Electronic Journal of Biotechnology 21 (2016) 18–25

HOSTED BY

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Development, characterization and use of genomic SSR markers for assessment of genetic diversity in some Saudi date palm (*Phoenix dactylifera* L.) cultivars

Sulieman A. Al-Faifi [a], Hussein M. Migdadi [a,*], Salem S. Algamdi [a], Mohammad Altaf Khan [a], Megahed H. Ammar [a], Rashid S. Al-Obeed [a], Mohammad I. Al-Thamra [a], Ehab H. EL-Harty [a], Jerenj Jakse [b]

[a] *Plant Production Department, College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia*
[b] *Agronomy Department, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia*

ARTICLE INFO

Article history:
Received 19 August 2015
Accepted 12 January 2016
Available online 17 February 2016

Keywords:
Expected and observed heterozygosity
Microsatellite markers
Polymorphic information content
Polymorphism
Primer discrimination power

ABSTRACT

Background: The present study was undertaken towards the development of SSR markers and assessing genetic relationships among 32 date palm (*Phoenix dactylifera* L.) representing common cultivars grown in different geographical regions in Saudi Arabia.
Results: Ninety-three novel simple sequence repeat markers were developed and screened for their ability to detect polymorphism in date palm. Around 71% of genomic SSRs were dinucleotide, 25% tri, 3% tetra and 1% penta nucleotide motives. Twenty-two primers generated a total of 91 alleles with a mean of 4.14 alleles per locus and 100% polymorphism percentage. A 0.595 average polymorphic information content and 0.662 primer discrimination power values were recorded. The expected and observed heterozygosities were 0.676 and 0.763 respectively. Pair-wise similarity values ranged from 0.06 to 0.89 and the overall cultivars averaged 0.41. The UPGMA cluster analysis recovered by principal coordinate analysis illustrated that cultivars tend to group according to their class of maturity, region of cultivation, and fruit color. Analysis of molecular variations (AMOVA) revealed that genetic variation among and within cultivars were 27% and 73%, respectively according to geographical distribution of cultivars.
Conclusions: The developed microsatellite markers are additional values to date palm characterization tools that can be used by researchers in population genetics, cultivar identification as well as genetic resource exploration and management. The tested cultivars exhibited a significant amount of genetic diversity and could be suitable for successful breeding program. Genomic sequences generated from this study are available at the National Center for Biotechnology Information (NCBI), Sequence Read Archive (Accession numbers. LIBGSS_039019).

1. Introduction

Date palm (*Phoenix dactylifera* L., 2n = 36) is a dioecious perennial fruit plant, belonging to the family of Arecaceae (Coryphoideae) [1]. It is the most important fruit crop of arid climate region in North African and Middle East in general and in Saudi Arabia in particular. It is a traditional crop in Saudi agriculture, most of the yield is locally consumed, and most Saudi dates are recognized as high quality products. It ranks among the top fruit trees in terms of the number of trees, area and production (approximately 23.7 million trees, 156 thousand hectares and 992 thousand tons) [2]. More than 400 Saudi date palm cultivars have been characterized based on tree morphology and fruit characteristics [3]. Some date palm orchards under Kingdom of Saudi Arabia conditions were planted with seeded palms. The selection of superior palms from these seeded plantations depend on the evaluation of the morphological characteristics and fruit physical and chemical properties as a start point for developing date palm orchards. However, morphological traits are often variable influenced by environmental conditions or varying with the developmental stage of plant [4]. Furthermore, molecular markers based on polymorphisms at DNA level, are used to assess genetic diversity in date palm. Several DNA based techniques are used such as Random Amplified Polymorphic DNA (RAPD) [5], ISSR, [6,7,8] combination of RAPD and ISSR [9,10,11,12], Amplified Fragment Length Polymorphism (AFLP) [13,14,15,16,17,18].

The genome size of date palm is estimated to be approximately 658-Mbp long [19] and genomic resources available for date palms are insufficient to be used in genetic studies, assessing genetic variability,

* Corresponding author.
E-mail addresses: hmigdadi@ksu.edu.sa, h.migdadi@gmail.com (H.M. Migdadi).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2016.01.006

cultivar identification and marker assisted selection. Microsatellites or simple sequence repeats (SSR) have been widely used in many crops, due to their abundance, high degree of polymorphism, locus specificity, reproducibility, and most of all, they are a co-dominant in nature. These make microsatellites an attractive option for genetic studies in date palms.

Sixteen (GA)n SSR markers have been developed and used to analyze Tunisian [20] and Sudan [21] germplasms. Further, 17 microsatellite loci were developed by constructing two microsatellite-enriched libraries of date palm using (GA) n and (GT)n repeats [22]. Microsatellite markers have been used to assess the genetic diversity and relationships of date palm varieties in Tunisia [7,23,24], in Oman [25], in Qatar [26,27,28], in Iraq [18,29], in Morocco [21,30], in Libya [31] and across regions [32] or different *Phoenix* species [33]. Recently, the Cornell Medical College in Qatar published assembly of the date palm genome 'Khalas' generated by whole genome shotgun next generation DNA sequencing [19]. One thousand microsatellite motifs across the date palm genome were analyzed [29]. GA (48.7%) followed by AT (37%) were most common type of dinucleotide repeats. TAA and GAA were the most abundant trinucleotide repeats (28.1 and 27.1%, respectively).

This study aimed towards the development of new set of SSR markers for *P. dactylifera*, defined their variability parameters and used these SSRs in assessment of genetic variation for some Saudi date palms.

2. Materials and methods

2.1. Tissues collection, DNA isolation, purification and quantification

For microsatellite development total genomic DNA was extracted from young fresh leaves of Saudi date palm cultivar (Sukary) according to SDS protocol as described by Al-Faifi et al. [34]. For genotyping, total genomic DNA was extracted from leaves of 5 plants/each cultivar grown at Agricultural Research Station in Dirab (24°:42 N, 46°:44 E, Alt. 660 m a.s.l). List of date palm cultivars analyzed in this study, their geographical distribution, Tamar color and maturity class grown at the station date palm gene bank are presented in Table 1. DNA concentrations were measured spectrophotometrically at 260 nm, and the extracts were electrophoresed on 1% agarose to confirm the quality. The purified DNAs were standardized at 100 ng/μl and stored at -20.

