DNA Fingerprinting of Egyptian Dry Date Palm Cultivars Using Amplified Fragment Length Polymorphism Technique

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Abstract

Amplified Fragment Length Polymorphism (AFLP) DNA analysis of date palm cultivars is based on a multi-step process with DNA digestion, fragment ligation to adapters, pre-selective PCR, selective PCR amplification and fragment separation. DNA analysis of date palm cultivars Sakkoty, Malakaby, Gondeila, Shamia and Bartamuda with a single PCR primer set based on the EcoRI and MseI cut site plus three selective nucleotides from each end of the DNA template. MseI is a 4-bp frequent cutting endonuclease which is typically mixed together with the less frequent 6-bp cutting endonuclease known as EcoRI. The use of these two restriction enzymes typically digests dry date palm cultivars genomic DNA into fragments with a size range of 56 –390 bp. DNA was isolated from frozen date palm tissues . About 50 mg leaf tissue placed in a capped tube sandwiched between 500 mg of garnet and two ceramic beads. Modified lysis buffer (400 ml) was added from the DNeasy plant system. The plant tissue was completely macerated using a fast prep 120 (Q-Biogen) shaker/ basher at oscillation speed of 5.0 rpm for 40 seconds. Further DNA clean up followed manufactures instructions for DNA isolation protocol used. The PicoGreen DNA quantitation kit was used to determine DNA content in the final preparation using fluorescence measurement from a Fluoroskan Ascent microplate reader equipped with 485 /538 exitation/ emission filter settings. The strength of AFLP technique lies in the fact that multiple primers can be run from the pre-selective amplification mixture to determine if they can distinguish between unknown dry date palm samples. Six primer pairs were used to detect differences in AFLP DNA fragments patterns for dry date palm cultivars and we found three (1- E-AAC / M- CAC .2- E-ACA / M-CAA. 3- E-ACT / M-CAT.) of them is the better to distinguish amongst date palm cultivars. The dendogram produced by the Jaccard measure cluster analysis of the pooled AFLP data from three primers pairs showed that date palm dry cultivars are arranged according to DNA fingerprinting as following: Sakkoty, Malakaby, Gondeila, Shamia and Bartamuda. It means that Sakkoty and Malakaby are very closed, Shamia and Bartamuda are very closed too, while Gondeila is in between two cluster cultivars but more related to that involved Sakkoty and Malakaby . The use of DNA cultivar typing in dry group samples are useful for cultivar identification and breeding programs.

Key words: Date palm, dry cultivars, DNA fingerprinting, PCR

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Introduction

Date palm is one of the most important fruit crops in Egypt and Arabian countries. It is difficult to identify date palm cultivars based on morphological characteristics, isozymes or RAPD markers. DNA fingerprinting, a tool that has been widely used in forensic science, is also useful in a cultivar of applications with plants. It is used to identify genetic diversity within breeding populations, to positively identify and differentiate accessions cultivars and species that might be difficult to characterize due to similar morphological characteristics or indistinct traits, and to identify plants containing genes of interest such as the confirmation of transformation events. A number of molecular tools and procedures are being employed to establish DNA fingerprinting profiles and each of these procedures has its strengths and weaknesses, Amplified restriction Fragment Length Polymorphic (AFLP) DNA analysis, which is the focus of this research work, is a useful procedure for DNA fingerprinting, especially when very little information is known about the genome of the plant under studies .AFLP method is a PCR –based fingerprinting technique that has been successfully applied to a wide range of organisms with a broad application in systematics, pathotyping, population genetics and quantitative trait loci mapping (Vos *et al.,* 1995; Mueller and Wolfenbarger, 1999). Amplified Fragment Length Polymorphism AFLP DNA analysis of dry date palm plants is a useful procedure for quickly assessing the genetic background of' selected lines or populations. AFLP techniques produce a much higher percentage of polymorphic bands per analysis than the earlier procedures of RFLP (restriction fragment length polymorphisms) or RAPD analysis (random amplified polymorphisms). For example, in a comparative study of AFLP, RFLP, and RAPD analysis of inbred lines of soybean. Lin *et al.* (1996) reported that AFLP gave a number of discriminatory polymorphic bands or probes that was 8 to 10 times higher than the other two procedures (Table 1). AFLP DNA analysis can be used in a cultivar of other DNA fingerprinting and genetic mapping procedures to provide markers for traits of interest in plants when very little preliminary knowledge of gene sequence is available. This type of information is especially important in the genetic analysis of tree crops due to the long lead times required to determine actual field performance of breeding results (Saunders *et al.*, 2001). The AFLP technique is an efficient and useful tool for detecting genetic diversity compared to other molecular techniques , such as random amplified DNAs (RAPD) and simple sequence repeats (SSR). AFLP is a reliable and effective marker technology for delineating genetic relationships and estimating genetic diversity (Jones *et al.,* 1997; Capo-chichi *et a*l., 2003). The main objective of this study was to estimate genetic diversity within and between Egyptian dry date palm cultivars (Sakkoty, Malakaby, Brtamuda, Shamia and Gondeila) using AFLP DNA fingerprinting.

