Genetic Diversity Analysis in Date Palm (*Phoenix dactylifera* L) Germplasm using Microsatellite Markers

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Abstract: The Date Palm (*Phoenix dactylifera* L), germplasm commonly cultivated in Egypt, shows a wide range of repining periods and fruit quality and is an unexploited resource for breeding programs. The main purpose of this study was to genetic diversity analysis and relationships for 45 date palm genotypes and to construct a molecular database including the cultivars commonly grown in Egypt. An analysis of thirty three microsatellite simple sequence repeat (SSR) loci out of thirty five markers was performed to define allele diversity, heterozygosity and genetic structure. The average number of alleles per locus was 32.3. Heterozygosity per locus was 0.64 (Dpalm-103) to 0.96 (Dplam-100, 256, mpdCIR08 and mpdCIR078) with an average of 0.89. The highest heterozygosity (0.768) was detected in Avanda, followed by Amhat (0.758) while Khalas had lowest heterozygosity value (0.455).The genetic similarity values ranged from 0.041 to 0.260. The obtained dendrogram showed three main clusters and generally, a good structuring of 45 genotypes and accessions. The use of 33 polymorphic microsatellite markers and the level of genetic variability detected within Egyptian date palm germplasm suggested that this is reliable, efficient and effective marker system that can be used for diversity analysis and subsequently in crop improvement programs.

Key words: Microsatellite loci, Heterozygosity, Genetic similarity, Cluster.

INTRODUCTION

Date palm (*Phoenix dactylifera* L., 2n = 36) is a perennial monocotyledonous fruit plant, belonging to the family of Arecaceae (Coryphoideae). The genome size is estimated to be approximately 658-Mbp long (Al-Dous *et al*., 2011). 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 indifferent 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). Date palm is an important economic crop in Egypt where the world's largest producer over the last two years is Egypt with 1,470,000 Mt followed by Islamic Republic of Iran (1,066,000 Mt) and Saudi Arabia (1,050,000 Mt) (FAO stat, 2012). As with many other plants, genetic diversity of date palm is threatened by habitat loss due to population pressure and clearance for agriculture development. Moreover, developing elite cultivars using a few genetic materials from gene pool and using off-shooting propagation intensively in date palm breeding could cause loss of genetic diversity (Zhao *et al.*, 2013). A variety of morphological characters of date fruits (viz., shape, size, weight, color, aspects of fruit skin, consistency, texture, etc.) and biochemical markers like isozymes and proteins (Abdulla and Gamal, 2010) have earlier been employed for the identification of date fruits. However, these traits are greatly influenced by environmental factors as well as the developmental stages of the plant.

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. Among molecular markers, microsatellites, also known as Simple-Sequence Repeats (SSRs), because of their particular features such as their codominant nature and their typically high levels of allelic diversity at different loci, represent a suitable tool for genotyping. The usefulness of microsatellite markers for measuring the genetic variability in a wide range of plants has been recently reviewed (Elsheikh *et al.,* 2014). Because of their high mutation rates and the ease of the analysis microsatellite markers were proved useful and effective for phylogenetic studies genetic fingerprinting and cultivar identification among different date palm accessions in Egypt (Adway *et al.*, 2005).

However, genetic variation is a basic requirement for plant breeding, whereas a high genetic variation is needed for genetic improvement of date palm. In recent years, genetic markers are increasingly for the study of genetic diversity. Therefore, the polymorphism determined by these markers is one of the valuable parameters for studying cultivars and understanding their genetic difference. 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. Microsatellite markers were used for the analysis of genetic differentiation among date palm cultivars, in which 33 loci out of 35 microsatellite loci for 45 date palm genotypes and accessions allowing the estimation of genetic diversity within.

MATERIALS AND METHODS

Plant materials:

The date palm materials were collected from different locations in Egypt. Forty-five genotypes and accessions were chosen for their good fruit quality Table (1). Three trees per each genotype were selected; most of them were vigorous vegetative propagated using off shooting.

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Table (1): List of date palm genotypes and accessions, geographic origin and consistency in the present study.