2.2. Development of date palm simple sequence repeat enrichment library

A microsatellite-enriched library was obtained using adapted protocols from Karagyozov et al. [35] and Billotte et al. [36] with modifications. 10 μg of DNA was restricted with several combinations of restriction enzymes: *Eco*RI, *Hae*III, *Hae*II, *Mse*I, *Rsa*I, *Bgl*II, *Eco*RV and *Hin*fI (New England Biolabs, USA). Fragments were end polished by Mung Bean Nuclease and dephosphorylated using alkaline phosphatase (CIAP). SNX reverse primer (GCT TCT GCT AGC AAG GCC TTA GAA AA) was ligated as a linker and pre-amplification was carried out using linker-specific primers for confirmation of ligation. Two complementary oligos were combined (GA15 + TC10, GT15 + AC9, ACA10 + TTG6, AGA10 + TTC6) and PCR product was precipitated and cleaned up with one round of chlorophorom:isoamylachohol, then with ethanol precipitation. For each microsatellite motif to fish, 5 μl of long microsatellite probe were pipetted to the (5 × 5 mm) nylon membrane and cross linked onto UV source for 2–3 min, then washed in 0.1× SSC at 65°C for one day. The membranes bounded microsatellite motifs that are already to the (GA, GT, ACA/AGA, ACT/ATC, and ACCT/ACTC/AAAAC) were hybridized following Fischer and Bachmann [37]. After last washing, the membrane was transferred in 220 μl of sterile dH_2O and caught fragments were eluted at 98 °C for 5 min. PCR amplification quality was checked on a 1.2% agarose gel and PCR reactions were cleaned using Sephadex G-50 chromatography.

Table 1
List of date palm cultivars used in this study, their name, geographical distribution, their color in Tamar stage and their maturity class.

No.	Cultivar	Geographical distribution*	Tamar stage color*	Maturity* class
1	Ajwa	West (Al-Madinah)	Dark	Mid-season
2	Anbra	West (Al-Madinah)	Maroon	Late
3	Barhi	Central (Riyadh, Qassim), Eastern Region, West (Al-Madinah)	Brown	Mid-season
4	Bintzamil	Central (Riyadh)	Brown	Mid-season
5	Dekhaini	Central (Riyadh)	Dark-brown	Early
6	Ghur	Eastern Region	Brown	Early
7	Hatmi	Eastern Region	Brown	Mid-season
8	Hilali	Central (Riyadh), Eastern Region	Brown	Late
9	Halawa	Central (Riyadh)	Dark-red	Mid-season
10	Kashkash	Central (Riyadh)	Brown	Mid-season
11	Khalas	Central (Riyadh, Qassim), Eastern-Region, Northern-Border	Brown	Mid-season
12	Khenaizy	Eastern Region	Dark-brown	Mid-season
13	Khesab	Central (Riyadh), Eastern Region	Dark	Late
14	Khodry	Central (Riyadh, Qassim), West (Makkah, Al-Madinah), South (Aseer, Jazan)	Dark-brown	Late
15	Meneifi	Central (Riyadh, Qassim)	Brown	Late
16	Nabtatali	Central (Riyadh, Qassim)	Maroon	Mid-season
17	Nabtatsaif	Central (Riyadh, Qassim), Northern Border	Golden-brown	Mid-season
18	Quatarah	Central (Qassim), West (Al-Madinah)	Dark	Mid-season
19	Rabeaa	West (Makkah, Al-Madinah)	Dark-brown	Early
20	Rushodia	Central (Riyadh, Qassim)	Brown	Mid-season
21	Ruthana	West (Al-Madinah), South (Aseer)	Yellowish-brown	Early
22	Ruzeiz	Eastern Region	Dark-brown	Mid-season
23	Safri	Central (Riyadh), South (Aseer, Al-Baha,Jazan), West (Makkah)	Brown	Mid-season
24	Sari	Central (Riyadh, Qassim)	Brown	Early
25	Segae	Central (Riyadh, Qassim), Northern Border, South (Aseer)	Reddish-brown	Mid-season
26	Shahal	Eastern Region	Reddish-brown	Late
27	Shaishi	Eastern Region	Reddish-brown	Mid-season
28	Sukkari	Central (Riyadh, Qassim), Northern Border	Brown	Mid-season
29	Sullaj	Central (Riyadh), Northern Border	Maroon	Mid-season
30	Thawee	Central (Riyadh)	Dark-red	Mid-season
31	Wannana	Central (Qassim)	Dark-brown	Mid-season
32	Wesaili	Eastern Region	Dark-red	Mid-season

* Source — http://www.moa.gov.sa. Famous date palm cultivars grown in Saudi Arabia.

Cleaned elutions of the SSR motifs were used for TA cloning using pGEM Easy cloning kit (Promega, Madison, WI, USA) following manufacturer's recommended protocol.

Transformed bacterial colonies were plated onto agar LB solid media supplemented with (100 μg/ml) carbenicillin final concentration as a selectable marker. Plasmid DNA was isolated from white colonies using the QIAprep Spin MiniPrep Kit (QIAGEN. Hilden, Germany). Eluted plasmids were subjected to sequencing. The sequencing reaction was carried out in 10 μl total volume, 0.5 μl mini prep. plasmid DNA, 1 μM T7 primer, 1× sequencing Buffer, 1 μl ABI BigDye terminator v3.1 and 6 μl Milli-Q water. The 40 cycle sequencing was 1 min at 96°C, 20 s at 96°C, 30 s at 50°C, 4 min at 60°C, and sequencing reactions were EtOH precipitated. Samples were sent to the sequencing facility at Lujubljna University (Slovenia). Sequences were viewed; vector trimmed and edited using Codon Code aligner software.

2.3. Genomic SSR primer development

CodonCode aligner software (CodonCode Corporation, Dedham, MA, USA) was employed for base calling and vector removal.

Sequences were assembled according to the TIGR Plant transcript assemblies' database [38]. To check the efficiency of our methodology of catching date palm SSR motives, we sequenced 192 bacterial colonies randomly selected from enriched library. 133 sequences were of good quality for further SSR primer development using BatchPrimer3 software [39]. Out of 133 input sequences we were able to pick 93 putative SSR primers. 71% 0f them were dinucleotides, 25% were trinucleotides, 3% tetra and 1% penta nucleotide motives.

2.4. SSR marker validation

The 93 microsatellite primers were tested on a panel of eight randomly selected DNA samples from date palm cultivars. Results indicated that the primers had the ability to amplify the date palm DNA and detect good allelic amplification for all the markers across the tested cultivars. Twenty two of the genomic primer pairs were randomly selected and labeled to screen all date palm cultivars. The PCRs were performed in 20 μl reaction volume containing 1× *GoTaq* green master mix (Promega Corporation, Madison, USA), 0.1 μM from each forward and reveries primers, 50 ng DNA template and nuclease-free water to 20 μl. The thermal cycler profile for PCR amplification was set on a TC-5000 thermal cycler (BIBBY Scientific — UK) as follows: an initial denaturation of 5 min at 94°C followed by 35 cycles of 45 s at 94°C, 45 s at the proper annealing temperature 55°C; 60 s at 72°C followed by 30 min at 72°C for final extension. One microliter of the PCR amplified product was diluted 1:40 and mixed with 0.5 μl of the GeneScan 500 LIZ size standard (Applied Biosystems) and 8.5 μl of Hi-Di formamide (Applied Biosystems). The mixture was denatured and loaded on 16-capillary system of the Applied Biosystems 3130*xl* Genetic Analyzer. A 36 cm capillary array (Applied Biosystems) and 3130 POP-7 polymer (Applied Biosystems) were used.

