Sex Determination of Iraqi Date Palms Based on DNA Markers

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Abstract

Date Palm tree (*Phoenix dactylifera* L.), is a dioecious monocotyledonous plant, with separate male and female trees and it is impossible to distinguish tree sex at an early stage until reaches the time of first flowering, which takes about 5-10 years. Date palm trees are propagated either from seed or vegetative offshoot. Although propagation with seeds is an economically desirable objective, which enhance breeding programs for genetic improvements of the date palm but it is hard to differentiate the male and female plants at early stages and that makes hard to employ the genetic diversity. There have been numerous attempts to use the biochemical and molecular markers at an early stage to discriminate among male and female seedling. Here, we tested sex- specific PCR-based markers that may assist in early gender determination at seedling stage. Four male varieties (Ghnami Ahmer, Ghnami Akhdhar, Khkry and Khkry-Semesmy) and 4 female varieties (Breem, Khidrawi Mandli, Maktoum and Teberzal) were subjected to PCR amplification using 7 Random Amplified Polymorphic DNA (RAPD) and 8 Simple Sequences Repeat (SSR) primers to identify sex-linked markers. Four SSR markers exhibited differential fragments between males and females which indicate marker to detect the sex in date palm seedling in the early stage. In order to identify the genome segments unique to male, selected bands at 582 pb for mPdIRD80 for Ghnami Akhdhar and at 926 bp for RAJF1R1 for Khkry have been sent for sequencing. Nucleotide sequences revealed high degree of similarity with Phoenix dactylifera L. sex-determination region sequence and now are available in GenBank NCBI under the Accession no. MK542703.1 and MK542705.1 for Ghnami Akhdhar and Khkry varieties respectively.

Keywords: Phoenix dactylifera, RAPD, Sex determination, SSR.

Introduction

Date palm progenies consist of male and female individuals in equal proportions which have been directed to the hypothesis that sex is found genetically (Daher et al., 2010). Propagation of date palm through seeds or zygotic embryos is desirable for improvement of the cultivars and for selection of biotic and abiotic tolerance, fruit quality and high yield. The identification of male and female plants in dioeciously species is becoming the priority for plant breeders, especially prior to propagation since this would result in crop improvement and increased profits (Sarkar et al., 2017). Morphological screening between male and female could be applied but it can be too late, particularly after first flowering, which takes 5 and 10 years after planting. A long juvenility presents a real challenge in date palm breeding programs, that means breeding programs have not progress because there is no accurate and easy way to distinguish the sex of the plant prior to first flowering (Bendiab et al., 1993; Juarez and Banks, 1998; El Hadrami and El Hadrami, 2009; Aberlenc-Bertossi et al., 2011). An extensive efforts have been used a wide range of methodologies to discrimination between male and female date palms at an early stage of seedlings, biochemical studies included the uses of isozymes (Torres and Tisserat, 1980; Qacif et al., 2007) have yielded a little information in the identification of male and female at their early development stage. Molecular markers have gave an efficient method for sex identification at an early stage of plant, these markers include Random Amplified Polymorphic DNA (RAPD) (Younis et al., 2008), PCR-based Restriction Fragment Length Polymorphism (PCR-RFLP) (Al-Mahmoud et al., 2012), RAPD-SCAR (Dhawan et al., 2013), Inter Simple Sequence Repeat (ISSR) marker (Younis et al., 2008), Simple Sequences Repeat (SSR) markers (Cherif et al., 2013) and PCR-based approaches SCoT (Adawy et al., 2014).

In an attempt to identify sex-specific markers in Iraqi date palm varieties, two DNA markers including SSR and RAPD can be employed in early sex determination.

