Genetic variability studies and molecular fingerprinting of some Egyptian date palm (*Phoenix dactylifera L.*) cultivars

I.A preliminary study using RAPD markers

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ABSTRACT

Determination of genetic variability and proper cultivar identification in date palm would be of major importance in improvement programs and in germplasm characterization and conservation to control genetic erosion. In a preliminary study for fingerprinting Egyptian date palm cultivars, the reliability of the RAPD technology for the identification of five cultivars (Zaghloul, Samany, Hayany, Siwi and Amhat) has been assayed. Two different protocols for DNA extraction have been tested and optimization of the PCR conditions was performed. Intra and inter varietal variations were investigated using ten random decamer primers. Only five primers generated reproducible, pertinent RAPD profiles. Due to the dioceous nature of date palm trees intravarietal polymorphism was detected among the sixteen individual samples representing each cultivar. The different RAPD profiles displayed within each cultivar and among cultivars were analyzed to identify markers that are characteristic for each cultivar. The five selected primers revealed 16 RAPD polymorphic markers across the five date palm cultivars. Similar markers were also detected in the RAPD profiles generated by five bulked DNA samples representing the different cultivars. This represents a first step towards the molecular identification and fingerprinting of these cultivars.

Key words: *Date palm, RAPD, molecular markers.*

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is a long-lived monocotyledonous dioecious diploid (2n=36) and is mainly cultivated in the old world (Moore, 1993). It is of great economic importance in oasis agriculture and in arid regions of the world due to its tolerance to environmental stresses including salinity, drought and high temperature. The average number of date palm trees in the Arab countries is estimated to be about 62 million trees, of which 7.5 million trees are cultivated in Egypt.

Exploitation of genetic variability and studying its pattern of distribution would be of a major importance in the improvement of date palm. They are also essential tasks in germplasm characterization and conservation to control genetic erosion, to design sampling strategies and to establish breeding programs.

As in many other perennial crops, proper cultivar identification in date palm traditionally relied on morphological characters that cannot be assessed until tree maturity. With the development of electrophoretic techniques, isozyme markers have been identified by some authors (Torres and Al-Jibouri, 1989; Al-Jibouri and Adham, 1991; Bannaceur *et al.*, 1991; Booij *et al.*, 1995). The advent of molecular biology has provided new genetic markers, which can be used to detect differences in the genetic information carried by different individuals. These DNA markers result from base-pair changes, rearrangements, insertions or deletions at the homologous DNA region. Therefore, molecular markers offer many advantages when compared to conventional markers, especially that genotypes could be successfully distinguished at the seedling stage. Among the molecular markers, restriction fragment length polymorphism (RFLP) has been used by Corniquel and Mercier (1997) for the identification of four date palm cultivars using DNA probes. Randomly amplified polymorphic DNA (RAPD) analysis, first described by Williams *et al.* (1990) and Welsh and McClelland (1990), has proven to be an easier and useful tool for genotyping and mapping. Sedra *et al.* (1998) used RAPD markers to detect the genetic variation among date palm varieties from Morocco.

The present investigation is a preliminary study to test the reliability of the RAPD technology for the identification of Egyptian date palm cultivars and to investigate the level of *intra* and *inter*-cultivar polymorphism.

MATERIALS AND METHODS

Plant material

Five Egyptian date palm cultivars, i.e., Zaghloul, Samany, Hayany, Siwi and Amhat were included in this investigation.

Extraction and purification of genomic DNA

Genomic DNA was extracted from leaf samples taken from sixteen individual date palm trees for each cultivar amounting to a total of 80 samples.

Isolation of DNA was performed using two different CTAB-based protocols. The first method was described by Aitchitt *et al.* (1993). This method is a modified CTAB protocol, which utilizes an increased CTAB concentration (3%), a single extraction with chloroform-isoamyl alcohol and an additional precipitation of DNA with sodium acetate and ethanol. The second protocol was adopted from Porebski *et al.* (1997). In the latter protocol, polyvinyl pyrrolidone (PVP) is added to the CTAB extraction buffer to remove polyphenols and an additional phenol-chloroform step was performed to remove any excess proteins.