2.5. Data analysis

Fragment analysis was performed with GeneMapper analysis software v3.7 (ABI). The Jaccard similarity matrix was used for cluster analysis using the unweighted pair group method arithmetic average to study the genetic relationships among the cultivars [40]. These coefficients were used to construct dendrogram using the unweighted pair group method with arithmetic average (UPGMA) and the robustness of internodes was assessed by bootstrap analysis with 100 replicates and principal coordinate analysis (PCoA) was performed. Analysis of similarities (ANOSIM) was used to test the significant levels of the groping patterns in PCoA coordinates. Function ANOSIM operates on a dissimilarity matrix using the PAST program [41]. For each primer pair, Microsatellite-Toolkit for Excel [42] was used for estimating mean number of alleles, (Ho, He) observed and expected heterozygosities [43], polymorphism information content (PIC) according to Botsteinet al. [44], power of discrimination was calculated with the formula $PD = 1 - \sum g_i^2$, where g_i is the frequency of the ith cultivar [45]. The genetic diversity was also estimated by the F-statistic of Wright [46] (F_{IS}, F_{ST}, F_{IT}) and the analysis of molecular variance (AMOVA) by using GenAlex 6.5 software [47]. The cultivars were grouped and analyzed according to three ways of grouping. The first analysis was according to geographical distribution of cultivar where the regions were divided into east, west, north, south and middle with distances among regions that exceeded 500 km. In the second analysis cultivars were grouped according to date of maturity and were divided into three groups: early, mid and late season maturity groups. The fruit color was the third group and were divided into (brown group included yellow and golden brown, dark group, dark brown group included dark red and maroon group included reddish brown).

3. Results

Constructing genomic libraries of *P. dactylifera* enriched for GA, GT, ACA and AGA repeats resulted in hundreds of clones containing SSR loci. Of the 192 clones sequenced from the libraries, 133 were of good quality for further SSR primer development gave sequences contained dinucleotides, trinucleotides, tetranucleotides and pentanucleotides. In forty out of 133 unique sequences, regions flanking the microsatellite were ambiguous or too short to allow designing of suitable PCR primers. As a consequence, primers were designed for 93 of these loci, 71% of them were dinucleotides, 25% were trinucleotides, 3% tetra and 1% pentanucleotides. These primers were tested for amplification and polymorphism on a set of 8 date palm cultivars. We randomly selected 22 primers and they were labeled to amplify the 32 date palm cultivars (Table 2).

The 22 genomic SSR primers generated a total of 91 alleles, all were polymorphic. The number of alleles varied from 2 (KSU-PDL39) to 6 in (KSU-PDL4 and KSU-PDL21) with a mean of 4.14 alleles *per locus*. Expected heterozygosity *per locus* ranged from 0.508 for the KSU-PDL73 to 0.823 KSU-PDL4 with an average of 0.676. The highest values for observed heterozygosity was 1.00 recorded for KSU-PDL2, KSU-PDL3, KSU-PDL4, KSU-PDL5 and KSU-PDL6 while the lowest value was 0.286 recorded for KSU-PDL58 with an average of 0.763.

The average value of PIC for the primer sets was 0.595, ranging from 0.373 for KSU-PDL39 to 0.783 KSU-PDL4. Most primers showed PIC values greater than 0.5, therefore, these primers have power for analyzing the genetic variability of date palm cultivars. PD varied from 0.062 for KSU-PDL4 to 0.938 for KSU-PDL73 with an average of 0.663. The variability parameters for 22 polymorphic loci are presented in Table 3.

Pair-wise similarity values ranged from 0.06 to 0.89 and the overall cultivar similarity showed an average of 0.41. The maximum similarity index (0.89) was recorded between cultivars Sullaj and BintZamil and the minimum value (0.06) was recorded between Kashkash and Anbarah.

The UPGMA cluster analysis of the cultivars based on genomic-SSR data exhibited weak clustering relationships, except for two cultivars, Sullaj and Bintzamail (bootstrapping value 93), two major groups were formed (with bootstrapping values of 5 and 10) and genetic similarity coefficients ranged from 0.25 to 0.88 (Fig. 1). Although, weak bootstrap supports were revealed for branches separating cultivars from the same geographical distribution, Cluster 1 contained seven cultivars of Quatarah, Hatmi, Wannana, Ruzeiz, Anbra, Halawa and Rushodia, all classified under the class of mid-season maturity cultivars regardless of their geographical distribution or fruit color. Cluster 2 was further subdivided to subgroups that compassed the 25 cultivars. In the Subcluster 1 cultivars grouped mostly corresponding with their geographical distribution. Except for Khenaizy, and Shaishi which are cultivated in the eastern part of Saudi Arabia, Khodry, Sullaj, Meneifi, Nabtatsaif, Barhi, NabtatAli, BintZamil, and Thawee were all cultivated in the central part of the kingdom and mostly classified under mid-season class of maturity. Subcluster 2 grouped cultivars under early class of maturity have brown fruit colors which are Safri, Ghur, Sari, Ruthana and Rabeaa. Kashkash, Khalas and Sukkari cultivars were grouped in Subcluster 3 corresponding to their fruit maturity (middle maturity class) and brown fruit color. The remaining cultivars Hilali and Dekhaini grouped in Subcluster 4 while Segae and Shahel cultivars were grouped in Subcluster 5 according to their fruit color regardless of the maturity class or geographical distribution. Khesab, Wesaili and Ajwa cultivars failed to form clusters and individually separated.

Principal coordinate analysis (PCoA) was performed to validate genetic relationships among 32 cultivars (Fig. 2). The first two axes explained 40% of the variation, where the first coordinate accounts for 25% variation, while the second axis explains 15% variation. Following the same trend of the dendrogram, weak relationships and no specific

Table 2
List of 22 new genomic SSR primer pairs designed for date palm, their name, working temperature, sequences, repeat motif and their size range.

