Development of 1000 Microsatellite Markers across the Date Palm (*Phoenix dactylifera* L.) Genome

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Abstract

Date Palm is a major environmental and economic factor in arid climates in many countries around the world. Microsatellite markers have been proven to be very powerful in plant genome analysis because they are locus-specific, codominant, highly polymorphic and highly reproducible. In date palm only few microsatellite markers have been developed so far. Recently, the Cornell Medical College in Qatar issued a draft assembly of the date palm genome ('Khalas') generated by whole genome shotgun next generation DNA sequencing. In this paper, we analyzed the microsatellite motifs across the date palm genome. The results indicated that the most abundant type of microsatellite repeats are dinucleotide repeats (52442 motifs) followed by trinucleotide (28503 motifs) and pentanucleotide repeats (12873 motifs). The frequencies of tetra-nucleotide and hexa-nucleotide repeats were less across the genome (5555 and 5810 motifs, respectively). The most common type of dinucleotide repeat was GA (48.7%) followed by AT (37%). Out of 28645 trinucleotide repeats, TAA and GAA repeats were the most abundant repeats (28.1 and 27.1%) respectively. More than 1090 new microsatellite markers could be designed. The primary test for 50 primer pairs revealed that 28 (56%) were functional and 19 (38%) yielded polymorphic PCR products. We wish that the results of our study will be a starting point for researchers making use of the markers for genetic mapping and diversity analysis of date palm.

INTRODUCTION

Date palm (*Phoenix dactylifera* L, $2n=2x=36$), is a dioecious perennial monocotyledon fruit plant from the *Arecaceae* family. The predicted genome size was estimated to be approximately 250 Mbp (Barakat et al., 1999). The origin of this tree is Iraq, and recently, thousands of cultivars have been reported (Hanachi et al., 1998). Date palms have always been clonally propagated to ensure the identity and uniformity of the cultivars.

Discrimination among closely related cultivars by using morphological traits (including fruit morphology) are often unreliable and extremely difficult, especially because of the influence of environmental conditions (Elhoumaizi et al., 2002). Therefore, the need for using DNA marker technology for DNA fingerprinting has become increasingly important in recent years. Several marker systems have been used to study the genetic diversity of date palm, in brief, randomly amplified polymorphic DNA (RAPD) fingerprints have been used to identify date palm accessions in Algeria (Benkhalifa, 1999), in Morocco (Sedra et al., 1998), in Tunisia (Trifi et al., 2000), in Saudi Arabia (Al-Khalifah and Askari, 2003), and in Egypt (Soliman et al., 2003; Adawy

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et al., 2006). Amplified fragment length polymorphic (AFLP) markers have been applied to study the polymorphisms of date palm cultivars from Egypt and California (Cao and Chao, 2002; El-Assar et al., 2005; Adawy et al., 2006). Microsatellite or simple sequence repeat (SSR) markers have been used in plant diversity analysis because they are locus-specific, codominant, highly polymorphic and highly reproducible. Microsatellite markers have been developed and used to investigate genetic diversity in *P. dactylifera* (Billotte et al., 2004). They used $(GA)_n$ microsatellite-enriched library to develop 16 microsatellite markers. More recently, 17 microsatellite loci were developed by constructing two microsatellite enriched libraries of date palm by using $(GA)_n$ and $(GT)_n$ repeats (Akkak et al., 2009). These microsatellite markers have been used to assess the genetic diversity and relationships of date palm varieties in Tunisia (Zehdi et al., 2004), in Sudan (Elshibi and Korpelainen, 2007), in Oman (Al-Ruqaish et al., 2008), and in Qatar (Ahmed and Al-Qaradawi, 2009). However, for a wider use of microsatellite makers evaluating DNA polymorphisms in date palm tree, the development of hundreds of microsatellite markers would be necessary.

Unlocking the date palm occurred in April 2009, when researchers in the Weill Cornell Medical College in Qatar (WCMC-Q) used the variety named 'Khalas' (one of the most popular varieties of the fruit) to issue an assembly draft of the date palm genome generated by whole genome shotgun next generation DNA sequencing. The available sequence is a start point to apply advanced genomic technologies to a better understanding of date palm genome. The objective of this research was to study the frequency of microsatellite motifs across the date palm genome, and to develop new microsatellite markers.