No.	Name	Location	Consistency	Origin
1	DegletNoor	Aswan	Semi-Dry	Algeria
2	Malkabi	Aswan	Dry	Sudan
3	Bartamoudawardy	Aswan	Dry	Sudan
4	BartamoudaAdia	Aswan	Dry	Sudan
5	Balady	Aswan	Dry	Egypt
6	Shamiya	Aswan	Dry	Egypt
7	Sakkoty	Aswan	Dry	Egypt
8	Gondailawardy	Aswan	Dry	Sudan
9	GondailaAdia	Aswan	Dry	Sudan
10	Maghal 1	Ismailia (elkasasen)	Soft	Egypt
11	Samany 1	Ismailia (elkasasen)	Soft	Egypt
12	Zaghlool 1	Ismailia (elkasasen)	Soft	Egypt
13	Kabooshy	Ismailia (elkasasen)	Soft	Egypt
14	Amry 1	Ismailia (elkasasen)	Semi-Soft	Egypt
15	Hayani 1	Ismailia (elkasasen)	Soft	Egypt
16	Amry 2	Sharqiya (elkoreen)	Semi-Soft	Egypt
17	Bent-ashaa	Sharqiya(elkoreen)	Soft	Egypt
18	Aglany	Sharqiy(elkoreen	Semi-Soft	Egypt
19	Hayani 2	Sharqiya (elkoreen)	Soft	Egypt
20	Khadrawi	KanaterKhairia	Semi-dry	Iraq
21	OmalDehn	KanaterKhairia	Semi-dry	Iraq
22	Nabotseaif	KanaterKhairia	Semi-dry	Iraq
23	Halawi	KanaterKhairia	Soft	Iraq
24	Galbi	KanaterKhairia	Soft	Iraq
25	Avanda	KanaterKhairia	Semi-dry	Iraq
26	Hayani 3	KanaterKhairia	Soft	Egypt
27	Zaghlool 3	KanaterKhairia	Soft	Egypt
28	Samany 3	KanaterKhairia	Soft	Egypt
29	Amhat 3	KanaterKhairia	Soft	Egypt
30	Hayani 4	Arish	Soft	Egypt
31	Maghal 4	Arish	Semi-dry	Egypt
32	Maghaltamr	Arish	Semi-dry	Egypt
33	Khalas	Ismailia (Al-RAJHI)	Soft	Saudi Arabia
34	Barhiisoidy	Ismailia (Al-RAJHI)	Soft	Saudi Arabia
35	NabtetSoltan	Ismailia (Al-RAJHI)	Semi-dry	Saudi Arabia
36	Sakey	Ismailia (Al-RAJHI)	Semi-dry	Saudi Arabia
37	Samany 5	Ismailia (Al-RAJHI)	Soft	Egypt
38	Zaghlool 5	Ismailia (Al-RAJHI)	Soft	Egypt
39	Medjool	Ismailia (Al-RAJHI)	Semi-Soft	Saudi Arabia
40	Hayani 5	Ismailia (Al-RAJHI)	Soft	Egypt
41	Agwet –Almadina	Ismailia (Al-RAJHI)	Soft	Saudi Arabia
42	Zaghlool 6	Rasheed	Soft	Egypt
43	Samany 6	Rasheed	Soft	Egypt
44	Hayani 6	Rasheed	Soft	Egypt
45	Araibee	Rasheed	Soft	Egypt

DNA Isolation:

For DNA extraction, three young leaves were collected from each adult tree and three plants per cultivar were subjected to molecular 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). A weight (0.2 g) from young leaves were ground in liquid nitrogen to fine powder and extracted using 10 ml preheated (65º C) cetyl hexadecyl-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 verified by Nano drop spectrophotometer model ND1000. DNA samples were then stored at 4º C. DNA samples of each cultivar were analyzed individually to detect intra-cultivar variations and bulked to detect inter-cultivar variations.

DNA amplification and PCR Conditions:

An initial screening of 35 SSR primer pairs (Successfully utilized in other date palm genotypes, was performed in order to test their readability and amplification profiles for polymorphism. After this screening procedure, 33 SSR primers were selected (Table 2), and these primers were synthesized by Oligo Macrogen, Seoul, Korea.

PCR reaction was 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 Master Cycler Gradient Eppendorf, Hamburg, and 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.

Primers used in the SSR analysis:

Thirty-three specific primers were used in this study; fourteen of these microsatellite markers selected from (Billotte *et al.,* 2004), and the others microsatellite markers issued by WCMC-Q was download from this web site address: http://qatar-weill.cornell.edu/research/datepalmGenome/download.html

These sequence file is named as (pdactyKAssembly1.0.fasta – 329328KB) and contained 271804 fast sequence clones.

Data Analysis

The similarity matrix was used in the cluster analysis. The cluster analysis was used to organize the observed data into meaningful structures, that is, to develop taxonomies. At the first step, when each genotype represents its own cluster, the distances between these genotypes defined by the chosen distance measure (Jaccard coefficient). However, once several genotypes have been linked together. All those analyses were computed by the program SPSS version 16.0.

Allelic composition of each genotype and the number of total alleles was determined for each SSR locus. Putative alleles were indicated by the estimated size in bp. The genetic information was assessed only for single loci SSRs using the following parameters: Observed number of alleles per locus (na), counts the number of alleles with nonzero frequency. The observed heterozygosity (Ho, direct count), expected heterozygosity (He) and polymorphic information content values for each locus (PIC) were calculated as follows:

He or PIC = 1- $\sum$ pi2 where pi is the frequency of the ith allele, and summation extends over n alleles (Nei, 1973), effective number of alleles (Ne) = (1/1-He), and heterozygosity level of Date palm genotypes assayed.

The computations were performed with the programs, GENEPOP version 1.31 (Raymond and Rouset, 1995), Quantity one, Irfanview and Microsoft Excel.

RESULTS AND DISCUSSION

Genetic variability at microsatellite loci

In order to reduce the number of samples processed, DNA or plant material of several individual plants may be combined into a single bulked sample. Thus, a certain loss of information of rare alleles has to be taken into account but on the other hand, bulked samples accumulate population specific markers (Doris, 2006). Since, a genotype is considered as a group of individuals that has been selected for expression of specific traits in a background of otherwise randomly distributed genetic variation. Therefore, the reason has led us to use bulk, instead of individual.DNA to characterize the date palm tree cultivars bulk sample of 3individuals in each geographic site may be expected to represent the markers linked to these traits (Yang and Quiros, 1993).