Materials and methods

1. Plant material and DNA isolation and quantitation

Genomic DNA was isolated from frozen Egyptian dry date palm tissues (cvs. Bartamuda, Gondeila, Sakkoty, Shamia and Malakaby grown at Aswan governorate, Egypt). About 50 mg leaf tissue placed in a capped tube sandwiched between 500 mg of garnet and two ceramic beads (Lysing matrix, P/N 6540-401 from the FastDNA kit, Q-Biogene, Carlsbad.

CA). Modified lysis buffer (400 ml) was added from the DNeasy Plant System (QIAGEN Inc. Valencia, CA). The plant tissue was completely macerated using a Fast Prep 120 (Q-Biogene) shaker/basher at oscillation speed of 5.0 rpm for 40 seconds. Further DNA clean up followed manufacturer's instructions for the DNA isolation protocol were used. The PicoGreen (Molecular Probes, Inc., Eugene, OR) DNA quantitation kit was used to determine the DNA content in the final preparation using fluorescence measurement from a Fluoroskan Ascent microplate reader equipped with 485/538 excitation/emission filter settings (Labsystems, Helsinki, Finland). (Saunders *et al*., 2001).

2. AFLP protocol and concept

The AFLP protocol initially described by Vos *et al*. (1995), was performed using components from various commercial AFLP kits (e.g. AFLP preamp primer mix I and AFLP core reagent kit from AFLP Analysis System I for plant genomes, Life Technologies, Rockville, MD; or AFLP amplification core kit, AFLP I pre-selective primer mix for regular genomes and AFLP EcoRl + Mse1 adaptors from Applied Biosystems, Foster City, CA). Alternatively, adaptors and pre-selective primers can be synthesized as described in the literature (Vos et al., 1995; Lin et al., 1996; Reineke and Karlovskv, 2000; and Saunders et al., 2001). AFLP DNA analysis is based on a multi-step process (Fig 1) with the following steps: DNA digestion, fragment ligation to adapters, pre-selective PCR amplification, selective PCR amplification, and fragment separation (Saunders *et al*., 2001). PCR amplification of restriction fragments is achieved by using the adapter and restriction site sequence as target sites for primers that extend into the restriction fragments, amplifying only those fragments in the primer extensions which match the nucleotides flanking the restriction site. From 64 primer combinations provided by the kit (EcoR1 / M se1) , we used the following 6 randomly chosen primer combination (E-AAC / M-CAC , E-AAC / M-CTC , E- AAG / M- CTA , E- ACA / M-CAA , E-ACC / M-CAT and E-ACT / M-CAT). Only three primers showed to be the most polymorphic ones. Therefore the results were only presented and discussed these three primers (E- AAC / M-CAC, E- ACT / M-CAT and E-ACA/M-CAA.

3. Preparation of DNA amplification fragments for separation by capillary electrophoresis and polymorphism detection

To prepare DNA fragments for separation by capillary electrophoresis, sample loading solution was prepared with a 400-base-pair (bp) DNA size standard labeled with WeIIRED dye Dl (approximately 100:1; Beckman Coulter 608082 and 608098). This solution was thoroughly mixed by vortexing for a minimum of two minutes. A 30-µL aliquot of this cocktail was added to 1.5 µL of the selective amplification product. Each well was over laid with a drop of Sigma mineral oil (M5904) and samples were analyzed in the CEQ™ 2000XL from Beckman Coulter.

Polymorphism was detected in AFLP analysis as the absence or presence of fragments due to a difference in restriction sites (a place on DNA molecule where a restriction enzyme acts (Krap *et al*., 1998).

4. Dendogram of Egyptian dry date palm cultivars

A similarity dendogram of the Egyptian dry date palm (*Phoenix dactylifera* L.) cultivars was produced from the cluster analysis software using unweighted pair-group method with arithmetic average (UPGMA).