Materials and Methods

Young leaf tissues were taken from 3- 5 plants of Iraqi female and male date palm trees that were randomly selected from the Date Palm Experimental Station at Al

Zufaranya, Ministry of Agriculture, Iraq. Four male cultivars (Ghnami Ahmer, Ghnami Akhdhar, khkhry and khkry-Semesmy) and 4 female cultivars (Breem, Khidrawi Mandli, Maktoum and Teberzal) were chosen and sampled. Genomic DNA from the collected leaves was isolated using CTAB (cetyl trimethyl ammonium bromide) method as reported by Yousif et al. (2014). Quality of DNA was assessed by electrophoresis on 0.8% agarose gel (stained with 0.5 μg ethidium bromide/ml) and its quantity was evaluated in Nano-Drop spectrophotometer (Bio-Rad, USA). The DNA samples were diluted to a working concentration (100 ng/μL) for PCR amplification. A total of 7 and 8 primers of Random Amplified Polymorphic DNA (RAPD) and Simple Sequences Repeats (SSRs) respectively (Table 1) manufactured by Bioneer-Korea were used as sex-linked markers. PCR was performed using an AccuPower©PCR Premix (Bioneer, Korea), containing 250 µM of each deoxyribonucleoside triphosphate, 30 mM of KCl, 10 mM of Tris- HCl (pH 9.0), 1.5 mM of MgCl2, and 1Unit of Top DNA polymerase. 200 ng of genomic DNA and 100 ng of primer were then add to a PCR Premix tube. Amplification was performed in Thermocycler (FlexCycler, Germany) using program: I cycle at 94°C for 4 min, 40 cycles as follows: 94°C for 45 sec, annealing at 36°C for RAPD and 53-56°C for SSR (based on melting temperature of used primers) for 1 min, 72°C for 2 min and the last cycle at 72 °C for 10 min. Amplification products were loaded on 1% agarose gels and stained with ethidium bromide (0.5 μg ethidium bromide/ml). The DNA banding patterns were visualized on an UV transilluminator and documented by using Gel Documentation System, E-Graph (AE-9000, Japan). Fragment length was estimated by comparison with standard size markers (100 bp DNA Ladder size range 100 – 2000 bp, Bioneer-Korea) and the molecular weight of the bands was analyses using the photo Capt MW program. Fragments (bands) were recorded numerically as (1) when present or (0) when absent. Fragments with the same mobility were considered as identical, irrespective of fragment intensity. The analyses were repeated twice to assure the reproducibility of the results.

The fragments that exhibited differential pattern between males and females were extracted from agarose gel using a Wizard® SV Gel and PCR Clean-Up System kit. Samples were sent to Macrogen (Korea) for sequences. Sequence manipulations were conducted in BioEdit Sequence Alignment Editor v.7.0.5.3

(http://www.mbio.ncsu.edu/bioedit/page2.html). Each sequence was queried in NCBI using Nucleotide BLAST search.

Parameters for calculating the marker efficiency and genetic characteristics were tested. Polymorphic information content (PIC) was calculated using the formula of Roldán-Ruiz et al. (2000:(

PIC = 2fi(1 - fi) where fi is the frequency of the amplified allele and 1 - fi is the frequency of null allele.

The % efficiency of a primer was calculated as a percentage of the total number of bands amplified by the primer out of the total number of bands amplified by all primers across all varieties.

Table1. SSR and RAPD primers.

Primer code	Primer sequences (5–3)	Sources
mPdIRDP50F	CATGGAAGTTGTTGGCAGAG	Cherif *et al*. (2013)
mPdIRDP50R	CATGCTCCTTGCCTCAATG	
mPdIRDP52F	TCGTGCTACAATGCCAAGAG	
mPdIRDP52R	CTAATGCTTGCATGGGAGGT	
mPdIRD80F	ATTGGGTGTTGGTCTCTAGGAA	
mPdIRD80R	TCGTGCTACTGCTTCTCCATTA	
PdMYB1F5	TTCTCAGCCCCTCAAACTTC	Cherif *et al*. (2016)
PdMYB1R1	GCACTTACTCTCACGGCCATC	
mPdCIR048F	CGAGACCTACCTTCAACAAA	Billotte *et al*. (2004)
mPdCIR048R	CCACCAACCAAATCAAACAC	
mPdCIR093F	CCATTTATCATTCCCTCTCTTG	
mPdCIR093R	CTTGGTAGCTGCGTTTCTTG	
K1	TGGCGACCTG	Bekheet *et al*. (2008)
K2	GAGGCGTCGC	
K3	CCCTACCGAC	
K4	TCGTTCCGC	
K5	CACCTTTCCC	
K6	GAGGGAGAG	
D10	GGTCTACACC	Younis *et al*. (2008)