MATERIAL AND METHODS

Sequence Analysis

The multi-fasta file of date palm sequence issued by WCMC-Q was downloaded from this web site address: http://qatar-weill.cornell.edu/research/datepalmGenome/download.html. The sequence file is named as (PdactyKAssembly1.0.fasta - 329328 KB) and contained 271804 fasta sequence clones.

Isolation of Microsatellites

The microsatellite motifs were classified as perfect, imperfect, compound perfect, or compound imperfect repeats according to the classification of Weber (1990), modified by Hüttel et al. (1999). A microsatellite is referred as 'simple', if a single type of repeat unit repeats several times (e.g., $(CA)_n$; etc.); a 'compound' microsatellite consists of stretches of more than one type of repeat unit (e.g., $(GA)_n{\cdot}(TA)_k$; $(GT)_k{\cdot}(TAA)_l{\cdot}(TA)_m$, etc.); a 'perfect' microsatellite does not contain mutations or interruptions (e.g., $(CA)_n$; $(TAA)_k$; $(CT)_m{\cdot}(GAA)_n$, etc.); an 'imperfect' microsatellite contains mutations or interruptions (e.g., $(CA)_nCC(CA)_m$; $(TA)_kAA(TA)_l{\cdot}(GA)_m$, etc.), subscripts k, l, n and m denote number of times the particular microsatellite motif repeats.

Short script was written by us in Perl software to collect the microsatellite motifs from the assembly draft of the date palm genome and categorize them as di-, tri-, tetra-, penta-, and hexa- nucleotide repeats. The percentage of accepted non-repeated nucleotides within the microsatellite motifs was fixed between 10-20% and called as error rate. For instance, in the following trinucleotide micosatellite $(TTA)_3$ –CAC – $(TTA)_3$ – GAC – $(TTA)_4$ – CCGG – $(TTA)_3$, consisted of 50 represent nucleotides, whereas ten (20%) nucleotides are not repeated sequence. However, only 10% of error rate was selected for further analysis in this study.

Plant Material

A total of 30 well-defined reference Iraqi date palm varieties were collected from two date palm stations belonging to the Ministry of Agriculture in Baghdad, Iraq. These female varieties are: 'Ashrasi', 'Barhi', 'Bream', 'Chipchab', 'Guntar', 'Helawi', 'Jamal

Al-Dean' and 'Khedrawi'. Total cellular DNA was extracted from young and healthy leaves as described by Rogers and Bendich (1985) with minor modifications. After purification, the obtained DNA was quantified and its integrity checked by using agarose gel electrophoresis (1%).

Primer Design and PCR Amplification of Microsatellites

Primer pairs were designed close to the microsatellite repeats in the flanking regions by using Primer3 v. 0.4.0 web base application available at this site address (http://frodo.wi.mit.edu/primer3/). The expected product size was limited to 200 bp, the length of the primers varied between 18 and 23 bases, the melting temperature (Tm) is fixed to be around 60°C, and all other parameters were kept as default values without change.

The PCR reactions were performed in a total reaction mixture of 20 µl containing: 50 ng of total cellular DNA (2 µl) as template, 1X PCR buffer (Roche, Manheim Germany), 0.2 mM of dNTP PCR mix (Roche), 0.5 U of *Taq* DNA polymerase (Roche) and 10 pmol of each primers (forward and reverse primers). Amplifications were performed in a Applied Biosystem Thermocycler (Applied Biosystem) with the following conditions: a denaturation step of 5 min at 95°C followed by 35 cycles of 15 s at 95°C, 15 s at 58°C and 30 s at 72°C, and a final extension step at 72°C for 5 min. Amplification products were separated on 8% polyacrylamide gels stained by ethidium bromide. The DNA banding patterns were visualized on an UV transilluminator and documented by using Gel Documentation System (Alpha Innotech).

RESULTS AND DISCUSSIONS

Microsatellite Motifs

The assembly draft of the date palm genome was analyzed and screened for microsatellites motifs using a script in Perl software (supplemented date). The results indicated that the draft sequence of date palm consisted of 321,278,327 bases including 94,386,304 adenine "A", 57,044,647 cytosine "C", 57,187,022 guanine "G", 94,100,785 thymine "T", and 18,559,569 unspecified nucleotides "N" (Table 1). Microsatellite motifs varied according to the three error rates (10, 15, 20%), less error rate showed less microsatellite motifs counted (Fig. 1). However, we used a 10% error rate in this study to increase the efficiency of microsatellite assessment.

