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DETERMINATION OF SEX-SPECIFIC DNA MARKERS DATE PALM (*Phoenix dactylifera L.*) GROWN IN EGYPT UTILIZING NUCLEAR MICROSATELLITE MARKERS

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ABSTRACT

This study conducted in the genetic laboratory, department of agricultural botany, in cooperation with laboratory of biotechnology, Faculty of Agriculture, Suez Canal University, Egypt during the period from 2014 to 2015. Fife date palm cultivars in the production period were used in this study as females (Barhiisoidy, Samany, Zaghlool, Nabtet Soltan and Sakey). In addition, three males recognized as superior pollinators (Sakey, Samany, and Zaghlool). These experimental trees are grown at private orchard (Al-raghy) at Ismailia governorate, Egypt. This study aimed to searching for sex-private DNA markers for some date palm cultivars grown in Egypt with the help of DNA marker (nuclear microsatellite or SSRs) to make their election more ease and accuracy, moreover; determine the good pollinators for more uses in the genetic improvement for some quality traits of fruits and increase the yield of palm tree. Eight date palm microsatellite markers were used for gender identification. All eight microsatellite markers analyzed were polymorphic, revealing a total of 205 fragments. The highest number of alleles was featured with Zaghlool cultivar (37 fragments). However, Barhiisoidy cultivar generated the lowest number (17 fragments). From total of the fragments, 65 fragments were scored as private/specific alleles (alleles were unique that genotype alone), among them 64 were as a positive markers and one only as a negative marker. This work is one of the earliest reports for study of the gender genetic determination in date palm cultivars utilizing SSR analyses which gave six positive specific markers for males only. In addition, four positive specific markers for females only. And from another side; the polymorphism level across cultivars was 100 % as revealed by SSR markers.

Keywords: *Phoenix dactylifera* L., Date palm, DNA markers, Gender identification, SSR markers.

INTRODUCTION

The date palm fruit (*Phoenix dactylifera* L., 2n = 36) is a perennial monocotyledonous fruit plant, one of the members family of Arecacea (Barrow 1998). Palm tree is an excellent candidate for cultivation in arid and semi-arid regions of the world due to its high tolerance to environmental stresses. In Egypt, date palm is one of the most important fruits and widely distributed in different districts. There are 3 main types of dates based on fruit moisture content, i.e., soft, semi - dry and dry cultivars (Adway *et al.,* 2005). Nowadays, date palm is an important economic crop in Egypt where the world's largest producer over the last two years is Egypt with 1,470,000Mt followed by Islamic Republic of Iran (1,066,000 Mt) and Saudi Arabia (1,050,000 Mt) (FAO stat, 2012).

Probably, there are risks in any production process that plants produced may not conformity to the correct type, i.e. similar genetically to the mother plant. A number of different methods are available for detecting true-to type plants. The means vary in their sensitivity, technical complexity, ease to use. For the date palm, the traditional technique is the morphological screening, but it can be used in fruit ripening stage. However, in the last period a numerous of molecular applications have been applied to correct identify the genotypes, Nowadays, molecular markers, based on polymorphisms at DNA level, are increasingly used and proved effective to assess genetic diversity. Data based on molecular markers such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Restriction Fragment Length Polymorphism (RFLPs), have been used to characterize date palm genotypes [Sedra *et al*. (1998); Ben Abdallah *et al* (2000); Trifi *et al*. (2000); Trifi (2001)]. Among molecular markers, microsatellites, also known as Simple-Sequence Repeats (SSRs), because of their particular features such as their co dominant nature, highly polymorphic, highly reproducible and their typically high levels of allelic diversity at different loci, represent a suitable tool for genotyping. Because of their high mutation rates and the ease of the analysis, Microsatellites were proved useful and effective for phylogenetic studies, genetic fingerprinting and accurately identifying among different date palm cultivars and accessions in Egypt. [Billotte *et al.* (2004); Hamama *et al*. (2003); Adway *et al.*, (2005)].

It is known, that the pollen of the date palm has been found to exert a direct influence on the morphological and fruits traits; size, shape, color of the seed, size of the fruit, speed of development the fruit and on the time of ripening. These direct effects of the male parent on the development of the date fruit is controlled, definite and varies with the male used for fertilize the female flowers, each male lead approximately the same effect on fruit of all cultivars and lead the same effect during all the years. This direct effect of pollen on the parts of the seed and fruit lying outside the embryo and endosperm is called metaxenia (Walter 1928). For those, it is important to select and identify superior male for the fertilization process. Nowadays, there are major problem for farmers is to discriminate the sex of saplings at an early stage so that they can planting in their orchards a large number of female trees. However, Experiences to determine the sex of the dioecious species at early stage have unsuccessful. In the last years, there have been serious efforts to understand and study the genetic basis of sex determination in plants and to develop methods to identify sex in the early stage, using molecular markers tools [Mulcahy *et al.* (1992); Hormaza *et al*. (1994)].