RAJF1	CGTCCATTCAGGCTGCGTTGC	The forward (RAJF1) and reverse primers (RAJR1 and RAJR5) were designed base on the Phoenix reclinata alcohol dehydrogenase (AdhA) gene, partial exons 4-10 and partial cds (Gene Bank Data Base Accession number U58362.1
RAJR1	AAGGCCGACAGCACCCAATC	
RAJR5	TCCAATTCTAGCTCCTGCACC	

Results and Discussion

The primers examined in this study effectively generated clearly amplified SSR and RAPD bands (except primer K4) with different sizes ranging from 50 to 1600 bp (Table 2). The different band size range was reported by other studies (Zehdi et al., 2004; Ahmed and Al-Qaradawi, 2009; Jaskani et al., 2016). The number of alleles (bands) per locus detected in this study was between 2 (for mPdIRDP50, RAJF1R1, K3, K5 and D10) and 9 bands (for RAJF1R5) with a mean of 4.25 and 3.67 alleles per locus for SSR and RADP respectively (Table 2). On the other hand limited number of bands (2 bands) generated with k5 primer which gave monomorphic bands in all genotypes. Zehdi et al. (2004) recognized 7.14 alleles per locus when examining 46 Tunisian date palm accessions using 14 microsatellite loci, while Elshibli and Korpelainen (2007) identified 21.4 alleles per locus, this may be a result of using a greater number of microsatellite loci in addition to using different genotypes. In this study mPdCIR48 produced 4 bands ranged from 80-250pb (Table 2 and Fig1), Jaskani et al. (2016) found that this primer successfully amplified while Zehdi et al. (2004) did not detect any bands when using mPdCIR48, the loss of alleles perhaps due to using different genotypes.

Table 2. Genetic information by locus.

Primer code	Allele (bands) range (bp)	Allele no.	Monomor-phic bands	Polymer-phic bands	% efficiency of primer	PIC*	Mean allele
mPdCIR048	80-250	4	1	3	7.14	0.45	4.25
mPdIRDP50	200-210	2	1	1	3.57	0.38	
mPdIRDP52	100-1600	6	3	3	10.71	0.50	
mPdIRD80	100-582	3	2	1	5.36	0.28	
mPdCIR093	80-310	4	2	2	7.14	0.40	
PdMYB1F5B1R1	100-1000	4	1	3	7.14	0.48	
RAJF1R1	900-926	2	0	2	3.57	0.50	
RAJF1R5	100-1600	9	3	6	16.07	0.50	
K1	50-300	5	1	4	8.93	0.50	3.67
K2	100-300	7	0	7	12.50	0.48	
K3	300-500	2	0	2	3.57	0.22	
K5	310-600	2	2	0	3.57	0.00	
K6	100-600	4	0	4	7.14	0.48	
D10	350-600	2	0	2	3.57	0.22	

*PIC: Polymorphic information content

All primers gave monomorphic bands (ranging between 1-3 bands) except the primers RAJF1R1, K2, K3, K6 and D10. It's to be noticed in table 2 that polymorphic band per each primer ranging between 1-7 bands except K5 primer. The efficiency of amplification depends on the total number of bands amplified by the primer, the results showed that the highest % efficiency of primer value was 16.07% for RAJF1R5 primer, The efficiency of the primer indicates that there is an area of the genomic DNA that complements the sequence of the primer and allowing a pairing between the primer and the genome.