Microsatellite motifs were frequently identified across the genome in about 105,183 microsatellite motifs (approximately one microsatellite per 3054.5 bases). Most of the repeats in date palm were of the simple/imperfect type (55,425 motifs) comparing to the simple/perfect (48,868 motifs). In contrast, many studies of plant species reported simple/perfect motifs to be the most abundant repeats in *Brassica napus* L. (Kresovich et al., 1995), in chickpea (*Cicer arietinum* L.) (Hüttel et al., 1999; Winter et al., 1999), and in lentil (Hamwieh et al., 2009). Among the microsatellite repeats, dinucleotide were the most abundant repeats across the date palm genome (52,442 motifs) followed by trinucleotide repeats (28,503 motifs) (Table 1). The most abundant repeat type in date palm genome was AG/TC (25,903 motifs) followed by AT/TA (20,160 motifs) then AC/TG (6,756 motifs). Billotte et al. (2004) developed 16 microsatellite primers from $(GA)_n$ microsatellite-enriched library by using GA. Later, Akkak et al. (2009) developed 17 microsatellite primers from two enriched libraries by using $(GA)_n$ and $(GT)_n$ repeats. Approximately 94.1% of their microsatellite motifs published were identified by the $(GA)_n$ library. This supports our finding that GA is the predominant repeat across date palm genome. However, the GT repeat is one of the most frequently occurring microsatellites in human and many mammals (Toth et al., 2000). This is also the case in some plant species, such as wheat (Varshney et al., 2000) and *Pinus radiata* (Smith and Devey, 1994), but this repeat (GT) is comparatively less frequent in other plants (Lagercrantz et al., 1993; Morgante and Olivieri, 1993). However, the dinucliotide repeats varied even within the genome itself between linkage groups or chromosomes. For

example, among the dinucleotide repeats across *Arabidopsis thaliana* genome, GT was most abundant in chromosome 1 followed by chromosome 4, 2 and 3 respectively, but this repeat was not found in chromosome 5 (Tamanna and Khan, 2005).

Trinucleotide repeat motifs have also been identified in plant genomes, the most frequently identified are $(TAA)_n$ and $(GAA)_n$ (Akkaya et al., 1992; Lagercrantz et al., 1993; Morgante and Olivieri, 1993; Winter et al., 1999). In the present study, trinucleotide repeats $(TAA)_n$ and $(GAA)_n$ were recovered in only 14,997 motifs of microsatellite representing 52% of the 28,645 trinucleotide motifs obtained across the date palm genomic sequence. Nonetheless, the isolation of trinucleotide repeats in date palm is expected to be important since the detection of polymorphisms involving trinucleotide microsatellite motifs may be easier compared with dinucleotide motifs, due to the presence of an extra base pair in the repeat unit (Hearne et al., 1992). Finally, less compound microsatellites were detected across the genome, included 702 dinucleotide motifs and 141 trinucleotide motifs.

Development of Microsatellite Markers

In total 1091 primer pairs could be designed in the flanking regions of simple/perfect microsatellite motifs. The expected sizes of these primers ranged between 113 and 345 bp with an average of 208 bp. Out of these primer combinations, 377 flanked dinucleotide, 352 primer pairs flanked trinucleotide, and 362 primer pairs flanked tetranucleotide repeats. Out of 33 published microsatellite primers only 22 (10 out of 16 microsatellite primers published by Billotte et al., 2004, and 12 out of 17 primers published by Akkak et al., 2009) could be detected in the sequence (Table 3).

To estimate the functional capacity of these primers, 50 primer pairs were tested with 8 Iraqi date palm varieties. The results revealed that 28 primers combinations were functional (56%) and 18 (36%) revealed polymorphic alleles (Table 4). If we extrapolate these results we would expect to obtain out of the 1091 primers at least 350 polymorphic microsatellite across the date palm genome. Certainly, these new co-dominant markers will be a starting point for researchers making use of the markers for genetic mapping and diversity analysis of date palm.