Locus	T (°C)	Primer sequence (5′-3′)	Repeat motif	Expected size	Size range
KSU-PDL2	55	F: TTGGAGTAGGAGACGACAATA R: GGGAGTGAGAGGGATATGTAG	(CT)14	147	131–140
KSU-PDL3	55	F: GGCAACACAATCAAGACTCTA R: TTGTTTGAAATCTGTAGTTAGCA	(AG)16	135	140–153
KSU-PDL4	55	F: CAACATAAGGAAAAATGATGC R: TGCATCACTCTGGGTATAAAT	(AG)19	151	147–200
KSU-PDL5	55	F: CCAAACAGAACATGAAGAGAG R: TGAGGAATTTGTCATAGTTGAA	(TC)13	163	160–200
KSU-PDL6	55	F: GCTTTTGCAAATAACAACATC R: CATGGAAAAGGCTCCTATC	(AG)25	116	139–246
KSU-PDL16	55	F: AGATGATTCCATAAGGTTGGT R: CAGAGCTCTCTCTCTCTCTC	(GT)15	173	134–159
KSU-PDL17	55	F: TGATCTATAGCAGCCACAAGT R: GTTTTATTGGGGTGGTATTCT	(GT)13	140	113–151
KSU-PDL18	55	F: TGTGGTCTATCCATTTTGTGT R: GTCATGCAGTTCTCAAAGAAA	(TG)10	147	135–148
KSU-PDL18_2	55	F: TCCATTTTGTGTATGTGTGTG R: TCAGTTTAGTTGGAACGTCAT	(GA)6	154	133–156
KSU-PDL20	55	F: ATGGTAGCTGATGGTCAAATA R: CTTGAACACTCTCCTCTCCTT	(GT)13	154	130–156
KSU-PDL21	55	F: GCTACTCCTTCTTCTTCTCCTT R: TGATGATTGGTTGAGATTAAGA	(CTT)6	116	104–177
KSU-PDL25	61	F: ACGGGAAGCTGGACCTTG R: CTACAAACCCAGCAGACATAG	(GGA)4	156	144–197
KSU-PDL29	55	F: AGCACATGGCAGTTACTCTAC R: AACAACAACAATCAGTCCAAA	(GTT)11	164	129–260
KSU-PDL39	55	F: AAACTGGTGACAACAAAACTG R: CATGATTACTCCTCATCATCA	(GTG)4	157	112–175
KSU-PDL42	55	F: GACCGTACAGTCACATGATTT R: TAGGAGAGAGAGAGGGTTTTG	(CT)18	189	145–158
KSU-DPL53	55	F: GAGGAGAGAGAGAGTGGAAGA R: CGTGAGAGAGACGAAAGATAA	(AG)12	138	127–135
KSU-PDL58	55	F: GAGAAGAGAAAGGGAGAGAGA R: GCCCTTCTTAATCAACAAAAT	(AG)9	150	104–154
KSU-PDL61	61	F: ATATAGGCATAGCGCCGACAC R: GGGGTAGGTAGATTTTCAAGA	(AC)11	153	104–348
KSU-PDL64	55	F: ACTCTTGTGGGACTCCTTTAC R: CCTAAATGTGCTTTCCTTCTT	(TC)17	141	140–152
KSU-PDL73	54	F: CAAATCCCAGTCACAAATTAT R: CTTATTGCGCGTTTTGTAATA	(TC)16	138	124–154
KSU-PDL74	54	F: ACCATATTCAGAAGCTACGAC R: TTAATGTTGATATGCACCTAACT	(AG)15	100	101–113
KSU-PDL76	55	F: TTGGAGTAGGAGACGACAATA R: AGAGAGAGATGGGGAAGAAG	(TC)12	148	120–378

geographic relationships were obtained ($R = 0.734$, $P < 0.001$). However, PCoA showed that all samples were divided into two major clusters. Cluster 1 included the mid-season maturity cultivars regardless of their geographical distribution or fruit color, while the remaining cultivars were grouped in the second cluster. Khalas and Sukkari cultivars were separated away from the second cluster and formed Subcluster 3 in UPGMA method whereas Wesaili failed to form cluster in both methods.

Fixation indices (F_{IS}, F_{IT} and F_{ST}) were used for population differentiation for each *locus* (Table 3). The mean values for the three parameters over cultivars were -0.132, -0.029 and 0.107 for F-statistics (F_{IS}, F_{IT} and F_{ST}) respectively. Fixation Index or Inbreeding Coefficient (F_{IS}) ranged from -0.779 for KSU-PDL3 to 0.578 for KSU-PDL58 *locus* and F_{IT} ranged from -0.763 to 0.763 for the same *loci*. Six out of 22 *loci* showed high degree of genotypic differentiation which had F_{ST} higher than 0.15 which were: KSU-PDL42, KSU-DPL53, KSU-PDL58, KSU-PDL64, KSU-PDL73, and KSU-PDL76.

The estimated variation percentage among and within cultivars was also analyzed by AMOVA at $P < 0.05$ level. The results indicated that 73% and 71% of variation were due to differences within cultivars regardless of geographical distribution and fruit colors which were much higher than the variations present among cultivars 27%, 21% for geographical distribution and fruit color respectively. The percentage was 92% among and 8% within cultivars according to date of fruit maturity (Table 4).

4. Discussion

Development of highly informative markers, such as simple sequence repeats, for variety identification and germplasm characterization and management is essential for date palm genetic studies. Saudi Arabia still preserves vast richness of date palm germplasm confirmed by a great number of different cultivars and accessions, described based on the morphological characters of fruit and seeds. To manage date palm germplasm, it should be genetically characterized, which will help in their preservation, transmit a significant genetic richness and in their exploitation.

Constructing genomic libraries of date palm (cultivar Sukary) enriched for GA, GT, ACA and AGA repeats, allowed the development of 93 SSR primer pairs used for genetic diversity analysis of 32 commercially important date palm cultivars grown in Saudi Arabia. The library enrichment procedure adopted here yielded 69% of inserts containing microsatellite repeat motifs which are higher than the average number previously reported for 71 plant species [48]. However it was 82% which was less than that by Akkak et al. [22] and Arabnezhad et al. [49] who reported 86% in date palm.

In this study twenty two highly polymorphic microsatellite loci were used to analyze the genetic variability among the most common grown cultivars in Saudi Arabia selected for their good fruit quality. High values of observed variability parameters (polymorphism %, number of alleles, observed and expected heterozygosities, Shannon's information index

Table 3
SSR locus name, number of alleles, (Ho), (He) observed and expected heterozygosities, polymorphic information content (PIC), discrimination power (PD), Shannon's information index and the inbreeding coefficient values for 22 developed polymorphic microsatellite loci in a sample of 32 date palm cultivars.

Locus	No. of alleles	He	Ho	PIC	PD	I	Fis	Fit	Fst
KSU-PDL2*	5	0.794	1.000	0.739	0.527	1.047	-0.259	-0.232	0.022
KSU-PDL3	3	0.562	1.000	0.445	0.647	0.790	-0.779	-0.763	0.009
KSU-PDL4	6	0.823	1.000	0.783	0.062	0.693	-0.215	-0.215	0.000
KSU-PDL5	5	0.779	1.000	0.724	0.569	0.630	-0.284	-0.276	0.006
KSU-PDL6	4	0.581	1.000	0.473	0.609	0.693	-0.721	-0.721	0.000
KSU-PDL16	5	0.699	0.958	0.641	0.438	0.684	-0.371	-0.362	0.006
KSU-PDL17	4	0.613	0.526	0.535	0.647	0.595	0.142	0.230	0.102
KSU-PDL18	3	0.670	0.826	0.581	0.483	0.688	-0.233	-0.225	0.006
KSU-DL18_2	5	0.691	0.957	0.623	0.483	1.238	-0.385	-0.361	0.017
KSU-PDL20	5	0.762	0.727	0.681	0.882	1.017	0.046	0.149	0.108
KSU-PDL21	6	0.738	0.786	0.669	0.809	1.144	-0.065	-0.006	0.055
KSU-PDL25	5	0.697	0.895	0.640	0.647	1.181	-0.284	-0.208	0.059
KSU-PDL29	3	0.622	0.588	0.525	0.718	0.820	0.055	0.159	0.110
KSU-PDL39	2	0.518	0.417	0.373	0.847	0.654	0.195	0.224	0.036
KSU-PDL42	4	0.726	0.615	0.646	0.780	0.915	0.153	0.334	0.214
KSU-DPL53	3	0.645	0.818	0.542	0.882	0.722	-0.268	0.144	0.325
KSU-PDL58	3	0.677	0.286	0.580	0.780	0.780	0.578	0.660	0.194
KSU-PDL61	3	0.677	0.882	0.584	0.718	1.039	-0.303	-0.263	0.031
KSU-PDL64	5	0.751	0.786	0.685	0.809	1.082	-0.047	0.226	0.261
KSU-PDL73	3	0.508	0.500	0.428	0.938	0.513	0.016	0.387	0.377
KSU-PDL74	5	0.602	0.714	0.544	0.569	0.974	-0.186	-0.019	0.141
KSU-PDL76	4	0.730	0.500	0.652	0.750	0.941	0.315	0.507	0.280
Total	91	–	–	–	–	–	–	–	–
Mean	4.14	0.676	0.763	0.595	0.663	0.856	-0.132	-0.029	0.107
Max	6	0.823	1.000	0.783	0.938	1.238	0.578	0.660	0.377
Min	2	0.508	0.286	0.373	0.062	0.513	-0.779	-0.763	0.000