The results of using 35 SSRs markers developed for date palm (Billotte *el at.*, 2004) gave successful amplification across the 45 genotypes excluding loci mpdCIR044 and locus mpdCIR015 which produced smear bands with an unclear major product size. These loci (mpdCIR044 and mpdCIR015) were discarded in data analysis. In this case at least the two following hypotheses could be forward to explain this: 1- the appropriate complementary microsatellites sequences are in frequent in the date palm genome. 2- the corresponding microsatellites sites are distantly located

in date palm DNA in such a way that no amplification occurred.

Unfortunately, some primer pairs succeed in amplifying in some genotypes while fail in other genotypes, suggesting that null alleles exist. Null alleles might be some mutations, including the deletion of microsatellites, and indels or substitution in primer binding sites (Varshney, *et al*., 2005). However, null alleles may also be more common, leading to an underestimation of heterozygosity (Al-Ghaliya, 2013).

However, Date palm SSR markers revealed higher levels of genetic polymorphism in present study. 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). All 33 loci were successfully amplified and a total of 1067 alleles were detected (Table3) Dpalm-113, mpdCIR048 and 230 showed the highest number of alleles per locus (51) while Dpalm-103 showed the lowest (7) with mean of 32.3 allele. The total number of alleles detected in this study was also higher than those found by Zehdi *et al.* (2004), Bodian *et al.* (2012), who scored 100 and 107 alleles, respectively.

Table (2): A list of 35 SSR loci used in the present study.

No.	Locus	SSR primers sequence 5---»3	No.	Locus	SSR primers sequence 5---»3
1	DPALM_100	F: GCCACTATCACCATTGCTGT	19	DP 175	F: ACACACACACACACACACACC
		R: CAATGGAGGTCGTAGTGGTG			R: GTGGCTTCTTTTTGGCTGTC
2	DPALM_103	F: TTCCATCCCTGGAGAAAGG	20	mpdCIR010	F: ACCCCGGACGTGAGGTG
		R: AACCAAGACATCGTCCCAAG			R: CGTCGATCTCCTCCTTTGTCTC
3	DPALM_104	F: GGAAAGTTTCGGAACATTTTGT	21	mpdCIR015	F: AGCTGGCTCCTCCCTTCTTA
		R: AACCCAACCTAAGCCCTACC			R: GCTCGGTTGGACTTGTTCT
4	DPALM_107	F: GGAAGGCGTCAAGGTATCTC	22	mpdCIR016	F: AGCGGGAAATGAAAAGGTAT
		R:ACAACACGGGGAAAGAACAT			R: ATGAAAACGTGCCAAATGTC
5	DPALM_110	F: TGTCACATTTGAGCATAATCCA	23	mpdCIR032	F: CAAATCTTTGCCGTGAG
		R: ACCCTTTGTTGATGCACCTC			R: GGTGTGGAGTAATCATGTAGTAG
6	DPALM_112	F: AGCAGGTTCATGGTTTGCTT	24	mpdCIR035	F: ACAAACGGCGATGGGATTAC
		R: AGAACCAGGGAGGATGAGGT			R: CCGCAGCTCACCTCTTCTAT
7	DPALM_113	F: GGTCCCGACGCCTATTTTAT	25	mpdCIR044	F: ATGCGGACTACACTATTCTAC
		R: AGCAAAGTCCACCCCTTTTT			R: GGTGATTGACTTTCTTTGAG
8	DPALM_119	F: TGCGCTAAATAGTTCCCTTCA	26	mpdCIR048	F: CGAGACCTACCTTCAACAAA
		R: CACATTCACAAGGCCTGCTA			R: CCACCAACCAAATCAAACAC
9	DPALM_120	F: TTCAATTCATCCCACTGCAA	27	mpdCIR050	F: CTGCCATTTCTTCTGAC
		R: CACCAACATGAGCAAATGGA			R: CACCATGCACAAAAATG
10	DP 151	F: TTGCTGGTTGAAATGGTGTT	28	mpdCIR057	F: AAGCAGCAGCCCTTCCGTAG
		R: GCAACAGATGCTCTTGCTCA			R: GTTCTCACTCGCCCAAAAATAC
11	DP 157	F: TGGACAATGACACCCCTTTT	29	mpdCIR063	F: CTTTTATGTGGTCTGAGA
		R: GCCCACACAACAACCTCTCT			R: TCTCTGATCTTGGGTTCTGT
12	DP 159	F: AGCTCCAATTTGCTGCAGAG	30	mpdCIR070	F: CAAGACCCAAGGCTAAC
		R: GCTGACCTGGAGTCCAAAAC			R: GGAGGTGGCTTTGTAGTAA
13	DP 160	F: AAGAGCGACAATCATGACCA	31	mpdCIR078	F: TGGATTTCCATTGTGAG
		R: GGAAATTGAAGGGCATCTTG			R: CCCGAAGAGACGCTATT
14	DP 168	F: GCAGCAAAAGCCCTTAGGC	32	mpdCIR085	F: GAGAGAGGGTGGTGTTATT
		R: GGTGTTATGTGCAGCCAATG			R: TTCATCCAGAACCACAGTA
15	DP 169	F: GCATGGACTTAATGCTGGGTA	33	mpdCIR090	F: GCACGAGAAGGCTTATAGT
		R: GGTTTTCCTGCCAACAACAT			R: CCCCTCATTAGGATTCTAC
16	DP 170	F:TCTTTGGGCTTACGACAACC	34	mpdCIR044	F: ATGCGGACTACACTATTCTAC
		R: GTATGGCCCAAGATGCAGAT			R: GGTGATTGACTTTCTTTGAG
17	DP 171	F: GTGGGAGTAGCGAGGTAT	35	mpdCIR015	F: AGCTGGCTCCTCCCTTCTTA
		R: GTCCGGCACTTTAGGAAGTT			R: GCTCGGTTGGACTTGTTCT
18	DP 172	F: ACCCCGGACGTGAGGTG			
		R: CGTCGATCTCCTCCTTTGTCTC			