DNA concentration was estimated electrophoretically by comparing the degree of fluorescence of the DNA samples with the different bands of the Lambda, Hind III digest /Phi X 174, Hae III digest size marker run on agarose gel. The DNA samples were diluted to contain 10 ng /µl DNA.

Randomly amplified polymorphic DNA (RAPD)

A total of ten random decamer primers synthesized at AGERI /Egypt, using a DNA synthesizer model ABI 392, were used for

RAPD analysis. RAPD reactions were conducted mainly according to Williams *et al.* (1990). RAPD analysis was carried out on individual samples (sixteen samples from each of the five cultivars) in addition to five bulked DNA samples representing the different cultivars. Reactions were performed in 50 ul volume. In a series of preliminary experiments, PCR amplification conditions were optimized using different concentrations of DNA (20, 40 and 60 ng), magnesium chloride (2.0, 2.5, 3.0 and 3.5 mM), RTS Taq DNA polymerase (2, 3 and 4 units). Based on the results, the final optimized amplification reaction conditions were used for subsequent RAPD analysis. The optimum thermal cycling conditions were: 94°C for 1 min, followed by 40 cycles with the following temperature profile: 94°C for 1 min, 37°C for 1min, 72°C for 2 min and a final extension at 72°C for 7 min.

Amplification products were analyzed by electrophoresis on 1.4% agarose gels in TBE buffer, stained with ethidium bromide, visualized on an UV transilluminator and photographed using Polaroid films. Fragment length was estimated by comparison with standard size markers (Lambda phage DNA, Hind III digested / Phi X 174 phage, Hae III digested) or 1 Kb ladder.

RESULTS AND DISCUSSION

DNA fingerprinting using molecular markers is a powerful method to analyze genetic variation in plants for improvement of agricultural species (Beckman and Soller, 1986; Williams *et al.*, 1990; Ben Abdallah *et al.*, 2000; Trifi *et al.*, 2000; Zuo *et al.*, 2000; Le febvre *et al.*, 2001). However, these methods require DNA that can serve as a good substrate for restriction enzymes (in RFLPs and AFLPs) and DNA polymerases (in PCR-based markers). There are many difficulties associated with the isolation of undegraded plant nucleic acids which are free from contaminating proteins, polysaccharides and polyphenols (Croy, 1993). Therefore, in the present investigation, two different protocols were tested for isolating purified, high-molecular- weight DNAs from the five date palm cultivars.

The protocol described by Porebski *et al.* (1997) proved to be more successful than that described by Aitchitt *et al.* (1993). PVP, a solid polymer that has high molecular weight and is water- soluble and chemically inert, was shown to remove the polyphenols while maintaining a high yield of high molecular weight DNA.

Optimization of randomly amplified polymorphic DNA (RAPD) analysis conditions

Despite the ease of RAPD methodology, empirical optimization of the reaction conditions is of a major importance to obtain reproducible and reliable amplification profiles. Therefore, initially the RAPD reaction conditions were optimized to obtain comparable amplification patterns before conducting routine analysis on date palm cultivars. Different experiments were carried out in which concentrations of template DNA, magnesium and Taq polymerase varied. To determine the optimum template concentrations, different DNA concentrations, viz., 20, 40 and 100 ng were added to the standard reaction mixture. The effect of magnesium ions (Mg^{2+}) on the efficiency of RAPD amplification was also examined using differrent $MgCl_2$ concentrations, viz., 2.0, 2.5, 3.0 and 3.5 mM. In addition, different Taq DNA polymerase concentrations (2, 3 and 4 units/50 µl reaction) were tested. It was shown that the best amplification profiles were produced with 40 ng template DNA, 3 mM $MgCl_2$ and 3 units of Taq polymerase. These