* KSU = King Saud University. PD = palm date, L = locus number.

and power of discrimination) were recorded which confirmed that these SSR markers are useful to detect genetic variability of date palm germplasm. The average number of alleles per locus detected in this study was comparable to that scored by Ahmed and Al-Qaradawi [26] and Arabnezhad [49]. While it was higher than that from the records of Mirbabaee et al. [50], it was lower than that previously described for Tunisian date palm germplasm [7,23,24], Sudanese date palm germplasms [21], Libyan date palm [22,31], Qatari date palm [27], Morocco date palm [30], Iraqi date palm [18] and across the genus *Phoenix* [20,33]. This may be due to differences in number of microsatellites and cultivars investigated, number of DNA samples used to represent the cultivars and number of replicates used in amplification and fragment analysis in this study compared with those previously studied.

A high genetic diversity was detected among Saudi date palm cultivars which was reflected in their wide range of heterozygosity values (H_{exp} ranged from 0.508 to 0.823, H_{ob} ranged from 0.286 to 1.000). These results were in concordance with those recorded by Hamza et al. [7], Khierallah et al. [18], and Akkak et al. [22]. While it was higher than that previously described by Bodia et al. [30],

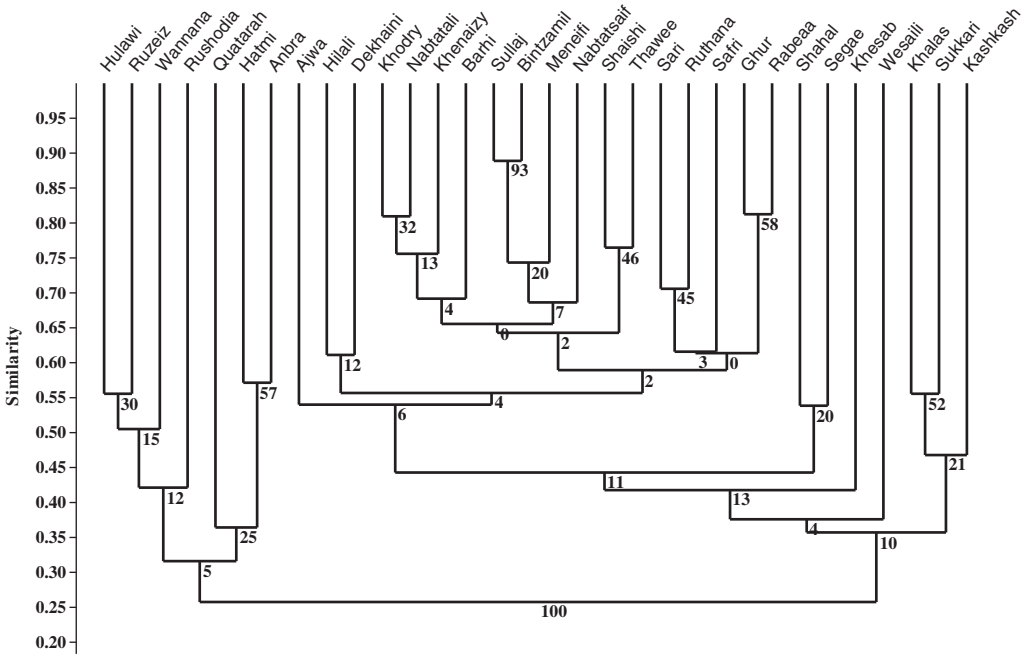

Fig. 1. Dendrograms generated using unweighted pair group method with arithmetic average (UPGMA) analysis, showing relationships between 32 date palm cultivars, using SSR data based on Jaccard genetic similarity index. Bootstrap support value is given above each branch.

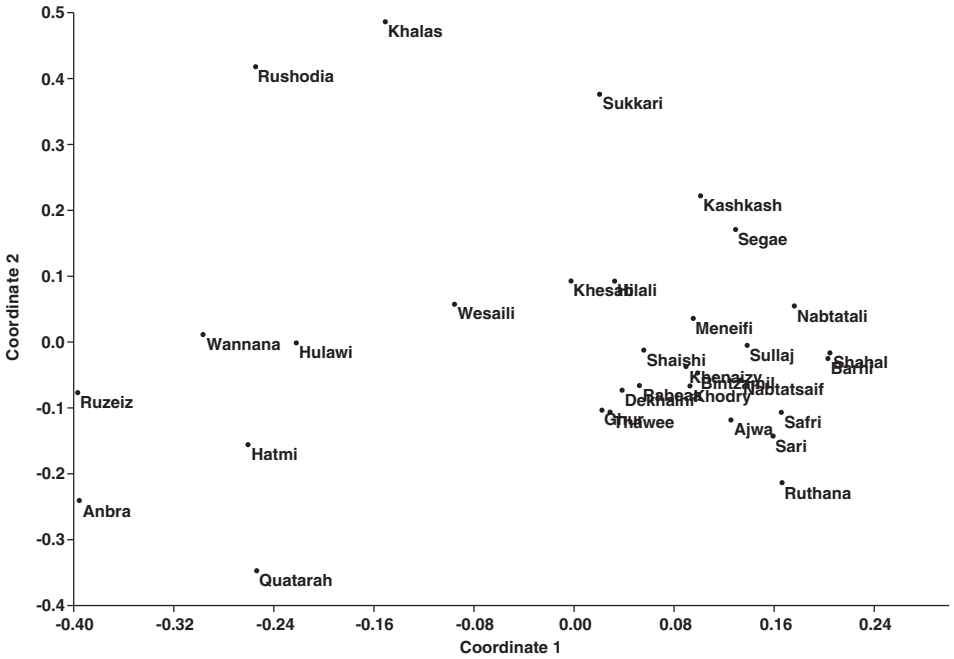

Fig. 2. Principal coordinate analysis (PCoA) of 32 Saudi date palm cultivars based on 22 microsatellite loci. The first and second coordinates accounting for 25% and 15% variation respectively.