It is important to mention that clone number (>PdactyK1.0Scaffold_1817710_length_7070C) showed 91% similarity to the *Washingtonia robusta* alcohol dehydrogenase (*adh*) gene, (reference number on NCBI is: U65972.1). This microsatellite motif can be found under DPALM1091 (in the supplementary file). The *adh* gene was reported previously as the genetic basis for sex determination in date palm (Rajendran and Al-Mssallem, 2007). They yielded two clear bands of 800 bp and 1000 bp for the female genotypes and a single fragment of approximately 800 bp in male genotypes. In this study, we identified one microsatellite motif $(ATG)_2(AT)_3C(ATG)(AT)_3$ which is located 158 bases away from the *adh* gene. It needs to be tested if derived microsatellite markers could be used to screen for sex.

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Tables

Table 1. The number of the nucleotides and its frequency within the date palm genome.

Nucleotide	Count	Frequency
Adenine(A)	94,386,304	0.294
Cytosine(C)	57,044,647	0.178
Guanine(G)	57,187,022	0.178
Thymine(T)	94,100,785	0.293
Unspecific (N)	18,559,569	0.058
C+G	114,231,669	0.356
A+T	188,487,089	0.587

Table 2. Frequency of various types of microsatellite motifs observed across the date palm genome.

		Dinucleotide	Trinucleotide	Tetranucleotide	Pentanucleotide	Hexanucleotide
Simple	Perfect	24256	13182	3729	5449	2252
	Imperfect	27484	15180	1807	7409	3545
	Total	51740	28362	5536	12858	5797
Compound		702	141	19	15	13
Total		52442	28503	5555	12873	5810

Table 3. Date palm genomic clones used by Billotte et al. (2004) and Akkak et al. (2009) to develop microsatellite primers.

Reference	Primes	Clone number
Billotte et al. (2004)	mPdClR010	>PdactyK1.0Scaffold_271028_length_2103
	mPdClR025	>PdactyK1.0Scaffold_375496_length_3618
	mPdClR032	>PdactyK1.0Scaffold_952984_length_2335
	mPdClR050	>PdactyK1.0Scaffold_1723771_length_10937
	mPdClR057	>PdactyK1.0Scaffold_1698674_length_1883
	mPdClR070	>PdactyK1.0Scaffold_961639_length_1935
	mPdClR078	>PdactyK1.0Scaffold_36821_length_10487
	mPdClR085	>PdactyK1.0Scaffold_1084952_length_3573
	mPdClR090	>PdactyK1.0Scaffold_830856_length_5891
	mPdClR093	>PdactyK1.0Scaffold_1742274_length_2028
Akkak et al. (2009)	PDCAT10	>PdactyK1.0Scaffold_1871877_length_11760
	PDCAT11	>PdactyK1.0Scaffold_332319_length_10772
	PDCAT14	>PdactyK1.0Scaffold_1406391_length_898
	PDCAT15	>PdactyK1.0Scaffold_1377333_length_946
	PDCAT17	>PdactyK1.0Scaffold_1077978_length_376
	PDCAT18	>PdactyK1.0Scaffold_1165340_length_2957
	PDCAT1	>PdactyK1.0Scaffold_1498949_length_13938
	PDCAT20	>PdactyK1.0Scaffold_317589_length_3777
	PDCAT21	>PdactyK1.0Scaffold_836398_length_3971
	PDCAT3	>PdactyK1.0Scaffold_1661598_length_4767
	PDCAT6	>PdactyK1.0Scaffold_1657267_length_1165
	PDCAT8	>PdactyK1.0Scaffold_1541464_length_3258

Table 4. Forward and reverse primer sequences of the primers revealed polymorphic loci in mini core collection of Iraqi date palm varieties.