conditions resulted in the most reliable and reproducible amplification products. In this context, Munthali *et al.* (1992) reported that the control of the amount of template DNA in the reaction is critical for obtaining reproducible results. In addition, Innis and Gelfand (1990) claimed that Mg^{2+} concentration may affect all of the following: primer annealing, strand dissociation temperatures of both template and PCR product, product specificity, formation of primer-dimer artifacts and enzyme activity and fidelity. Similarly, Bagheri *et al.* (1995) stated that one factor known to affect the stringency of PCR amplification is the magnesium concentration. Concerning the concentration of Taq polymerase, Innis and Gelfand (1990) claimed that the enzyme requirements might vary with respect to individual target templates or primers. Therefore, they suggested testing an enzyme concentration ranging from 0.5 to 5 units/100 µl, when optimizing PCR reactions.

Moreover, to ensure reproducibility and genetic pertinence of RAPD markers data, the primers generating weak or complex patterns were discarded. Therefore, among the ten tested primers, only five were selected for further analysis. The sequences of these primers are presented in Table (1).

Table (1): Primers used in RAPD analysis.

Primer	Sequence (5`-3`)	GC%
OPB07	GGTGACGCAG	70
OPB12	CCTTGACGCA	60
OPB15	GGAGGGTGTT	60
OPB17	AGGGAACGAG	60
OPB18	CCACAGCAGT	60

Due to the dioceous nature of date palm trees, intra-varietal variations are expected. Therefore, a preliminary study was carried out to investigate *intra* and *inter*-varietal variations among the five date palm cultivars using the five selected primers in RAPD reactions with sixteen samples from each cultivar. Polymorphism was detected among individuals within the same cultivar. The degree of variability differed according to the primer used for amplification in the RAPD reaction. Also, the varieties differed in the degree of variability present among them. For example, the Samany cultivar exhibited the least variability among its individuals, where the amplification profiles revealed a high level of homogeneity with each primer.

From the sixteen DNA samples examined in each cultivar, ten samples were chosen which gave consistent and reproducible results with the five primers. These samples were run on agarose gels to detect and score polymorphic bands within each cultivar as shown in Fig. (1). Moreover, in order to detect RAPD markers characteristic for each cultivar, different RAPD profiles within each cultivar and among cultivars were compared. For fingerprinting purposes, care was taken that the RAPD markers identified to characterize each cultivar were common among the different amplification profiles of the same cultivar. In addition, these markers were compared with the RAPD profiles generated by the bulked DNA samples. Similar markers were identified for each cultivar. Table (2) represents the RAPD markers showing polymorphism among the five date palm cultivars under investigation. In this context,

Sedra *et al.* (1998) were able to distinguish 43 date palm accessions using 19 selected primers and concluded that RAPD markers should be of high value for date palm germplasm characterization and genetic maintenance. Alternatively, Corniquel and Mercier (1994; 1997) reported a clear-cut discrimination among five and four date palm cultivars by RFLP analysis, with a single DNA probe restriction enzyme combination.