Aberlenc-Bertossi et al. [33], and Mirbabaee et al. [50], it was lower than that of Billotte et al. [20], Elshibli and Korpelainen [21], Zehdi et al. [23], Hammadi et al. [24], and Racchi et al. [31]. This result probably reflects the homogeneous nature of the date palm cultivars used in this study comparing to very divergent sets of cultivars studied earlier. Moreover, variability parameter values of KSU-PDL2, KSU-PDL4, KSU-PDL5, KSU-PDL16, KSU-PDL18_2, KSU-PDL21, KSU-PDL25 and KSU-PDL64 loci exceeded the averaged variability values of the other tested loci, which can be considered promising; and thus a high level of polymorphism is expected once a larger number of cultivars is analyzed.

As a result of the number of SSR alleles revealed, a relatively higher degree of genetic variability in this study for date palm is permitted. A 100% polymorphism percentage was obtained and pair-wise similarity values ranged from 0.06 to 0.89. These results confirm that the great diversity observed probably due to mating system (out crossing), as already found in other studies [26,30,31,49]. Khierallah et al. [18] reported wide genetic distance among Iraqi date palm that varied from 0.171 to 0.938, indicating diverse relationships among the cultivars. Based on the results of Bodia et al. [30] polymorphic loci ranging from 80 to 100%, (mean of 96.11%) and genetic similarity from 0.165 to 0.681 in Morocco date palm cultivars were obtained. Ahmed and Al-Qaradawi, [26] recorded a range of 0.00 to 0.75 and Elmeer et al. [27] from 0.00 to 1.00 similarity level among Qatari date palm cultivars. The mean genetic distances between Sudan cultivars and males (0.123), Sudan and Morocco cultivars (0.748), and Sudan males and Morocco cultivars (0.894) were recorded by Elshibli et al. [21].

Table 4
Summary of AMOVA. Estimated variance percentage and the inbreeding coefficient values for 32 date palm cultivars using 22 developed polymorphic microsatellite loci.

Source	Location % variance	Fruit maturity % variance	Fruit color % variance
Among pops	0.0001%	0.0001%	7%*
Among cultivars	27%	92%	21%
Within cultivars	73%	8%	71%
Total	100%	100%	100%
F_{ST}	0.004	0.000	0.073**
F_{IS}	0.270**	0.922**	0.229**
F_{IT}	0.267**	0.922**	0.286**

* Significant at $P < 0.01$.
** Significant at $P < 0.05$.

In this study, UPGMA cluster analysis and principal coordinate analysis (PCoA) exhibited weak clustering relationships (weak bootstrapping), which illustrated that cultivars tend to group mainly according to their class of fruit maturity and in some cases according to region of cultivation and fruit color. The clustering showed weaker bootstrapping than that of Elshibli et al. [21], however our results were partially coincide with what [21] found, were clustering of Sudan cultivars did not follow a clear geographic pattern. Also, the effect of the pollination methods (farmers depend on few selected males for the pollination of female trees and exchanged between farmers and pollen grains of more than one male are sometimes mixed and used then for pollination in addition toexchange of propagules, which are a mix of vegetative and seed-propagated materials, is conducted between farmers) and cultivar selection on the genetic structure was clearly detected by weak clustering association that was observed for the majority of accessions originating from Sudan and Morocco as well. The results of Zehdi et al. [23] showed a high level of polymorphism with little geographic structure within 49 Tunisia accessions from three main oases. However, Arabnezhad et al. [49] found that date palm cultivars were clustered in agreement with their geographical origin and the analysis significantly distinguished African from Iranian and Iraqi cultivars. In a recent published study, Yusuf [51] used six SSRs to separate Nigerian cultivars from Saudi Arabia cultivars confirming that variation observed among the cultivars follow a geographical pattern.

The moderate to high values of F_{IS} and F_{IT} obtained in this study indicated a considerable inbreeding within and among cultivars, and also point towards high genetic differentiation among cultivars. An excess of heterozygosity manifested by negative F_{IS} values was observed. The average value of F_{ST} (0.107) indicated that the index of genetic differentiation was high among all cultivars. F-values close to zero (KSU-PDL3, KSU-PDL4, KSU-PDL5, KSU-PDL6, KSU-DL16 and KSU-PDL18) are expected under random mating, while substantial positive values indicate inbreeding or undetected null alleles. Negative

values indicate an excess of heterozygotes, due to negative assortative mating or selection [52]. These results indicated considerable genetic differentiation within the 32 studied cultivars. Generally; higher level of genetic diversity may result from larger populations, geographic distribution, and the number of sampling and biological traits [53,54]. Here, five populations were widely distributed in five regions, where there is more than 500 km between their geographic distances. Gene flow is limited by big geographic zone which induces higher genetic variation within populations. In a study of Zehdi et al. [55], using ISSR markers to assess genetic diversity among a set of Tunisian date palm varieties, concluded that since all date palm ecotypes originated by hybridization, it may have a common genetic basis. Nevertheless, varieties diverged from others by mutational events that arise during selection and hence, accession grouping in relation to their geographical origin is not well defined.

The association between morphological and molecular analysis was also studied. Hammadi et al. [24] detected a significant positive correlation between the SSR data and fruit consistency but not with the maturity. He concluded that using microsatellite markers with high polymorphism supported cultivars clustering according to their fruit traits. However, Hamza et al. [7] showed that distances based on quantitative morphological traits were not correlated with genetic distance.

The result of this study also coincides with that of Srivashtav et al. [11] who detected good associations between molecular using ISSR and RAPD data and some date palm fruit traits where early maturing cultivars with red fruits and high resistance to rain water formed one subcluster and late maturing with yellow fruit and medium resistance to rain water formed the second subcluster.

Genetic diversity indicates good potential for further improving the agronomic and commercial characters of date palms [21] and for allowing them to adapt to new environments and to climate change [56]. The genetic diversity seems to be high in Saudi date-palms. This could be attributed to the dioecious nature of this crop. The high level of diversity is expected because of the unique mechanism responsible for generating SSR allelic diversity by replication slippage. Replication slippage is thought to occur more frequently than single nucleotide mutations and insertion/deletion events, which generated the polymorphisms detected by RAPD analysis [57]. The codominant nature of SSR markers also permits the detection of high number of alleles per locus and contributes to higher levels of expected heterozygosities being reached than what would be possible with dominant markers.

5. Conclusions

In this research, SSR marker technology has been used to increase the number of available molecular markers that are suitable for the molecular characterization, and the investigation of phylogenic relationships among Saudi date palm germplasm. The development of 93 SSRs together with their characterization will facilitate mapping studies and integration of date palm maps. The markers developed herein will be a valuable resource for breeding, genetic, diversity, and genomic studies of date palm germplasm.