This study aimed to determine sex- private/specific DNA markers for some date palm cultivars using microsatellites markers (SSRs) to facilitate the selection and to determine the good male pollinators for further using in the genetic improvement for some quality traits of fruits and increase the yield of palm tree.

MATERIALS AND METHODS

Plant materials: Eight date palm cultivars were used in this study. Fife cultivars of them (Barhiisoidy, Samany, Zaghlool as soft cultivars and Nabtet Soltan, Sakey as semi- dry cultivars), and three males (Sakey, Samany, Zaghlool) were recognized as superior pollinators. Leafs samples of the date palm cultivars were obtained from the private farm (Al-raghy) at Ismailia governorate, Egypt, during the period from 2014 to 2015.

DNA extraction: Three young leaves were collected from each tree and three trees for each cultivar were used to SSR analysis. Total genomic DNA was extracted according to the basic DNA extraction protocol of Dellaporta *et al*. (1983) with slight modifications by Porebski *et al*. (1997). young leaves (0.2 g) were ground in liquid nitrogen to fine powder and extracted using 10 ml preheated (65º C) cetylhexadecyl-trimethyl ammonium bromide (CTAB) extraction buffer [3% CTAB (w/v), 100 mMTris- HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% (w/v) PVP (Polyvinyl pyrrolidone)], then 1% (v/v) of β-mercaptoethanol (15 mM) with further grinding.. The mixture was incubated at 65º C for 60 min, followed by two extractions with chloroform/isoamyl alcohol (24:1). The nucleic acids were precipitated with cold isopropanol, and the pellet was dissolved in 1 mL TE 0.1X (Tris-EDTA) buffer (10 mMTris-HCl, pH = 8 and 1 mM EDTA, pH = 8). Co-precipitated RNA was removed by digestion with RNAase A. 4 µl (10 mg/mL). The DNA was further purified by 300 µl phenol:chloroform: isoamyl alcohol (25:24:1), then left overnight at (-20º C) using 1/10 vol. from 2 M sodium acetate (pH = 8.0) and one volume of cold isopropanol alcohol. The precipitate was washed twice with 10 mM ammonium acetate in 76 % ethanol, and the pellet was dissolved in 0.1 XTE buffer. The purified total DNA was quantified by gel electrophoresis, and its quality was verified and concentrations by Nanodrop spectrophotometer model ND1000. DNA samples were then stored at 4º C. The stock DNA samples of each genotype were diluted with tris-EDTA and analyzed individually to detect intra-cultivar variations and bulked to detect inter-cultivar variations.

DNA amplification and PCR Conditions: An initial screening of 10 SSR primers (Successfully utilized in other date palm genotypes, (Billotte *et al*.2004) was performed in order to test their readability and amplification profiles for polymorphism. After this screening procedure, eight SSR primers were selected (Table 1) and these primers were synthesized by Oligo Macrogen, Seoul, Korea.

PCR reactions were performed in 25 µl volume contained 2µl (20 ng) of template DNA, 1µl (20 pmol) forward primer, 1µl (20 pmol) reverse primer, 12.5 µl Master Mix , and 8.5 µl PCR water . The amplification was carried out in a thermocycler (Eppendorf Mastercycler Gradient Eppendorf, Hamburg, Germany). After a first denaturation step at 95º C for 5 min, the reaction went through 35 cycles at 95º C for 15 sec., 51º C for 15 sec., 72 º C for 30 sec. followed by a final extension step of 5 min at 72º C. The analyses were repeated at least twice to assure the reproducibility of the results.PCR products were detected by electrophoresis on Multi-capillary electrophoresis

QIAxcel (QIAGEN Germany) system for DNA fragmentation analysis. For each amplification, a negative control reaction (without DNA samples) was used. However, PCR reaction, that generated high level of polymorphism using this type of markers, repeated twice at least in order to verify the reproducibility of scored polymorphic bands.

Table1. A list of eight SSR primers used in this study which developed by Billotte *et al*. (2004).