A low number of alleles of Dpalm-103 locus was detected It may suggest that this locus concentrated in preserved regions, with low mutation rate. According to Pavia *et al.* (2014), the number of alleles per locus is related to the number of replicates in the microsatellite, which explains the polymorphism found in the three loci (Dpalm-113, mpdCIR048 and 230) that presented a greater number of alleles. Therefore, the results revealed that for a few markers (Dpalm-104, Dpalm-107 and Dpalm-110) a narrow range of variation was found in the number of observed alleles. When this situation is found the most frequent alleles are likely to be the oldest, the other being the results of mutation process through insertion-deletion mechanisms However, the level of variation depicted by the number of alleles at each locus serves as a measure of genetic variability having direct impact on differentiation of breeds within the populations.

In this study, mpdCIR SSRs usually had more alleles than Dpalm loci as they were previously reported (Hamwieh *et al.*, 2010). This could probably be due to differences in the SSR origins. According to Billotte *et al.* (2004), mpdCIR markers might be extracted from either small-insert genomic libraries or bacterial artificial chromosome end sequences. These loci therefore were more likely to include non-coding regions than the Dpalm SSRs that are mainly developed from low-copy RFLP probe sequences located primarily near or in genes (Hamilton *et al.,* 1999).

However, the number of alleles per locus detected in this study was higher than those scored by Elmeer and Mattat (2012) who recognized 8.86 alleles per locus when examining 34 date palm cultivars grown in Qatar using 14 microsatellite loci. It may be a result of using a greater number of microsatellite loci (33) in addition to using different genotypes-45 Egyptian date palm accessions in this study. Thompson *et al.* (2009) and Kaushik *et al.* (2011) confirmed that the number of alleles detected by a single SSR locus varied from 1 to 31 depending upon the fingerprinting techniques and materials used in the studies.

Effective number of alleles (Ne) is the measure of allelic evenness. In this study, the results showed that the effective number of alleles (Ne) for the polymorphic markers ranged between 2.81, for Dpalm-103, and 23.29, for Dpalm-100A, with average value of 12.85. The total number of effective alleles produced by the 33 SSR loci was 424.14. According to the selective standard of the microsatellite loci, it ought to have at least four alleles to be considered useful for the evaluation of genetic diversity. Bases on this criterion, the 33 microsatellite loci used in this study were useful for the evaluation of genetic diversity in 45 date palm genotypes. These results imply that abundant genetic polymorphism exist in date palm cultivars.

In Table (3), as expected, the range of sizes amplified by each primer pair across different date palm genotypes was considerable (73bp in Dpalm-103 up to 4689 bp size range in mpdCIR035). This difference was probably due to particular set of loci test. Actually, in the case of such widely divergent sizes, the actual number of nucleotides in the allele would need to be established by sequencing. There are several reports that

the allelic variation might correlate with the number of repeats within a particular locus (Cole, 2005). However, the allele size is not only dependent on the number of nucleotides but there are several factors affecting the allele size including: the mobility of the fragment in the electrophoresis, the distance of the allele from the standard used, the type of fluorescent label used, and the use of different instruments using different software (Stewart *et al.*, 2011).

For some SSR loci, the bands observed in this study were larger than the expected size, based on the predicted location of the primers on the sequence in previous reported in studies of the same loci in other date palm cultivars (Bodian *et al.*, 2014). Since the primers for PCR were based on cDNA from sequences while the PCR reaction for amplification of SSR was carried out on genomic DNA, these larger bands most likely indicate the presence of introns in the corresponding genomic regions (Rui Liu *et al.,* 2013) and/or variation in the repeat numbers. This suggests that the date palm genotypes studied may be exceptionally polymorphic providing more size variation with 45 genotypes to other studies (Elmeer and Mattat, 2012). This could also be due to the inclusion of interracial cultivars in this study.

Our study confirms that the mentioned microsatellite markers are able to generate higher number of allele per locus. The markers produced higher number of alleles could have better application to find out the polymorphisms in heritability of date palm cultivars.

Generally the mean number of alleles is highly dependent on the sample size because of the unique alleles in populations, which occur in low frequencies and also because the number of observed alleles tends on the population size. All 1067 individuals in 45 date palm genotypes were considered in this study.