Results and discussion

The molecular basis for the AFLP polymorphism is most frequently a sequence polymorphism at the nucleotide level .Single nucleotide changes will be detected by AFLP when either restriction site themselves or nucleotide adjacent to the restriction site are affected, causing AFLP primers to mispair at the 3´ end and preventing amplification since the selective nucleotide will not exactly match the sequence next to the restriction site. In addition, deletion, insertion and rearrangements affecting the presence or size of restriction fragments contribute to polymorphism detected by AFLP. Three primer combinations (*E* -AAC / *M*- CAC, *E*- ACT / *M*- CAT and *E* -ACA / *M* -CAA) were found the best primers amongst 6 primers to polymorphism detected by0 AFLP. The three primers combinations amplified a total of 161 fragments including 77 polymorphic (47.9 %) prevailing one or another genotype (Table 1). The sizes of AFLP fragments ranged from 56 to 390 base pairs and the polymorphic fragments were distributed across the entire size range. Table (1) illustrates the products of each primer combination and the level of polymorphism detected by each of these primer combinations. The number of fragments produced by the different primer combinations ranged from 95 (E -AAC / M -CAC) to 220 (E- ACA /M -CAA) and the average number of scorable fragments was 162 (Table 2) . Meanwhile, the level of polymorphism ranged from 45.9 % to 50.9% in primer combination *E* -ACA / *M* -CAA and *E*- ACT / *M* –CAT, respectively. The high level of in formativeness represents one of the most important advantages of the AFLP; hence, this type of markers provides wide range coverage of the date palm genome. The primer combination E –ACT / M- CAT was the most informative one (50.9% polymorphism). These results confirmed the high multiplex ratio expected with this type of markers (Table 1). The use of AFLPs, allows the screening of a large number of loci (several hundreds) with a few primer combinations, therefore, helps to rapidly identify unknown accessions and to establish genetic relatedness with high certainly. Genetic similarity measured on the basis of AFLP results is more reliable than other markers since they are based on the analysis of a large number of unbiased genetic markers. These markers represent a random sample of genetic loci distributed along the genome, and thus reduce the variance of similarity estimate (Capo chichi *et al.*, 2003). In the present investigation , the fragment size among the five genotypes ranged0 from 66 to3 21 base pair (bp) for the first primer (E -ACA / M -CAA) and from 71 to 390 for the second primer (E- ACT / M -CAT) and from 56 to 360 for the third primer (E -ACA / M -CAA) . Number of AFLP DNA fragments was affected by AFLP primer used and genotype as shown in Table (1 and 2). It ranged from 16 (Sakkoty cv with the first primer) to 53 (Bartamuda with the third primer). Data in Table (1 and2) show that the 1st AFLP primer E- AAC/ M-CAC showed a total of 16 polymorphic fragments. (47%), 9 monomorphic (26.5%) and conserved fragments (26.5%) over the five tested date palm cultivars which were observed from a total of 34 fragments. Meanwhile, the fragments ranged between 66 to 321