Primer No.	Primer name	Sequence
1	mPdCIR010	F: ACCCCGGACGTGAGGTG
		R: CGTCGATCTCCTCCTTTGTCTC
2	mPdCIR016	F: AGCGGGAAATGAAAAGGTAT
		R: ATGAAAACGTGCCAAATGTC
3	mPdCIR032	F: CAAATCTTTGCCGTGAG
		R:GGTGTGGAGTAATCATGTAGTAG
4	mPdCIR050	F: CTGCCATTTCTTCTGAC
		R: CACCATGCACAAAAATG
5	mPdCIR063	F: CTTTTATGTGGTCTGAGAGA
		R: TCTCTGATCTTGGGTTCTGT
6	mPdCIR070	F: CAAGACCCAAGGCTAAC
		R: GGAGGTGGCTTTGTAGTAT
7	mPdCIR085	F: GAGAGAGGGTGGTGTTATT
		R: TTCATCCAGAACCACAGTAT
8	mPdCIR093	F: CCATTTATCATTCCCTCTCTTG
		R: CTTGGTAGCTGCGTTTCTTG

This Confirmation allowed that the fragments present in all replicated experiments were scored as markers. The size of fragments was estimated using 250 bp DNA standards as a size marker (Bioron, Germany).

Data Analysis: Reproducible DNA bands were visualized on the automatic multi-capillary electrophoresis QIAxcel and scored as a binary code (1 and 0) for their presence or absence for microsatellite markers, respectively (Nei and Li 1979).

RESULTS AND DISCUSSION

The results of using eight SSRs markers developed for date palm (Billotte *el at.*, 2004) gave successful amplification across the eight cultivars. The difference between the number of alleles in each locus and number of effective loci showed the existences of private/specific alleles. Private alleles occurred on one or more genotypes (Kohpayegani and Behbahani, 2008). In this study SSR markers used for sex determination analysis of palm germplasm; for science, this is one of the earliest studies related to exposure the gender genetic determination in date palm using SSR markers.

Identification of SSR Markers: Of the many molecular techniques available to researchers, microsatellites, or simple sequence repeats (SSRs), are

becoming increasingly widespread. SSRs are tandemly repeated bases of DNA composed of 1–6 base pairs (bp) long units spread throughout the genome of different organisms. Compared to other markers, the high reproducibility of Microsatellite markers may be because of their large number, distribution throughout the genome, co-dominant inheritance, neutrality with respect to selection and easy automation of analytical procedures of SSR technique. Thus, they are extremely useful for fine-scale genetic analysis [Gupta *et al*., (1996); T´oth*et al*., (2000); Powell *et al*., (1996); Jones *et al*., (1997)]**.** However, Date palm SSR markers revealed high levels of genetic polymorphism in present study. (Figure 1 and Table 2). All eight loci were successfully amplified and a total of 205 alleles were detected. All of them were polymorphic (100 %). The highest levels of polymorphism for SSRs markers compare to other markers also reported in the previous studies [Belaj *et al*. (2003); Rajora and Rahman (2003); Ferreira *et al*. (2004)]. The high level of polymorphism associated with SSR markers may be a function of the unique replication slippage mechanism, loss or gain of specific nucleotide/s during evolution responsible for generating SSR allelic diversity (Morgante *et al.,* 2002). Our study confirms that the mentioned microsatellite markers are able to generate higher number of allele per locus. The markers produced number of alleles could have better application to find out the polymorphisms in heritability of date palm being reached than would be possible with RAPD markers.

The highest number of alleles was generated with Zaghlool cultivar (37) while Barhii soidy cultivar generated the lowest (17 alleles). CIR 063 showed the highest number of alleles per locus (47), while CIR 010 showed the lowest (7 alleles) with mean of 25.6 allele (Table 2).