Polymorphic information content (PIC) value was from 0.5 in mPdIRDP52, RAJF1R1, RAJF1R5 and K1 to 0.22 in K3 and D10 while the PIC value in K5 was zero. PIC describe genotypic variation and if there is no allelic variation (monomorphic), the PIC value will be almost zero and reaches a maximum of one if there is a very highly discriminative, so PIC provides an estimate of the discriminatory power of a locus. Many primer pairs showed closely related to male-specificity, and it is interesting to find four primers produced a specific band in all male plants whereas the same locus was absent in female plants (Table 3), these primers were mPdCIR48 (Fig1), mPdIRD80 (Fig2) and RAJF1R1 (Fig3) and mPdIRDP50 (Fig4) which produced a specific band at 180pb for mpdCIR48, 582bp for mPdIRD80, 900 and 926 bp for RAJF1R1 and mPdIRDP50 amplified a

specific fragment at 270 bp in all male plants. The minimum number of sex-linked SSR markers were detected in Ghnami Ahmer and the highest number of sex-linked SSR markers were detected in Ghnami Akhdhar where 11 bands were scored from these primers whereas the same band were absent in female plants (Table 3). Cherif et al. (2013) identified sex-linked markers using three microsatellite (SSR) markers and reported three genetically linked loci that are heterozygous in males only, Jaskani et al. (2016) found that primer mpdCIR48 produced a specific locus (250/250) in all male samples whereas the same locus was absent in female samples. For RAPD markers we did not find Sex-linked RAPD markers for all tested male; however maximum RAPD markers associated with sex were detected in khkry, these markers were K1, K2 and K6. Primers K1 and K6 characterized all male varieties except Ghnami Ahmer, while primer K2 characterized only khkry variety (Table 3). These data proved that SSR markers are more influential, powerful and key identifiers of date palm sex for speeding up the breeding programs.

In order to identify the genome segments unique to male, selected bands at 582 pb for mPdIRD80 for Ghnami Akhdhar and at 926 bp for RAJF1R1 for Khkry have been sent for sequencing. DNA sequence analysis of the 2 clones showed high similarity with sex-determination region sequence in date palm after BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Sequences of RAJF1R1 at 926 pb for Khkry variety (clone S2_SRr) and at 582 pb for Ghnami Akhdhar (clone S1_SRr) showed particularly high similarity with the date palm sex-determination region sequence (P. dactylifera clone dpS5560CH2 sex-determination region sequence; MH680991.1 and P. dactylifera clone dpS5560CH1 sex-determination region sequence; MH680988.1). Clones submitted to the GenBank database are available under the Accession no. MK542703.1 and MK542705.1 for S1_SRr and S2_SRr respectively.

Table 3. Bands (bp) which identified male date palm.

Markers	Ghnami Ahmer	khkry	khkry-Semesmy	Ghnami Akhdhar
RAJF1R1	900, 926	900, 926	900, 926	900, 926
mPdCIR048	180, 230	180, 230	180	180, 230
mPdIRDP50	270	270	270	270
mPdIRDP52				400, 1000,1200
mPdIRD80	582	582	582	582
PdMYB1F5B1R 1	500	500		500,900
K1		700	700	550,700
K2		550		
K6		300,600	300, 600	600

Figure 1. Agarose gel electrophoresis of mPdCIR048 of different date palm female and male cultivars, from right to left; Molecular marker (bp) (lane 1); Maktoum (lane 2); Teberzal (lane 3); Khidrawi Mandli (lane 4); Ghnami Ahmer (lane 5); Khkry-Semesmy (lane 6); Ghnami Akhdhar (lane 7); Khkry (lane 8) and Breem (lane 9).