bp. Differential amplified number of fragments were detected; 16, 23, 19, 17 and 20 for the cultivars; Sakkoty, Malakaby, Bartamuda, Shamia and Gondeila, respectively. The 2nd AFLP primer ACT/CAT showed a total of 27 (50.9%) polymorphic fragments, 11 monomorphic (20.7 %) and 15 (28.3 %) conserved fragments out of 53 fragments and the fragment size ranged from 71 to 390 bp. Differe0ntial amplified number of fragments were detected; 39, 33, 26, 30 and 43 for the cultivars; Sakkoty, Malakaby, Bartamuda,Shamia and Gundeila respectively. The 3rd AFLP primer ACA/CAA showed a total of 34 (45.9 %) polymorphic fragments, 21 (28.3 %) monomorphic and 19 (25.7 %) conserved fragments and the fragment size ranged from 56 to 360 bp. Differential amplified number of fragments were detected; 34, 51, 53, 44 and 38 for the cultivars; Sakkoty, Malakaby, Bartamuda, Shamia and Gondeila, respectively. Among the 161 DNA fragments that were scored across all cultivars, 43 DNA fragments (26.7%) were conserved throughout all cultivars (samples) tested. The remaining 118 DNA fragments (73.3%) were polymorphic (77 fragments, 47.9%) and monomorphic (41 fragments, 25 .4%). Meanwhile, the fragments size ranged between 56 – 390 bp. The number and pattern of conserved and polymorphic fragments within the dry cultivars which are used as a function of the genetic variability within the plant collection (Aswan governorate) being analyzed. The specificity of amplification is determined by the three additional selected nucleotides that correspond to the first base pairs of the genomic DNA beyond the restriction site. Changing these three base pairs in the primer, or using less than 3 selective base pairs to reduce the selectivity of the primer set can be used as a dynamic tool to examine the genetic diversity within dry cultivars. While the three primer pair combinations used in this study *E*-AAC/*M*-CAC, *E*-ACT/*M*-CAT and *E*-ACA/*M*-CAA, clearly differentiated the cultivars tested. Subtle differences in the dissimilarity dendrogram may have occurred with different primer pair tested with the composite dendrogram of all the three primer pairs, a more comprehensive analysis was achieved. Although these primer sets were chosen at random, we assumed that not all primer pairs provide the same degree of genetic information about the genome under study. The dendrogram produced by the Jaccard (1908) measure cluster analysis of the pooled AFLP data from three primer pairs is presented in Fig. (1). Date palm dry cultivars are arranged according to AFLP DNA fingerprinting as following; Sakkoty, Malakaby , Gondeila, Shamia and Bartamuda . It means that Sakkoty and Malakaby are very closed, Shamia and Bartamuda are very closed too, while Gondeila is in between the two cluster cultivars but more related to that involved Sakkoty and Malakaby (Fig. 1). Fig (2) is presented as one example of the fifteen capillary electrophoresis DNA samples of the five dry date palm cultivars with each of the three primer combinations. Data presented in Table (2) showed the number and size of amplified fragments for each dry date palm cultivars with three AFLP primers (E- AAC /M-ACA, E- ACT / M- CAT and E-ACA/ M-CAA).Unique fragments for each cultivar were underlined for dry date palm cultivar identification. The total number of fragments and the polymorphic fragments were found to be genotype and primer combination dependant . The obtained results confirm the usefulness of the proposed modification of the AFLP techniques for diversity studies and identification of Egyptian dry date palm cultivars .The AFLP technique was successfully used in localization of resistance genes in economical crops like potato (Li *et al*., 1998) as well as measuring the variation in wild barely germplasm (Pakniya *et al*., 1997) . In this respect, Microslaw (2002)

studied AFLP method for fingerprinting of common wheat cultivars. The simplified AFLP method was developed for iden0tification and genetic diversity studies of wheat cultivars .Selective primers exploited in AFLP assay based on a single cutting enzyme Pst1 (Pst1 AFLP) generated total of 111 robus0t fragments including 67 (60%) monomorphic and 12 (11%) cultivar specific markers. The level of genetic variation within the most dry date palm cultivars is fairly substantial despite rigorous selection pressure aimed at cultivar purity in breeding programs. However, a lot of information still needs to be collected and the genome of the dry date palm need to be studied in more details. This will facilitate the improvement of dates quality and the development of date palms with better adaptability to the environmental conditions. Studying the genomes of important organisms was done in different parts of the world, such as the human genome project, the rice genome project, etc. Starting a genome project of the date palm will be the ideal method to improve it in the near future. Some of the main objectives of such project would be: studying the genetic information of date palm inducing the resistance to pests (Salma and Saker , 2002 ; Saker and Moursy, 2003). Moreover accurate identification and classification of cultivars would be achieved using neutral markers (molecular probes) which are not influenced by the environmental conditions or physiological state. Establishment of long term breeding programs in which the selections carried out based on molecular markers and the recognition of the best heterotic combinations. Determination of genotypes can be easily propagated through tissue culture techniques (El-Karbotly *et al.*, 1998). It is worthy to mention that he AFLP markers generate large number of polymorphic fragments among different date palm cultivars and allow easy identification of each cultivar. The identification of genetic strains of date palm cultivars using AFLP markers will enhance the decision for future date palm germplasm collection and preservation .The information can be used in future date palm breeding and improvement.

Table (1): Number, size range and type of AFLP fragments scored for three primer sets consisting of *EcoRI*-AAC/*Mse*1. CAC, *E*-ACT/*M*-CAT and *E*-ACA/*M*-CAA for date palm cultivars (Sakkoty, Malakaby, Bartamuda, Shamia and Gondeila).