Heterozygosity refers to the presence of different alleles at one or more loci on homologous chromosomes. Heterozygosity per locus was 0.64 (Dpalm-103) to 0.96 (Dplam-100, 256, mpdCIR08 and mpdCIR078) with an average of 0.89 (Table 3). In this study, heterozygosity values were high except for the locus ssr Dpalm-103. The heterozygosity observed at some of the loci could also be due to high mutational rate and mutational bias at SSR loci. The loci with large number of repeat units (SSR units) tend to show high mutational rate. As a result, any mutations in any one of the alleles may create a heterozygous condition (Bharathi, 2011). The measure of level of heterozygosity across loci can be used as an indicator of the amount of genetic variability (Zulkifli *et al.,* 2012). However, Allelic diversity and heterozygosity are important features for the establishment of microsatellite markers for linkage studies (Raffaella *et al.*, 2002).

Polymorphism Information Content (PIC) values in Table (3), were quite high and varied (range 0.64 to 0.96, average value 0.89) considerably as an estimate of the discriminatory power to study the degree of gene variance among 33 SSR loci in the present study. This broad range of PIC values was indicative of the

presence of unique alleles in some cultivars which facilitates their differentiation from another.

The lower PIC value (0.64) observed for Dpalm-103 locus can be attributed to the concentration of gene frequencies, which leads to deviation from the condition of maximum information content of a locus. This occurs when all alleles have similar frequencies (Paiva *et al.,* 2014). MpdCIR048 and 230 loci had higher PIC value (0.96) than locus Dpalm-113 (0.87) for the similar number of alleles (51). This result indicated that PIC values depend not only on the number of alleles but also shared frequencies of those alleles (Smith *et al.,* 2000). Generally, PIC values increased proportionally with increasing heterozygosity at a locus.

The mean PIC value of 0.89 reflected the high level of polymorphisms of the used set of microsatellites

and heterogeneity in 45 date palm genotypes. These PIC values are in a slightly higher compared to other studies which mean of PIC value was 0.66 (Elmeer and Mattat, 2012). This difference might be linked with selection of different markers and more diverse set of cultivars.

However, the high estimates of PIC further substantiated the suitability of the used set of markers to applications such as linkage-mapping programs in addition to genetic studies in Egyptian date palm. The high average number of alleles per locus (na = 32.3), heterozygosity value per locus (He = 0.89), and polymorphism information content (PIC = 0.89) confirmed that these SSR markers are useful tools to detect genetic variability of date palm germplasm.

Table (3): Descriptive statistics and genetic diversity of Egyptian date palm genotypes and accessions at thirty three microsatellite loci

SSR loci	N	(*na)	Ne	Alleles size rang	He	Pic
Dpalm_100	45	42	23.2975	19 – 359	0.96	0.957
Dpalm_103	45	7	2.8106	24 – 97	0.64	0.644
Dpalm_104	45	11	5.4173	24 – 225	0.82	0.815
Dpalm_107	45	12	4.9469	55 – 4531	0.80	0.798
Dpalm_110	45	13	4.6084	52 – 728	0.78	0.783
Dpalm_112	45	24	7.3911	46 – 2044	0.86	0.865
Dpalm _113	45	51	7.6650	26 – 3925	0.87	0.870
Dpalm_117	45	28	7.3842	46 – 3310	0.86	0.865
Dpalm_119	45	25	10.6913	23 – 3060	0.91	0.906
Dpalm_120	45	20	9.0023	31 – 4036	0.89	0.889
DP151	45	16	9.2860	43 – 4577	0.89	0.892
DP157	45	41	13.7041	22 – 4335	0.93	0.927
DP159	45	34	14.6602	19 – 4009	0.93	0.932
DP160	45	14	7.3414	40 – 748	0.86	0.864
DP168	45	20	7.3934	46 – 3236	0.86	0.865
DP169	45	21	9.0482	35 – 232	0.89	0.889
DP170	45	36	12.2650	46 – 1283	0.92	0.918
DP171	45	15	5.3581	61 - 3156	0.81	0.813
DP 172	45	28	7.5852	37 - 3310	0.87	0.868
mpdCIR035	45	44	26.1514	32 – 4343	0.96	0.962
mpdCIR015	45	39	20.7804	50 – 4515	0.95	0.952
256	45	47	22.6527	20 – 4449	0.96	0.956
269	45	33	10.0416	22 – 433	0.90	0.900
mpdCIR035	45	49	18.1165	28 – 4707	0.94	0.945
mpdCIR057	45	49	14.6867	23 – 2066	0.93	0.932
mpdCIR08	45	35	22.6992	23 – 4410	0.96	0.956
mpdCIR032	45	44	20.9514	51 – 4410	0.95	0.952
mpdCIR048	45	51	20.6647	21 - 885	0.95	0.952
mpdCIR070	45	46	17.5896	18 – 4515	0.94	0.943
mpdCIR078	45	50	22.2262	20 – 448	0.96	0.955
mpdCIR063	45	23	5.6403	40 – 4135	0.82	0.823
mpdCIR044	45	48	12.6183	24 – 4378	0.92	0.921
230	45	51	19.4676	17 - 4460	0.95	0.949
Total		1067	424.1428		29.433	29.458
Mean		32.3	12.85		0.892	0.893

Genetic Variability within Genotypes

Heterozygosity is an appropriate measure of genetic variability within cultivars because genetic diversity can be measured as the amount of actual or potential heterozygosity. Table (4) showed that the highest heterozygosity (0.768) was detected in "Avanda" cultivar, followed by "Amhat" (0.758). This finding seems to indicate the two cultivars have had greater than average external gene flow, which is likely the cause of the excess heterozygosity (Bodian *et al.*, 2012). "Khalas" cultivar had lowest heterozygosity value (0.455). This suggests that Saudi cultivar may be, to some extent, isolated and has not received free external gene flow. *P. dactylifera* is an obligate out-crossing species and its recessive alleles tend to be maintained in a heterozygous state, as it was observed in Khalas (Al-Mssallem *et al.,* 2013). The weak differentiation among heterozygosity values of Zaghlool cultivars from different regions as noted in this study could be attributed to transit of date palm via human practices coupled with date seed between regions.