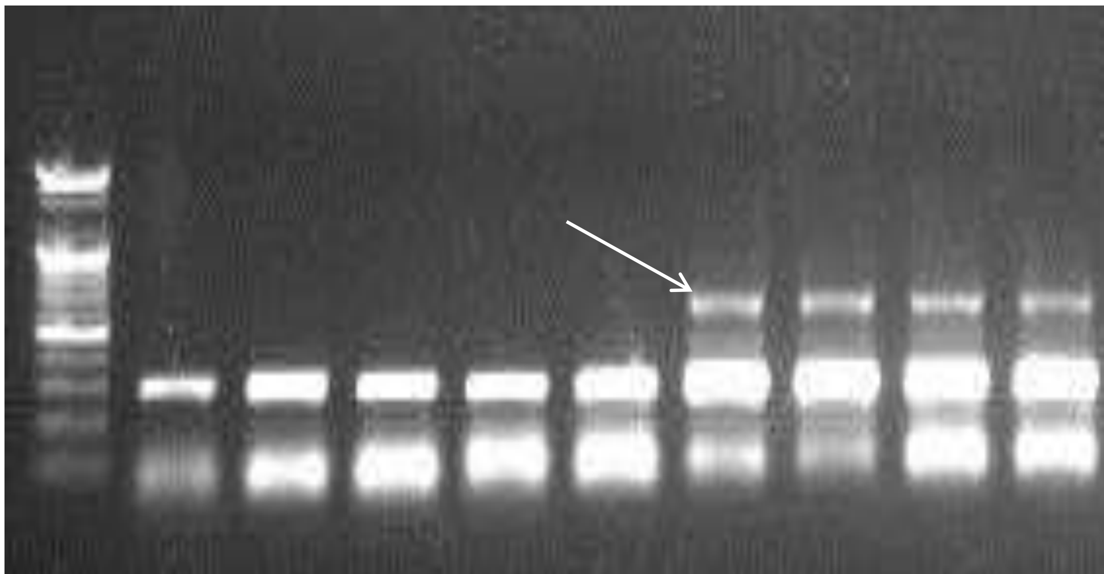

Figure 2. Agarose gel electrophoresis of mPdIRD80 of different date palm female and male cultivars, from left to right; Molecular marker (bp) (lane 1); Maktoum (lane 2); Teberzal (lane 3); Khidrawi Mandli (lane 4); Breem (lane 5); Breem (lane 6); Ghnami Ahmer (lane 7); Khkry-Semesmy (lane 8); Ghnami Akhdhar (lane 9) and Khkry (lane 10).

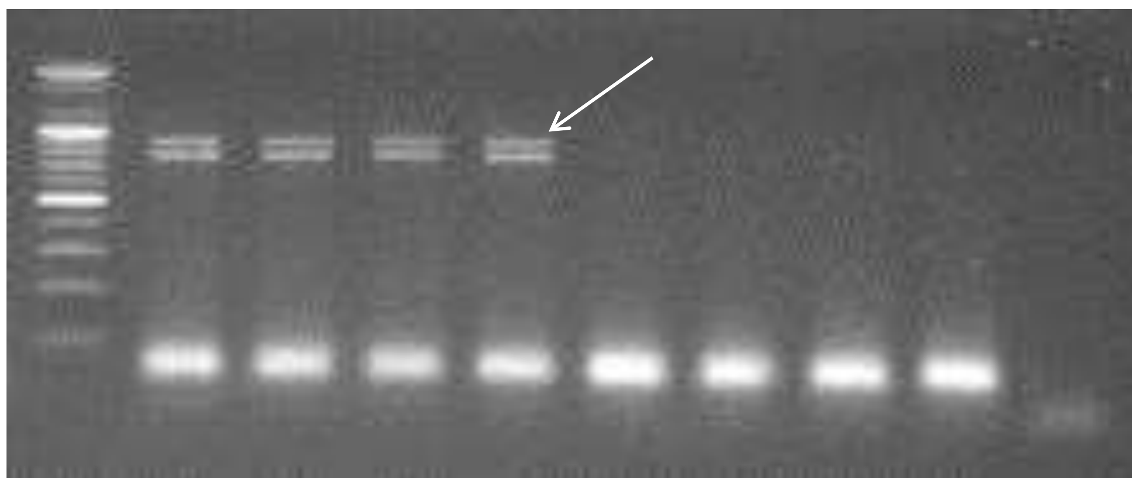

Figure 3. Agarose gel electrophoresis of RAJF1R1 of different date palm female and male cultivars, from left to right; Molecular marker (bp) (lane 1); Ghnami Ahmer (lane 2); Khkry-Semesmy (lane 3); Ghnami Akhdhar (lane 4); Khkry (lane 5); Maktoum (lane 6); Teberzal (lane 7); Khidrawi Mandli (lane 8); Breem (lane 9) and blank(lane 10).