Fragment Parameters	AFLP primer set			Combined Fragment
	***E*-AAC/*M*-CAC**	***E*-ACT/*M*-CAT**	***E*-ACA/*M*-CAA**	
Fragment size (bp.) range scored	66 – 321	71 – 390	56 – 360	56 – 390
Total fragments	34	53	74	161
Conserved fragments	9	15	19	43
Polymorphic fragments	16	27	34	77
Monomorphic fragments	9	11	21	41
% Polymorphism	47 %	50.9 %	45.9%	47.9 %

Table (2): Number and size (bp.) of amplified fragments for each dry date palm cultivar over each AFLP primer combination set

AFLP Primer set	Fragment size (bp.)	DRY DATE PALM CULTIVAR										Total Fragments
		SAKKOTY		MALAKABY		BARTAMUDA		SHAMIA		GONDEILA		
		F. No.	F. Size (bp.)	F. No.	F. Size (bp.)	F. No.	F. Size (bp.)	F. No.	F. Size (bp.)	F. No.	F. Size (bp.)	
E – AAC / M – CAC	66 – 321	16	66-71-94-96-97-101-109-165-166-167-177-179-180-252-255-267	23	66-69-70-71-78-94-96-97-98-101-108-109-145-165-166-167-177-179-180-255-256-267-285	19	66-70-71-86-94-95-97-102-109-165-167-177-180-210-252-256-264-267-301	19	66-70-71-78-86-94-97-102-109-146-165-167-177-180-210-255-267	20	66-70-71-94-97-102-145-165-166-167-177-180-210-255-265-267-285-307-318-321	95
E – ACT / M – CAC	71 – 390	39	71-72-87-90-96-97-100-110-112-125-127-130-131-134-135-136-140-141-142-145-146-154-160-168-169-177-179-180-195-201-202-224-235-241-243-284-303-331-357	33	71-72-87-90-96-97-100-110-113-125-127-131-135-141-142-145-146-154-164-168-169-177-195-201-224-241-243-270-284-303-311-332-357	26	71-72-87-90-97-100-110-112-113-125-131-134-136-139-141-145-146-154-168-169-176-177-195-224-241-284	30	72-87-90-95-96-97-100-110-125-127-131-134-135 -140-141-142-145-146-154-164 -168-169-177-180-195-201-241 -244-285-311	43	71-72-87-90-96-97-100-110-125-127-130-131-132-134-135-140-141-142-145-146-154-164-168-169-179-180-200-201-202-224-235-241-243-244-270-277-284-285-303-311-319-357-390	171

E – ACA / M – CAT	56 – 360	34	64-66-68-70-80-84-91-92-96-97-102-109-112-124-126-128-138-148-149-157-164-178-179-184-229-230-238-239-249-251-258-278-309-322	51	62-63-64-66-68-70-80-84-91-92-96-97-99-102-109-112-119-120-121-124-125-126-128-134-138-148-149-157-158-164-177-179-184-199-207-224-228-229-230-234-238-239-249-251-258-272-277-278-309-322-337	53	56-61-70-80-84-88-91-92-96-97-102-109-112-120-121-124-125-126-128-132-134-148-144-150-157-158-166-170-177-184-188-191-218-224-229-230-231-233-234-238-240-244-252-258-272-275-278-328-337	44	62-64-66-68-70-81-84-91-92-96-97-102-109-112-126-121-124-125 -126-128-134-148-149-150-157 -166-168-179-184-188-207-218 -224-228-229-234-239-224-251 -272-278-309-328-360	38	62-64-66-68-70-76-80-83-91-92-96-97-102-109-112-119-121-124-125-126-138-148-157-159-164-179-184-188-229-230-238-239-249-252-258-272-277-278	220
Total Fragments	56 – 390	89		107		98		91		101		486

Unique fragment for each cultivar were underlined.

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البصمة الوراثية لبعض الأصناف الجافة لنخيل البلح باستخدام تقنية التعدد الشكلي لأطوال القطع (AFLP)

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الملخص العربي

تقنية التعدد الشكلي لأطوال القطع (AFLP) تم إستخدامها لتحليل الحمض النووي (DNA) لأصناف نخيل البلح سكوتي، ملكابي، جنديلة، شامية و برتمودا وذلك بإستخدام بادئ مع إنزيمي قطع EcoRI و MseI. أظهرت النتائج من هذا التكنيك مع الاحماض النووية المعزولة لاصناف النخيل انها تقع في مدى ٥٦- ٣٩٠ قطع زوجية. كما أظهرت نتائج التحاليل والرسم البياني لإظهار مدى درجة التقارب بإستخدام معامل Jaccard ان مجموعة تضم من صنفي سكوتي وملكابي على درجة تقارب عالية جدًا من بعض وكذلك مجموعة تضم صنفي شامية وبرتمودا على درجة تقارب عالية من بعضهما في حين ان صنف جنديلة كان بين المجموعتين السابقتين ولكن يقترب أكثر من مجموعة السكوتي والملكابي. وتعتبر نتائج هذه التقنية مهمة لإستخدام الاصناف في برامج التربية.

الكلمات الدالة: نخيل البلح، الأصناف الجافة، الحمض النووي، البصمة الوراثية