However, high levels of heterozygosity of Egyptian originated genotypes were observed in this study ranging from 0.515 to 0.727 for each marker. The high level of heterozygosity for these populations could be explained by one of the following reasons: long-term natural selection for adaptation, yield and quality or the mixed nature of the populations and historic mixing of individuals of different populations (Al-Ghaliya, 2013). As reported by Kimani *et al.* (2014) the differences in the genetic diversity could be attributed to traditional farming systems with agronomic, economic and cultural considerations that foster high levels of genetic diversity.

Table (4): Heterozygosity values of 45 date palm genotypes and accessions assayed with 33 SSR loci.

	Genotypes	Heterozygosity	Num.	Genotypes	Heterozygosity
1	**Deglet Noor**	0.697	24	Galbi	0.727
2	**Malkabi**	0.727	25	Avanda	0.768
3	**Bartamoudawardy**	0.727	26	Hayani	0.545
4	**BartamoudaAdia**	0.636	27	Zaghlool	0.697
5	**Balady**	0.515	28	Samany	0.606
6	**Shamiya**	0.727	29	Amhat	0.758
7	**Sakkoty**	0.667	30	Hayani	0.576
8	**GondailaAdia**	0.606	31	Maghal	0.697
9	**Gondailawardy**	0.485	32	Maghaltamr	0.636
10	**Maghal**	0.697	33	Khalas	0.455
11	**Samany**	0.606	34	Barhiisoidy	0.727
12	**Zaghlool**	0.576	35	NabtetSoltan	0.636
13	**Kabooshy**	0.636	36	Sakey	0.606
14	**Amry**	0.606	37	Samany	0.636
15	**Hayani**	0.606	38	Zaghlool	0.667
16	**Amry**	0.606	39	Medjool	0.697
17	**Bent-ashaa**	0.667	40	Hayani	0.515
18	**Aglany**	0.576	41	Agwet -Almadina	0.545
19	**Hayani**	0.515	42	Zaghlool	0.606
20	**Khadrawi**	0.545	43	Samany	0.576
21	**OmalDehn**	0.667	44	Hayani	0.667
22	**Nabotseaif**	0.576	45	Aloraiby	0.576
23	**Halawi**	0.727			

Genetic Structure for date palm genotypes

The genetic structure reflects interactions among cultivars with regard to their long-term evolutionary history, mutation and recombination, genetic drift, reproductive system, gene flow, and natural selection (Slatkin, 1987 and Schaal *et al.*, 1998). Thus, an understanding of the level and structure of the genetic diversity of a date palm is a prerequisite for the conservation and efficient use of the germplasm available for breeding (Laidò *et al*., 2013).

To further elucidate the relationships among 45 date palm genotypes, based on the results, a cluster analysis of the distance matrices based on an UPGMA algorithm was used to generate a dendrogram.

The genetic similarity ranged from 0.041 to 0.260, (data not shown). The highest similarity was observed between "Bent-ashaa" and "Hayani"/Al-raghy farm means that they are genetically the closest. It is possible over the years that many date palm cultivars have been transplanted to areas other than the area of their origin, and there may have been adapted with different names (Al-Khalifah and Askari, 2003). While "Khalas" and "Hayani/Kasaseen" had the smallest similarity value, means that they are the most genetically distance. As date palm is an obligate out-crossing species, cultivars will not be of identical genotype unless they are clonally derived from the same original palm (Al-Ruqaishi *et al.*, 2008).

Unsurprisingly, genetic similarity between the date palm cultivars was not comparatively high. Thus, SSR markers provide adequate power of resolution to discriminate between date cultivars and it could serve as a potential tool in the identification and characterization of genetically distant cultivars from various sources.