Conflict of interests

The authors have declared that no conflict of interest exists. All roles and conditions of publication are accepted by authors and their responsibilities to follow.

Financial support

The authors are grateful to the Deanship of scientific research at King Saud University, for the financial support through the research group project number RGP-VPP-090.

References

[1] Barrow SC. A monograph of Phoenix L. (Palmae: Coryphoideae). Kew Bull 1998;53: 513–75. http://dx.doi.org/10.2307/4110478.
[2] Ministry of Agriculture. [cited August 2015]. Available from Internet: http://www.moa.gov.sa; 2011.
[3] Hussain F, El-Zeid A. Studies on physical and chemical characteristics of eighteen date cultivars grown in Saudi Arabia. Indian J Horticult 1976;33:107–13.
[4] Elhoumaizi MA, Saaidi M, Oihabi A, Cilas C. Phenotypic diversity of datepalm cultivars (*Phoenix dactylifera* L.) from Morocco. Genet Resour Crop Evol 2002;49: 483–90. http://dx.doi.org/10.1023/A:1020968513494.
[5] Al-Khalifah NS, Askari E. Molecular phylogeny of date palm (*Phoenix dactylifera* L.) cultivars from Saudi Arabia by DNA fingerprinting. Theor Appl Genet 2003;107: 1266–70. http://dx.doi.org/10.1007/s00122-003-1369-y.
[6] Mansour MT, Hassawi DS, Migdadi HM, Brake M. Fingerprinting date palm genotypes (*Phoenix dactylifera* L.) using Inter Simple Sequence Repeat (ISSR) markers. J Genet Breed 2011;63:17–22.
[7] Hamza H, Elbekkay M, Ben Abderrahman MA, Ali AF. Molecular and morphological analyses of date palm (*Phoenix dactylifera* L.) subpopulations in southern Tunisia. Span J Agric Res 2011;9:484–93.
[8] Ahmed TA, Alhadidi SH, Al-Qaradawi AY, Radwan O. Determination of inter and intra-specific genetic variations among Qatari date palm cultivars using inter simple sequence repeat (ISSR) markers. Afr J Biotech 2013;12:2540–6. http://dx.doi.org/10.5897/AJB2013.12049.
[9] Munshi A, Osman G. Investigation on molecular phylogeny of some date palm (*Phoenix dactylifera*) cultivars by protein, RAPD and ISSR marker in Saudi Arabia. Aust J Crop Sci 2010;4:23–8.
[10] Haider N, Nabulsi I, Ali N. Phylogenetic relationships among date palm (*Phoenix dactylifera* L.) cultivars in Syria using RAPD and ISSR markers. J Plant Biol Res 2012;1:12–24.
[11] Srivashtav VS, Kapadia CV, Mahatma MK, Jha SK, Jha S, Ahmad T. Genetic diversity analysis of date palm (*Phoenix dactylifera* L.) in the Kutch region of India using RAPD and ISSR markers. Emir J Food Agric 2013;25:907–15.
[12] Khierallah HSM, Al-Sammarraie SKI, Haider IM. Molecular characterization of some Iraqi date palm cultivars using RAPD and ISSR markers. J Asian Sci Res 2014;4: 490–503.
[13] Elkhishin DA, Adawy SS, Hussein EHA, Elitriby HA. AFLP fingerprinting of some Egyptian date palm (*Phoenix dactylifera* L.) cultivars. Arab J Biotech 2003;6: 223–34.
[14] El-Assar AM, Krueger RR, Devanad PS, Chao CT. Genetic analysis of Egyptian date (*Phoenix dactylifera* L.) accessions using AFLP markers. Genet Resour Crop Evol 2005;52:601–7. http://dx.doi.org/10.1007/s10722-004-0583-z.
[15] Jubrael JMS, Udupa S, Baum M. Assessment of AFLP based genetic relationships among date palm (*Phoenix dactylifera* L.) varieties of Iraq. J Am Soc Hort Sci 2005; 130:442–7.
[16] Elhoumaizi MA, Devanandm PS, Fang J, Chao CCT. Confirmation of "Medjool" date as a landrace variety through genetic analysis of "Medjool" accessions in Morocco. J Am Soc Hort Sci 2006:403–7, e13.
[17] Rhouma S, Zehdi-Azouzi S, Ould MSA, Rhouma A, Marrakchi M, Trifi M. Genetic diversity in ecotypes of Tunisian date-palm (*Phoenix dactylifera* L.) assessed by AFLP markers. J Hort Sci Biotech 2007;82:929–33. http://dx.doi.org/10.1080/14620316.2007.11512328.
[18] Khierallah HSM, Bader SM, Baum M, Hamwieh A. Assessment of genetic diversity of some Iraqi date palms (*Phoenix dactylifera* L.) using AFLP markers. Afr J Biotech 2011;10:9570–5.
[19] Al-Dous EK, George B, Al-Mahmud ME, Al-Jabber MY, Wang H, Salameh YM, et al. De novo genome sequencing and comparative genomics of date palm (*Phoenix dactylifera*). Nat Biotechnol 2011;29:521–7. http://dx.doi.org/10.1038/nbt.1860.
[20] Billotte N, Marseillac N, Brottier P, Noyer JL, Jacquemoud-Collet JP, et al. Nuclear microsatellite markers for the date palm (*Phoenix dactylifera* L.): Characterization and utility across the genus Phoenix and in other palm genera. Mol Ecol Notes 2004;4:256–8. http://dx.doi.org/10.1111/j.1471-8286.2004.00634.x.
[21] Elshibli S, Korpelainen H. Microsatellite markers reveal high genetic diversity in date palm (*Phoenix dactylifera* L.) germplasm from Sudan. Genetica 2008;134:251–60. http://dx.doi.org/10.1007/s10709-007-9232-8.
[22] Akkak A, Scariot V, Torello Marinoni D, Boccacci P, Beltramo C, Botta R. Development and evaluation of microsatellite markers in *Phoenix dactylifera* L. and their transferability to other *Phoenix* species. Biol Plant 2009;53:164–6. http://dx.doi.org/10.1007/s10535-009-0026-y.
[23] Zehdi S, Trifi M, Billotte N, Marrakchi M, Pintaud JC. Genetic diversity of Tunisian date palms (*Phoenix dactylifera* L.) revealed by nuclear microsatellite polymorphism. Hereditas 2004;141:278–87. http://dx.doi.org/10.1111/j.1601-5223.2004.01855.x.
[24] Hammadi H, Vendramin GG, Ali AF. Microsatellite diversity among Tunisian date palm (*Phoenix dactylifera* L.) subpopulations. Pak J Bot 2011;43:1257–64.
[25] Al-Ruqaishi IA, Davey M, Alderson P, Mayes S. Genetic relationships and genotype tracing in date palm (*Phoenix dactylifera* L.) in Oman based on microsatellite markers. Genet Resour Crop Evol 2008:70–2. http://dx.doi.org/10.1017/S1479262108923820.
[26] Ahmed TA, Al-Qaradawi AY. Molecular phylogeny of Qatari date palm genotypes using simple sequence repeats markers. Biotechnology 2009;8:126–31. http://dx.doi.org/10.3923/biotech.2009.126.131.
[27] Elmeer K, Sarwath H, Malek J, Baum M, Hamwieh A. New microsatellite markers for assessment of genetic diversity in date palm (*Phoenix dactylifera* L.). 3Biotech 2011; 1:91–7. http://dx.doi.org/10.1007/s13205-011-0010-z.
[28] Elmeer K, Mattat I. Marker-assisted sex differentiation in date palm using simple sequence repeat. 3Biotech 2012;2:241–7. http://dx.doi.org/10.1007/s13205-012-0052-x.