No	Primer Name	Forward Primer	Reverse Primer	Expected size	Tm°
1	DPALM100	GCCACTATCACCATTGCTGT	CAATGGAGGTCGTAGTGGTG	203	59
2	DPALM103	TTCCATCCCTGGAGAAAGG	AACCAAGACATCGTCCCAAG	200	60
3	DPALM104	GGAAAGTTTCGGAACATTTTGT	AACCCAACCTAAGCCCTACC	228	59
4	DPALM107	GGAAGGCGTCAAGGTATCTC	ACAACACGGGGAAAGAACAT	200	59
5	DPALM110	TGTCACATTATTTGAGCATAATCCA	ACCCTTTGTTGATGCACCTC	178	60
6	DPALM112	AGCAGGTTCATGGTTTGCTT	AGAACCAGGGAGGATGAGGT	200	60
7	DPALM113	GGTCCCGACGCCTATTTTAT	AGCAAAGTCCACCCCTTTTT	255	60
8	DPALM119	TGCGCTAAATAGTTCCCTTCA	CACATTCACAAGGCCTGCTA	208	60
9	DPALM120	TTCAATTCATCCCACTGCAA	CACCAACATGAGCAAATGGA	222	60
10	DPALM121	CATATGATTGTGATGGGGACA	CACCTCTCCGAGAAAACCAG	210	59
11	DPALM123	GGCAGGTGGATTGTTCTTGT	CAGGGGTATGGAGAGAGAGAGA	207	59
12	DPALM125	TTATGCTGAGGCCAGGTTTT	CATGCTGCAGAACCTGAAGA	191	60
13	DPALM132	TCAGCTCAAAGCACACAACA	CCGGAGATTTTGTTTCGATG	226	60
14	DPALM133	CAGATGGGATCGTTTTACCTG	CCGTATCGGGAGAGAGAGAG	197	59
15	DPALM139	TCTCGATCTCGACCTTGGTT	CGGATCCGGTTCTCTCATTA	202	60
16	DPALM141	CATTGCTCAGAAGCATCCAA	CTCTCCCTCCCTCTCGTTCT	212	60
17	DPALM142	CAATGGACCACCAAAATCAA	CTCTCCGAGAAAACCAGGTC	179	59
18	DPALM144	ACACACACACACACGCGAAT	CTTGCAGCCATTTAGGCAAC	187	61
19	DPALM146	ATGATTGAGAGGCAGGCAAA	GACAAGAGGGAAGGGGAGAG	198	60

Figures

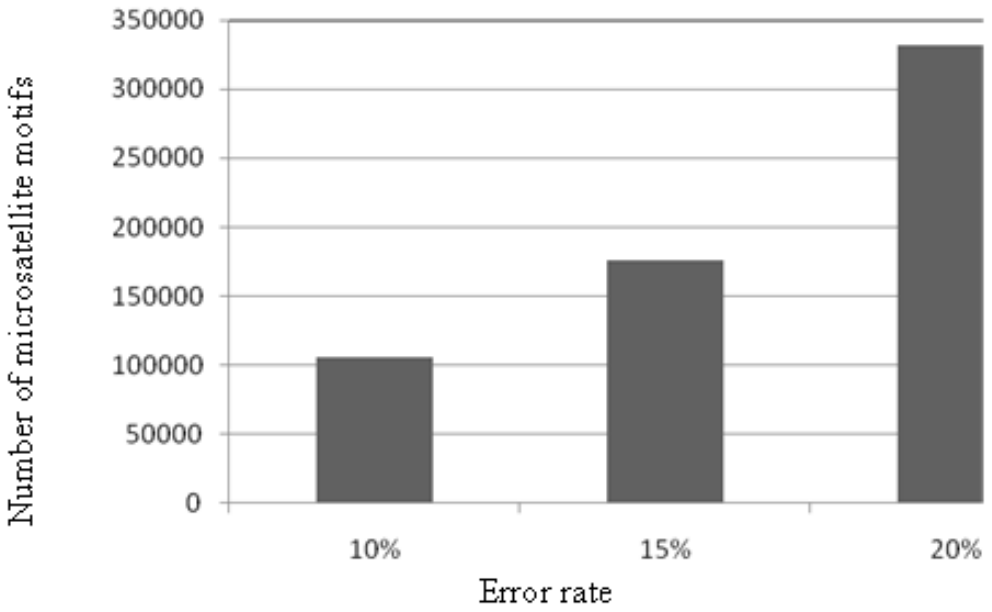

Fig. 1. Number of total microsatellite markers counted according to the different error rates (error rate: non-repeated nucleotides within the microsatellite motif). The figure shows that if more error in the microsatellite sequence is accepted then more microsatellite motifs could be counted.