In the present study, UPGMA dendogram indicated segregation of 45 date palm genotypes into three main clusters (Figure 1). NabotSeaf and UmAldehn formed a separated cluster (cluster1). Whereas, cluster 2 consisted of three genotypes (Khalas, Khadrwi and NabotSoltan). NabotSoltan was most distance.
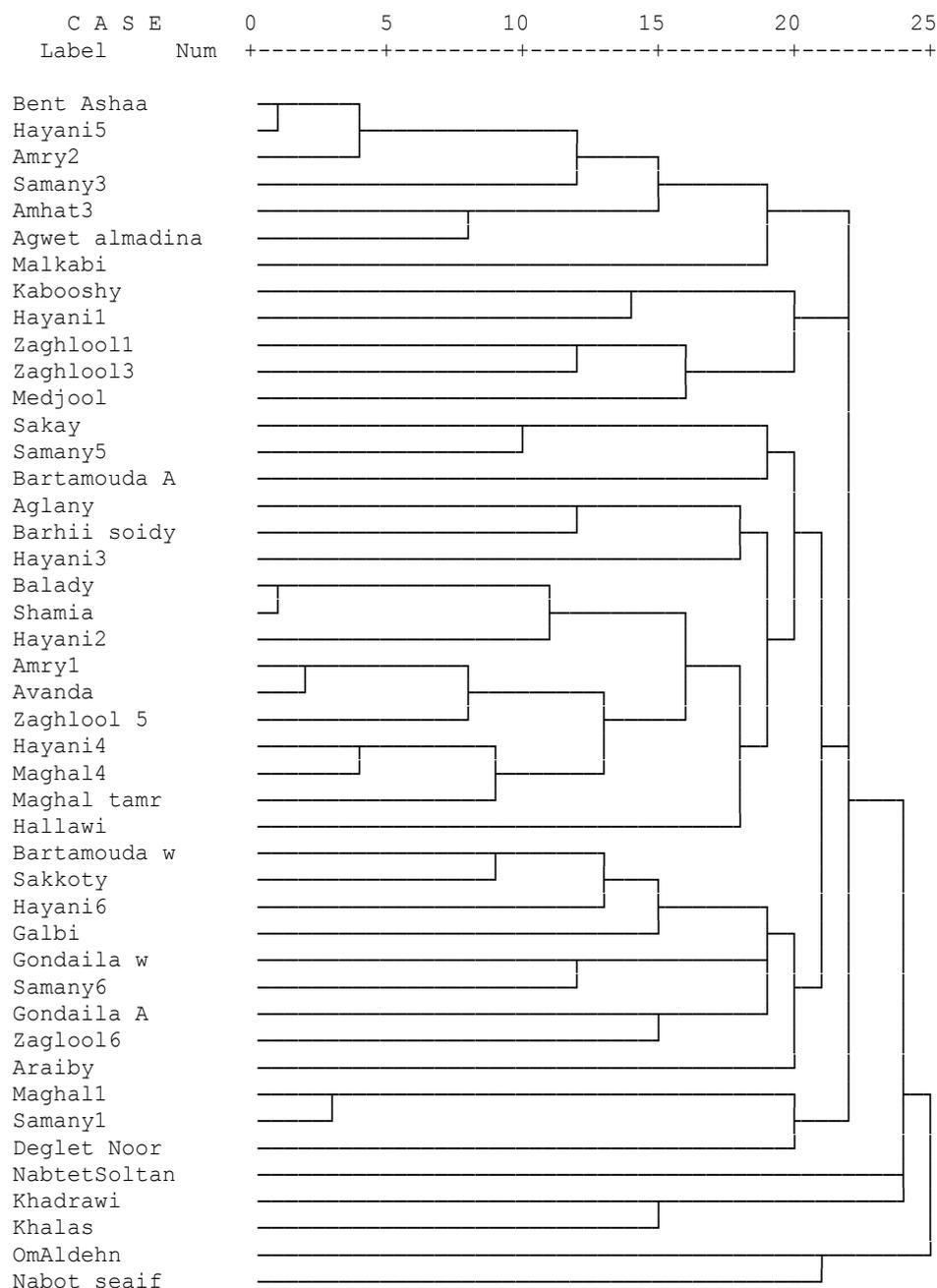
Figure (1): Dendrogram for forty five date palm cultivars and accession constructed from SSRs data based on average Linkage (Between Groups), using Similarity computed according to Jaccard's coefficient, with 1067 fragments.

Cluster 3 contained three main sub clusters. The first sub cluster consisted of Deglet Noor and other two genotypes namely, Samanay/ElKasasen and Magahal/ElKasasen were placed close to each other. Second sub cluster divided to two groups. First group contained 9 genotypes (Araibee, Zaghlol/Rashed, Gondila, Samany/Rashid, Gondilia Ward, Galbi, Hayani/Rahid, Sakkoty and Bartmotward). Whereas, Second group divided into further two sub clusters. First one contained ten genotypes (Halawi, Magahal, Magahal/Arish, Hayani/Arish, Zaghlol/ElKassen, Avanda, Amry/Elakassen, Hayany/Shriquia, Shamia and Baldy). Here, it was observed Magahal/Arish and Hayani/Arish were closed to each other. Avanda, Amry/Elakassen were also closed to each other and the same attitude with Shamia and Baldy. Other group contains five genotypes namely, Hayani/Kanater, Bahri, Aglany, Samany/Al-raghy and Saky. Third sub cluster contained the all remaining genotypes and divided into two groups. Madjool, Zaghlol/Kanter, Zaghlol/El-raghy, Hayani/Kasasen and Kabooshy formed together the first group. It was observed Zaghlol/Kanter, Zaghlol/El-raghy were placed close to each other. The other group contained Malkabi, AgwatAlmadaina, Amaht/Kanter, Samany/Kanater, Amry/Shriquia, Hayani/Rashid and BentAsha. The two genotypes Hayani/Rashid and BentAsha were closed to each other. Although some genotypes displayed different levels of dissimilarity but still were grouped with each other's. It is suggested that the variation or polymorphism of SSRs are a result of polymerase slippage during DNA replication or unequal crossing over (Levinson and Gutman, 1987).