Figure 4. Agarose gel electrophoresis of mPdIRDP50 of different date palm female and male cultivars, from left to right; Ghnami Ahmer (lane 1); Khkry-Semesmy (lane 2); Ghnami Akhdhar (lane 3); Khkry (lane 4); Maktoum (lane 5); Teberzal (lane 6); Khidrawi Mandli (lane 7); Breem (lane 8); blank(lane 9) and Molecular marker (bp) (lane 10).

Conclusion

The SSR markers that were tested in this study could be used to discrimination of male and female Date Palm plants that means SSR markers may be tightly linked to a sex of date palm at the seedling stage. Further these DNA markers can be apply to identify the sex of individual plants.

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تحديد جنس نخيل التمر العراقي بالاعتماد على مؤشرات الدنا DNA

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الخلاصة

يعد نخيل التمر (*Phoenix dactylifera* L) من النباتات أحادية الجنس ثنائية المسكن وحيدة الفلقة ، لذا يصعب تمييز جنس نخيل التمر في المراحل الاولية من النمو وحتى بلوغ النضج، في وقت الإزهار الأول، والذي يستغرق حوالي 5 –10 سنوات كي تثمر شتلات إناث النخيل، وحينئذ يمكن تمييزها عن ذكور النخيل. يتم اكثار نخيل التمر إما بالبذور أو الفسائل. ان التكاثر بالبذور غير مناسب للإنتاج التجاري لكون نصف الذرية هم من الذكور ونصفها الاخر من الاناث، لذا فإن تحديد الجنس المبكر في مرحلة الشتلات (تفريق الشتلات الصغيرة إلى ذكور وإناث) هو هدف مرغوب فيه اقتصاديا، مما يعزز برامج التربية للتحسينات الوراثية لنخيل التمر. كانت هناك محاولات عديدة لاستخدام المؤشرات البايوكيميائية والجزيئية في مرحلة مبكرة للتمييز بين الذكور والإناث في نخيل التمر. تم في هذه الدراسة اختبار مؤشرات جزيئية خاصة بالجنس تعتمد على تقنية تفاعل البلمرة المتسلسل (Polymerase Chain Reaction (PCR والتي قد تساعد في التحديد المبكر للجنس في مرحلة الشتلات. تم إخضاع أربعة أصناف من الذكور (غنامي احمر، غنامي أخضر، خكري وخكري – سميسمي) و 4 أصناف مؤنثة (بريم، خضراوي مندلي، مكتوم وتبرزل) باعتماد 7 بواديء من نوع التضاعف العشوائي المتعدد الاشكال لسلسلة الدنا (Random Amplifi polymorphic (RAPD) و 8 بواديء من نوع التسلسلات البسيطة المتكررة (SSR) لتحديد المؤشرات المرتبطة بالجنس. أظهرت خمس بواديء من نوع SSR حزم خاصة يمكن بها التفريق بين الذكور والإناث والتي تشير إلى امكانية الكشف بها عن الجنس في شتلات نخيل التمر في المراحل المبكرة للنمو. ومن أجل التأكد من كون تلك المؤشرات مرتبطة بالذكور، تم اختبار تسلسل حزم مختارة عند 582 زوج قاعدي للمؤشر mPdIRD80 في الصنف غنامي اخضر و 926 زوج قاعدي للمؤشر RAJF1R1 للصنف خكري. كشفت متواليات القواعد النايتروجينية درجة عالية من التشابه مع تسلسل منطقة تحديد الجنس لنخيل التمر *Phoenix dactylifera* L. وهي الآن متاحة في بنك الجينات GenBank NCBI تحت الرقم التصنيفي. MK542703.1 و MK542705.1 للصنفين غنامي أخضر و خكري على التوالي.

الكلمات المفتاحية: تحديد الجنس، نخيل التمر *Phoenix dactylifera*، RAPD، SSR