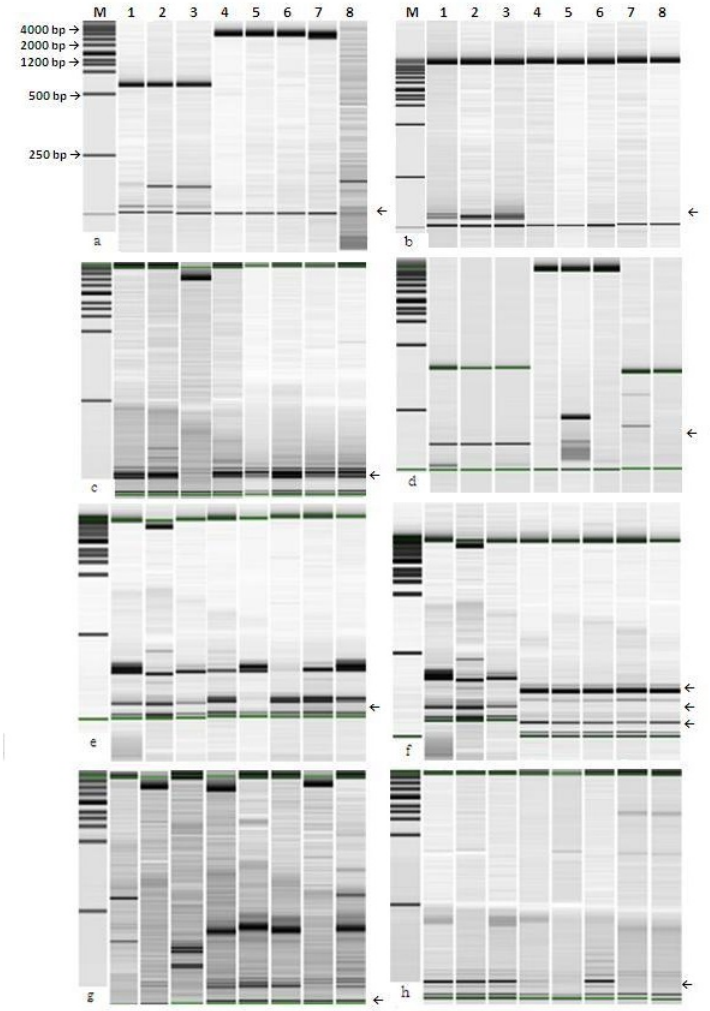

Fig. 1. SSR profiles of the eight Palm cultivars amplified using CIR 010 (a), CIR 016 (b), CIR 032 (c), CIR 050 (d), CIR063 (e), CIR 070 (f), CIR 085 (g) and CIR 093 (h) primers, M: Molecular weight marker. Lanes 1 up to 8 refers to Palm cultivars: Sakey male, Samany male, Zaghlool male, Barhii Soidy, Nabtet Soltan, Sakey, Samany, Zaghlool.

The difference between the number of alleles in each locus and number of effective loci showed the existences of private/specific alleles. Private alleles occurred on one or more genotypes (Kohpayegani and Behbahani, 2008). However, the specific alleles could be used to identify the genotypes by means of combination of some genetic loci. For example, using

CIR032 can be used as marker to identify cultivars which the gene loci were different from each other.

Table 2. Number of amplicons of eight palm cultivars based on SSR analysis.

Palm cultivars		SSR loci								Total
		CIR 010	CIR 016	CIR 032	CIR 050	CIR 063	CIR 070	CIR 085	CIR 093	
Sakey male	AF	2	3	3	3	8	3	3	4	29
	SM	1	1	1	1	0	3	2	2	11
Samany male	AF	2	2	3	1	7	3	2	2	22
	SM	0	0	1	0	2	2	1	0	6
Zaghlool male	AF	2	3	12	1	4	1	6	2	31
	SM	0	2	13	0	0	0	3	0	18
Barhiisoidy	AF	0	0	2	0	5	4	7	1	17
	SM	0	0	0	0	0	0	1	1	2
NabtetSoltan	AF	0	0	5	5	6	4	6	0	26
	SM	0	0	0	4	0	0	1	0	5
Sakey	AF	0	0	4	0	4	3	7	4	22
	SM	0	0	0	0	0	0	1	2	3
Samany	AF	0	0	4	1	5	4	3	2	19
	SM	0	0	0	1	2	0	0	1	4
Zaghlool	AF	1	0	4	0	8	3	10	11	37
	SM	1	0	0	0	1	0	5	9	16
	TSM	2	3	15	6	5	5	14	15	65
	TAF	7	8	37	11	47	25	44	26	205
	PB	7	8	37	11	47	25	44	26	205
%Polymorphism		100%	100%	100%	100%	100%	100%	100%	100%	

TAF = Total number of bands, PB = polymorphic bands, AF = Amplified bands, SM = Marker, including presence or absence of the band in Palm cultivar, TSM = Total number of private markers with Palm cultivars.

The Genetic analysis of the date palm genotypes based on 8 polymorphic SSR markers detected 65 distinct specific alleles across all loci, in which 64 of them were scored for the presence of a private band for a given cultivars (positive markers), while one only was scored for the absence of a common band (negative marker). 'Zaghlool' male had the most unique alleles (18 alleles). While the lowest number of genotypes specific alleles (2) was scored for 'Barhii soidy'(Table 3).

Table 3. Cultivars- private/specific markers obtained from SSR analysis.

