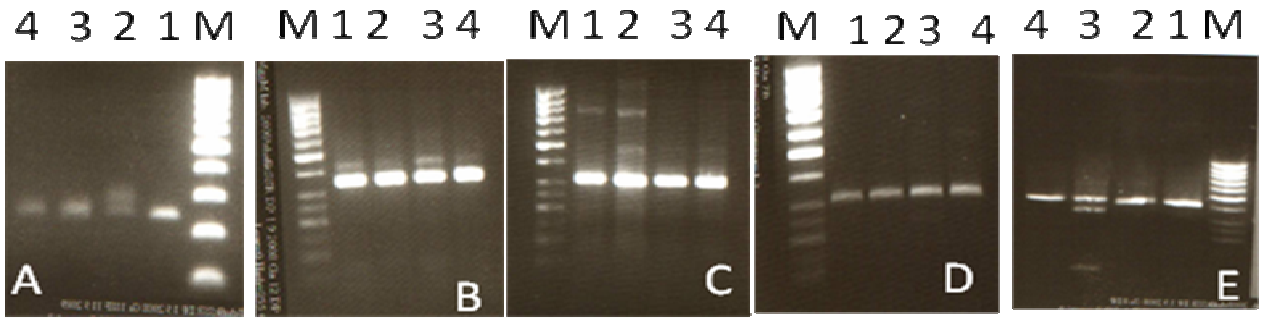

Fig 5. ISSR profile A: SSRDP118 primer with the four date palm cultivar, Lane 1- Med300lb1, 2- Sugay1 b1, 3- Khalasb1 and 4-Sukkar1 b1 , profile B: SSRDP12 primer with the four date palm cultivar lane 1- Med300lb1, 2- Sugay1 b1, 3- Khalas b1 and 4-Sukkar1 b1,profile C: SSRDP11 primer with the four date palm cultivar: lane 1- Med300lb1, 2- Sugay1 b1, 3- Khalas b1 and 4-Sukkar1 b1 , profile D: SSRDP7B primer with the four date palm cultivar lane : 1- Med300lb1, 2- Sugay1 b1, 3- Khalas B1 and 4-Sukkar1 b1 and profile E: SSRDP8 primer with the four date palm cultivar lane: 1- Med300lb1, 2- Sugay1 b1, 3- Khalas b1 and 4-Sukkar1 b1 and lane M in the five profiles is the 100 bpDNA Marker.

Apparently the RAPD markers must be an effective and powerful tool for identification and DNA fingerprinting of the date palm varsities, although polymorphism is low in comparison with other cultivated species (Farooq et al., 1994; Koller et al., 1993; Yang and Quiros, 1993). Despite of previously mentioned statements we suggest that utilization of RAPD markers could potentially have the high priority for characterization and genetic conservation of date palm germplasm. However, it is necessary to choose and include more varsities and the number of the primers to precisely assess the phylo-genetic relationship in populations. Low RAPD poly-morphism and the lack of evident organization observed among the date palm varieties could be due to the nature of introduction of different varieties in the country. Instead of relying only on RAPD markers, we also studied fast and effective ISSR marker as well. ISSR markers were found to be potentially useful for studying genetic diversity, introgression analysis and identification of germplasm. In the present study five ISSR primers were used (Fig 5). The ISSR markers also revealed the narrow genetic diversity among the four cultivars. This narrow genetic diversity probably might be due to several different reasons mainly exchanging of varieties between different plantation areas, clonal propagation of ecotypes, development of new recombinant by seedling selection and limited sexual reproduction. Arbitrary or random selection by farmers may represent only a small fraction of the date palm diversity.

In conclusion, present study indicate that protein, RAPD and ISSR marker individually have their own merits in the date palm cultivar fingerprinting, However a combination of all three markers can be used in date palm fingerprinting (Al-Khalifah and Askari, 2003).

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