[29] Hamwieh A, Farah J, Moussally S. Development of 1000 microsatellite markers across the date palm (*Phoenix dactylifera* L.) genome. Acta Horticult 2010;882:269–78.
[30] Bodia A, Elhoumaizi MA, Ndir KN, Hasnoui A, Nachtigall M, Wehling P. Genetic diversity analysis of date palm (*Phoenix dactylifera* L.) cultivars from *Figuig oasis* (Morocco) using SSR markers. IJSAT 2012;2:96–104.
[31] Racchi ML, Bove A, Turchi A, Bashir G, Battaglia M, Camussi A. Genetic characterization of Libyan date palm resources by microsatellite markers. 3Biotech 2014;4: 21–32. http://dx.doi.org/10.1007/s13205-013-0116-6.
[32] Zhao Y, Williams R, Prakash CS, He G. Identification and characterization of gene-based SSR markers in date palm (*Phoenix dactylifera* L.). BMC Plant Biol 2012;12: 237. http://dx.doi.org/10.1186/1471-2229-12-237.
[33] Aberlenc-Bertossi F, Castillo K, Tranchant-Dubreuil C, Chérif E, Ballardini M, Abdoulkader S, et al. In silico mining of microsatellites in coding sequences of the date Palm (Arecaceae) genome, characterization, and transferability. Appl Plant Sci 2014;2:1300058. http://dx.doi.org/10.3732/apps.1300058.
[34] Al-Faifi SA, Migdadi HM, Al-doss A, Ammar MH, El-Harty EH, Khan MA, et al. Morphological and molecular genetic variability analyses of Saudi lucerne (*Medicago sativa* L.) landraces. Crop Past Sci 2013;64:137–46.
[35] Karagyozov L, Kalcheva ID, Chapman VM. Construction of random small-insert genomic libraries highly enriched for simple sequence repeats. Nucleic Acids Res 1993;21:3911–2.
[36] Billotte N, Lagoda PJL, Risterucci AM, Baurens FC. Microsatellite-enriched libraries: Applied methodology for the development of SSR markers in tropical crops. Fruits 1999;54:277–88.
[37] Fischer D, Bachmann K. Microsatellite enrichment in organisms with large genomes (*Allium cepa* L.). Biotechniques 1998;24:796–802.
[38] Childs KL, Hamilton JP, Zhu W, Ly E, Cheung F, Wu H, et al. The TIGR plant transcript assemblies database. Nucleic Acids Res 2007;35:D846–51. http://dx.doi.org/10.1093/nar/gkl785.
[39] Rozen S, Skaletsky HJ. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz Misener S, editor. Bioinformatics methods and protocols: Methods in molecular biology. 1st ed. New Jersey: Humana; 2000. p. 365–86.
[40] Jaccard P. Nouvelles recherches sur la distribution florale. Bull Soc Vaudoise Sci Nat 1908;44:223–70. http://dx.doi.org/10.5169/seals-268384.
[41] Hammer O, Harper DAT, Ryan PD. PAST: Paleontological statistics software package for education and data analysis. Palaeontol Electron 2001;4:1–9.
[42] Park SDE. The Excel microsatellite toolkit; 2001[March 2015].
[43] Nei M. Molecular evolutionary genetics. New York, NY, USA: Columbia University Press; 1987.
[44] Botstein D, White RL, Skolnick M, Davis RW. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet 1980; 32:314–31.
[45] Kloosterman AD, Budowle B, Daselaar P. PCR-amplification and detection of the human DIS80 VNTR locus. Amplification conditions, population genetics and application in forensic analysis. Int J Legal Med 1993;105:257–64. http://dx.doi.org/10.1007/BF01370382.
[46] Wright S. The interpretation of population structure by F-statistics with special regard to systems of mating. Evolution 1965;19:395–420. http://dx.doi.org/10.2307/2406450.
[47] Peakall R, Smouse PE. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research — An update. Bioinformatics 2012;28:2537–9. http://dx.doi.org/10.1093/bioinformatics/bts460.
[48] Squirrell J, Hollingsworth PM, Woodhead M, Russell J, Lowe AJ, Gibby M, et al. How much effort is required to isolate nuclear microsatellites from plants? Mol Ecol 2003;12:1339–48. http://dx.doi.org/10.1046/j.1365-294X.2003.01825.x.
[49] Arabnezhad H, Bahar M, Mohammadi HR, Latifian M. Development, characterization and use of microsatellite markers for germplasm analysis in date palm (*Phoenix dactylifera* L.). Sci Hortic 2012;134:150–6. http://dx.doi.org/10.1016/j.scienta.2011.11.032.
[50] Mirbabaee SA, Mardi M, Mahmoodi P, Pirseyedi SM, Abbasi A, Farsi M, et al. Development of new microsatellite markers from an enriched genomic library of date palm (Phoenix dactylifera L.). J Hort Sci Biotech 2011;86:539–41. http://dx.doi.org/10.1080/14620316.2011.11512801.
[51] Yusuf AO, Culham A, Aljuhani W, Ataga CD, Hamza AM, Odewale JO, et al. Genetic diversity of Nigerian date palm (*Phoenix dactylifera*) germplasm based on microsatellite markers. J BioSci Biotechnol 2015;7:121–32.
[52] Hartl DL, Clark AG. Principal of population genetics. 3rd ed. Sunderland, MA: Sinauer Associates; 1997.
[53] Nybom H. Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Mol Ecol 2004;13:1143–55. http://dx.doi.org/10.1111/j.1365-294X.2004.02141.x.
[54] Trapnell D, Hamrick J. Mating patterns and gene flow in the Neotropical epiphytic orchid, *Laelia rubescens*. Mol Ecol 2005;14:75–84. http://dx.doi.org/10.1111/j.1365-294X.2004.02383.x.
[55] Zehdi S, Sakka H, Rhouma A, Ould MSA, Marrakchi M, Trifi M. Analysis of Tunisian date palm germplasm using simple sequence repeat primers. Afr J Biotechnol 2004;3:215–9.
[56] Hamrick J, Godt MJW, Sherman-Broyless SL. Factors influencing levels of genetic diversity in woody plant species. New For 1992;6:95–124. http://dx.doi.org/10.1007/BF00120641.
[57] Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S, et al. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol Breed 1996;2:225–38. http://dx.doi.org/10.1007/BF00564200.