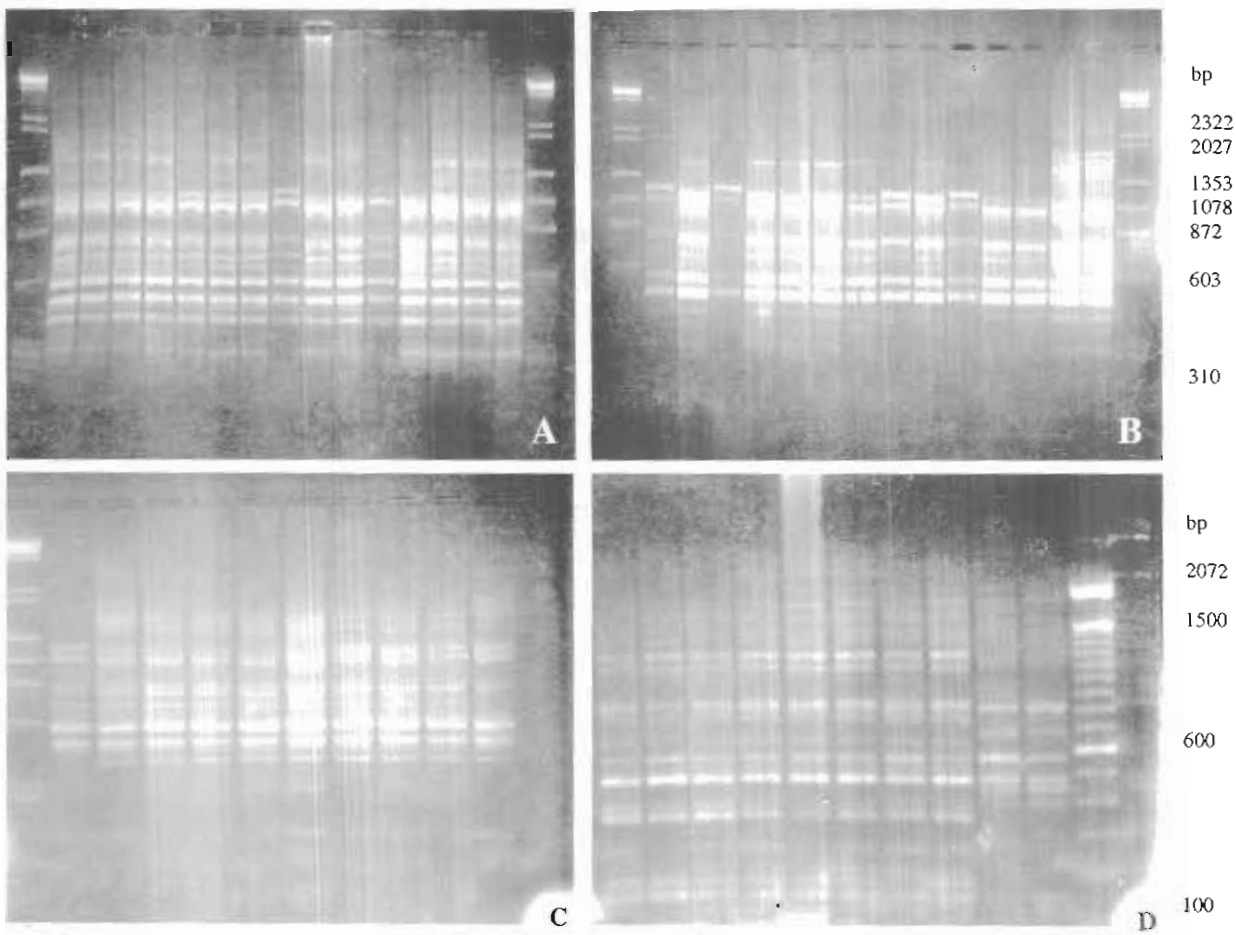

Fig. (1): RAPD profile for individual date palm samples, (A) Samany, (B) Hayany, (C) Zaghloul and (D) Amhat, amplified by OPB12 primer. M refers to Lambda Hind III digest, Phi x174 Hae11 digest; M1 refers to 100 bp DNA ladder.

As shown in Table (2), the five selected primers revealed 16 RAPD markers, which were polymorphic across the five date palm cultivars under study. These markers ranged in size from 450 to 1250 bp. The cultivar Samany was characterized by the absence of the $OPB07_{550bp}$ band, which was present in all the other cultivars. While, Zaghloul could be distinguished by the absence of the three bands $OPB12_{450bp}$, $OPB12_{500bp}$ and $OPB12_{570bp}$. The absence of the RAPD markers $OPB17_{550bp}$ and $OPB18_{650bp}$ identified the cultivar Siwi and the absence of the $OPB18_{900bp}$ marker characterized the cultivar Hayany, while in this work we were unable to find a unique marker for the cultivar Amhat, which needed more primers. Although we obtained unique markers characterizing four of the cultivars (Z, S, Si and H), all the five cultivars could still be identified through their overall banding