Palm cultivars	Positive marker	Negative marker	total
Sakey male	11	0	11
Samany male	6	0	6
Zaghlool male	17	1	18
Barhiisoidy	2	0	2
NabtetSoltan	5	0	5
Sakey	3	0	3
Samany	4	0	4
Zaghlool	16	0	16
Total	64	1	65

Table 4. Ten Sex-private/specific markers in Date Palm based on SSR analysis.

	Male positive markers	NO.	Female Positive markers	NO.
SSR Markers	CIR010-52,CIR016-49, CIR050--218, CIR063-54, CIR070-82, CIR093-42	6	CIR032-70, CIR070-159, CIR070-54, CIR085-22	4

These SSR markers that generate unique alleles will play a key role in creating a cost effective Egyptian date palm cultivars fingerprinting technique. However, the presence of many unique alleles can be taken as an indication of high and adaptation based genetic diversity in date palm.

In general, all SSR markers utilized in this study allowed for enough discrimination among the eight palm cultivars as used before in previous studies (Talaat and Asmaa 2009). Depending on the amplification of products, eight primers were selected and the amplification polymorphism for DNA samples from three males and five females were tested. Of the eight primers, only six primers CIR 010, CIR 016, CIR 050, CIR 063, CIR 070, CIR 093, yielded a clear and characteristic sex-specific markers (approximately 52, 49, 218, 54, 82 and 42 bp long), respectively, featured for males only and not in females. These fragments were consistent and present in all three males analyzed from the palm samples. While primers CIR 032, CIR 070, CIR 085 yielded a clear and characteristic amplification fragments, approximately 70bp with primer CIR 032, also 159 bp and 54 bp with primer CIR 070, in addition, 22 bp in size from primer CIR 085, featured only in females and not in males. These four specific markers were consistent and present in all five females used from the palm samples.

These results help us to address many of the issues, including developing DNA probes. Add to that it will allow the breeders to identify the sex in palm rapidly and to increase understanding the evolution of date palm.

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تحديد معلمات الحامض النووي DNA المتخصصة والمميزة لجنس نخيل البلح المنزرع في مصر باستخدام معلمات الميكروستاللیت

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أجريت هذه الدراسة في معمل الوراثة بقسم النبات الزراعي بالتعاون مع معمل التكنولوجيا الحيوية بكلية الزراعة جامعة قناة السويس خلال الفترة من 2014 الى 2015 م، باستخدام خمسة أصناف نخيل بلح كاناث في مرحلة الانتاج وهم (برحي سعودي، سماني، زغلول، نبتت سلطان و السقعي) بالاضافة الى ثلاثة ذكور مسجلة كملقحات جيدة وهم (السقعي، السماني و الزغلول) وجميع الأشجار التجريبية المستخدمه في الدراسة منزرعة في مزرعة خاصة (مزرعة الراجحى) بمحافظة الاسماعيلية، مصر. تهدف هذه الدراسة الى تحديد معلمات الحامض النووي DNA المتخصصة والمميزة للجنس وذلك لبعض أصناف نخيل البلح المنزرعة في مصر باستخدام التقنية الجزيئية المعروفة باسم الميكروستاللیت أو التتابعات المكررة البسيطة بهدف تسهيل عملية الانتخاب وتحديد الملقحات الذكرية الجيدة للمزيد من الاستخدامات في برامج التربية لزيادة المحصول ولتحسين جودة بعض صفات الثمار. تم استخدام ثمانية معلمات ميكروستاللیت متخصصة لنخيل البلح لتمييز الجنس. أظهر تحليل النتائج ان كل معلمات الميكروستاللیت الثمانية المستخدمة كانت متباينة وأعطت عدد اجمالي 205 حزمة أوأليل، وقد نتج أكبر عدد من الأليلات مع الصنف زغلول (37 حزمة). و على الجانب الأخر أعطي الصنف البرحي السعودي أقل عدد (17 حزمة). ومن اجمالي الأليلات المتحصل عليها عدد 65 حزمة أمكن الاستفادة منها كمعلمات متخصصة و مميزة للصنف منهم 64 كمعلمات متخصصة موجبة وواحدة فقط سالبة. وتعتبر هذه الدراسة من أوائل الدراسات التي تهتم نتحليل تحديد وتمييز الجنس في نخيل البلح على أساس وراثي باستخدام تقنية الميكروستاللیت، لذا فقد تم تحديد وتمييز ستة معلمات متخصصة موجبة مع الذكور فقط ومميزة لها عن الاناث بالأضافة الى أربعة معلمات متخصصة موجبة ومميزة للاناث دون الذكور. وعلى الجانب الأخر، كان مستوى التباين بين الأصناف المستخدمة بالدراسة 100% وذلك كما أظهرتها نتائج معلمات الميكروستاللیت.