However, dendrogram showed that genotypes grouping in relation to their geographical origin are not well defined. Consequently, since all date palm ecotypes are originated by hybridization, it may be assumed that they have a common genetic basis. Nevertheless, varieties diverged from others by mutational events that arise during selection (Zehdi *et al.*, 2004). According, Al-Qurainy *et al.* (2011) revealed that eight cultivars grouped into three clusters, Based on Chloroplast DNA Sequences rpoB and psbA-trnH. The cultivars Khodary, Sefri, Ajwa, Ruthana and Hilali clustered together (ii) the cultivars Sukkari and Khalas are clustered together and (iii) the sequence pattern of Segae was found different from the other cultivars.

In fact, a dendrogram typically denotes the genetic relationship among individuals in a population and may reflect the evolutionary history of the species if the population sample is representative enough (Tran, 2005). However, in this study the population was sampled from plantations rather than a wider gene pool, so the dendrogram was merely employed to assess the structure of genetic variation within the sample population, not to infer any evolutionary relationship. Concluding, the use of molecular markers, like microsatellite, is imperative to build a database for cultivar analysis, and for the appropriate management of date palm germplasm collections. However, this study, using microsatellite markers on Egyptian date palm genotypes and accessions showed considerable genetic diversity existing among the population. This is most likely to different condition under which the populations are grown and conserved. Evaluation of genetic diversity among germplasm, particularly of crops, is crucial in utilization genetic potential to improve traits needed for adaption to various conditions. Moreover, the results presented here regarding clustering are extremely useful for the selections of genotypes to be used for breeding programs and thus ensuring an optimal management of Egyptian palm date germplasm collection.

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تحليل التنوع الوراثي في نخيل البلح باستخدام معلمات الميكروستالليت

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تهدف الدراسة الحالية إلى تحليل الاختلافات الوراثية والعلاقات الوراثية لبعض أصناف نخيل البلح والتراكيب الوراثية المنزرعة في مناطق مختلفة من مصر. باستخدام المعلمات الجزئية المعروفة بالتتابعات المكررة البسيطة أوالميكروستالليت. استخدم ٣٥ زوجا من البوادئ المتخصصة لتحليل الاختلافات الوراثية الجزئية والعلاقات بين ٤٥ تركيب وراثي لنخيل البلح وللتمييز بينهم، تم اختيار ٣٣ زوج من البوادئ كلهم أعطوا حزم واضحة وثابتة، حيث كان العدد الإجمالي لهذه الحزم ١٠٦٧ حزمة أو (اليل) بمتوسط ٣و٣٢ ومتوسط عدد الأليلات الفعالة ١٢.٨٢ وتراوحت قيم التباين الوراثى للمواقع بين (٠.٦٤) للموقع DP103 و (٠.٩٦) للموقع DP100 بمتوسط (٠.٨٩). فى حين كانت قيمة محتوى معلومات التباين (PIC VALUE) منخفضة (٠.٦٤) للموقع الوراثى DP103 بينما كانت للمواقع pdCIR048 و mpdCIR016 عالية (٠.٩٦) وهذا يعنى القدرة العالية للمعلمات الجزيئية المختارة في التمييز بين الأصناف والتراكيب الوراثية تحت الدراسة.

تم دراسة التباين الوراثي بين خمسة وأربعين صنف نخيل بلح باستخدام معلمات الميكروستالليت أو التتابعات المكررة البسيطة. حيث تراوحت قيم التباين بين (٠.٤٥٥ : ٠.٧٦٨) حيث كانت ٠.٧٦٨ في الصنف أفندا يليه الصنف أمهات ٠.٧٥٨. وكان الصنف خلاص اقلها قيمة في التباين الوراثى ٠.٤٥٥. وكانت أعلى قيمة لمعامل التشابه (أو درجة التشابه) هي (٠.٢٦) بين بنت عيشة و حيانى (الرجحى) بينما كانت أقل قيمة لمعامل التشابه هي (٠.٤١) بين خلاص و حيانى(القصاصين).

وأظهرت نتائج التضاعف للـ ٣٣ موقعا من التتابعات المكررة البسيطة للحامض النووي دنا إمكانية التمييز الكامل لكل أو معظم أصناف نخيل البلح تحت الدراسة بواسطة حزمة منفردة أو أكثر. حيث تم تحديد عدد إجمالي ٤٢٨ كشاف متخصص (حزمة منفردة) بواسطة كل معلمات التتابعات المكررة البسيطة (الميكروستالليت)، جميعهم كواشف متخصصة موجبة. وكانت مفيدة ككشافات متخصصة لتحديد وتمييز الأصناف تحت الدراسة. وتم تسجيل أكبر عدد من الكشافات المتخصصة مع الصنف حيانى حيث تم تحديد ٥٩ كشافاً متخصصاً يليه صنف سمانى (تم تحديد ٣٨ كشاف). بينما كان أقل عدد من الكشافات المتخصصة (ثلاثة كواشف) تم تسجيله في صنف جنديلة وردى مع البوادئ DP160_4009-pdCIR063_110-mpdCIR063_422 وقد كانت جميع ال ٤٢٨ حزمة منفردة والمسجلة مع معلمات الميكروستالليت عبارة عن كشافات متخصصة موجبة.