profiles obtained by the different primers that yielded unique fingerprints, which characterized each cultivar. Primer OPB07 gave 5 different unique profiles characterizing each of the five cultivars. While primers OPB12, 15 and 17 gave 4 different profiles and primer OPB18 gave 3 different profiles. Primer OPB12 differentiated between cultivars Z, H and A and gave similar profiles for Samany and Siwi cultivars. Primer OPB15 differentiated between cultivars Z, Si and A and gave similar profiles for Samany and Hayany cultivars. Primer OPB17 gave unique profiles for Z, Si and H and similar profiles for both Samany and Amhat cultivars. Similarly, primer OPB18 differentiated between Si and H only and gave similar banding patterns for S, Z and A.

Table (2): RAPD markers showing polymorphism among the five date palm cultivars Samany (S), Zaghloul (Z), Siwi (Si), Hayany (H) and Amhat (A).

Primers	Approx. Length (bp)	Cultivars S	Z	Si	H	A
OPB07	550	0	1	1	1	1
	690	1	1	0	1	0
	800	0	1	1	1	0
	900	0	1	0	0	1
OPB12	450	1	0	1	1	1
	500	1	0	1	1	1
	570	1	0	1	1	1
	880	0	1	0	0	1
	1200	1	0	1	0	1
OPB15	900	1	1	0	1	0
	1250	0	1	0	0	1
OPB17	550	1	1	0	1	1
	700	1	0	0	1	1
	750	0	1	0	1	0
OPB18	650	1	1	0	1	1
	900	1	1	1	0	1

The results of this study are to learn and pave the way for DNA-based markers for identification of Egyptian date palm cultivars and for the investigation of the levels of *intra* and *inter*-cultivar polymorphism. This investigation represents a first step towards fingerprinting and the establishment of a catalogue of the Egyptian date palm cultivars, however, additional markers should be used to obtain definitive results.

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الملخص العربي

تقييم التباين الوراثي وتحديد البصمة الوراثية الجزيئية لبعض أصناف نخيل البلح المصري

أ- دراسة أولية باستخدام تقنية الرابد (RAPD)

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تعتبر دراسة التباينات الوراثية والتعريف الدقيق لأصناف نخيل البلح ذات أهمية قصوى في برامج التحسين الوراثي والحفاظ علي الأصول الوراثية. ولذا كان من الضروري تحديد البصمة الوراثية للأصناف المصرية لنخيل البلح. وفي دراسة أولية لتحقيق هذا الغرض تم اختبار ملائمة تقنية الرابد (RAPD) للتمييز بين خمسة أصناف من نخيل البلح وهي زغلول، سماني، حياني، سيوي وأمهات . وفي هذا الصدد تم اختبار بروتوكولين لاستخلاص المادة الوراثية (د ن أ) من أوراق نخيل البلح كما تم تحديد الظروف المثلي للتفاعل المتسلسل للبولميرات (PCR). ثم أجريت دراسة للتباينات داخل الصنف الواحد وبين الأصناف المختلفة باستخدام عشرة بادئات عشوائية حيث أوضحت النتائج أن خمسة فقط من هذه البادئات تعطي نتائج متكررة ونمط ثابت للرابد. وقد أظهرت الدراسة تبايناً بين الستة عشر عينة الممثلة لكل صنف وذلك يتوافق مع طبيعة أشجار النخيل حيث أنها ثنائية المسكن. وعند مقارنة نواتج الرابد داخل كل صنف وبين الأصناف المختلفة أمكن تحديد واسمات رابد فريدة مميزة لكل صنف. حيث أظهرت البادئات الخمسة المختارة ستة عشر واسم رابد في الخمسة أصناف من النخيل المدروسة. وكذلك أمكن التوصل إلى واسمات رابد باستخدام عينات من الـ د ن أ تمثل خليطاً من عشرة أشجار لكل صنف (bulked DNA sample). وتعتبر هذه الدراسة خطوة أولية نحو التمييز بين أصناف نخيل البلح المصري علي المستوي الجزيئي وتحديد البصمة الوراثية لكل منها.