Characterization of genetic diversity of Date palm (*Phoenix dactylifera* L.) cultivars collected from New Valley governorate (El-Kharga and Dakhleh) based on morphological variability and molecular markers

Ibrahim A. Ibrahim[1], Medhat H. Hashem[1], Alaa A. Hemeida[1]*, Mona M. Hassan[2] and Ahmed I.A. Maksoud[1]

[1.] Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Sadat City, Egypt
[2.] Central Laboratory of Date Palm for Research and Development, Agriculture Research Center (ARC), Egypt
*alaa.hemeida@gebri.usc.edu.eg

Abstract: The present work was designed to study the genetic back ground of three date palm (*Phoenix dactylifera* L.) cultivars (Siwi, Tamr, Hegazi) and one unknown female (Faleg, has high quality and desirable traits) which were collected from New Valley Governorate (El-Kharga and Dakhleh). To achieve this purpose, morphological variability; RAPDs; SSRs and AFLPs technologies were applied. Moreover, through the obtained data, the genetic relationships between the cultivars were determined. Also, for each cultivar, different genomic markers were identified. In addition, some specific markers for certain cultivars were screened. These results indicated that each cultivar has its own genetic makeup at the level of coding sequences. Concerning the data of the three DNA-markers, considerable genetic diversity for coding and non-coding sequences was indicated among the genomes. However, each technology exhibited different level of polymorphism and unique markers. This feature may be attributed to the limited number of AFLPs selective primers used in the present analyses; the amplification of different parts of the genomes or/and the reliability of each technique to react with Date palm genomes. SSRs were the most effective method for assessing the genetic diversity and the unique DNA-markers across the four Date palm genomes. The dendrograms of the three applied DNA techniques were partially different. Therefore, the data of RAPDs, SSRs and AFLPs analyses were combined to estimate the genetic relationships among the cultivars under study.

[Ibrahim IA, Hashem MH, Hemeida AA, Hassan MM and Maksoud AIA. **Characterization of genetic diversity of Date palm (*Phoenix dactylifera* L.) cultivars collected from New Valley governorate (El-Kharga and Dakhleh) based on morphological variability and molecular markers.** *Life Sci J* 2014;11(11):879-889]. (ISSN:1097-8135). http://www.lifesciencesite.com. 158

Keywords: Date palm; Genetic similarity; Molecular marker; Morphological analysis

1. Introduction

Date palm *(Phoenix dactylifera L.*) is a dioecious perennial monocotyledon plant that belongs to *Arecaceae* family. It is originated in Mesopotamia and thousands of cultivars have been reported. It is the main source of income of oases inhabitants and creates favorable conditions for improving secondary crop culture like barley, alfalfa and clover as forage. It's a common staple food in the Middle East and North African regions as well as many other tropical and subtropical regions (Amy *et al.*, 2012). In Egypt, the annual date production is on average 1373,570 metric tons. Date palms is a diploid (2n = 2x = 36), and the predicted genome size is estimated to be approximately between 550 and 650 Mbp long (Malek, 2010).

Date palm varieties can be differentiated using morphological markers viz., shape, size, weight, color, aspects of fruit skin, consistency, texture, *etc*. (Salem *et al.*, 2008). These characters are known to be strongly affected by environmental conditions and have limited discriminatory power. Accordingly, this has led to some cultivars with similar morphological characters being given the same varietal name. In addition, these characters have a strong genetic control when the environment component is discarded (Hamza *et al.*, 2011).

Worldwide, many markers have been used to identify almond genotypes such as RAPD (Random Amplified Polymorphic DNA) is possibly the simplest test of all recently applied DNA-based tests for date palm identification (Trifi *et al.,* 2000). AFLP (Amplified Fragment Length Polymorphism) provides an effective, rapid and economical tool for detecting a large number of polymorphic genetic markers that are highly reliable and reproducible, and are able to be genotyped automatically. The AFLP technique has been used extensively to detect genetic polymorphisms, evaluate and characterize breed resources, construct genetic maps and identify genes (Vos *et al.,* 1995).

Microsatellites or SSRs (Simple Sequence Repeats) are simple sequences made of short pattern of 1 to 6 nucleotides repeated in tandem, found in most genomes, which show exceptional variability in most species (Billotte *et al.*, 2004). The variability has made SSRs the genetic marker of choice due to their abundance, polymorphism and reliability compared to other types of DNA markers and for the vast majority of applications, including fingerprinting, analysis of

genetic structure and for investigating evolutionary links between species and populations. Codominant markers like SSRs are very useful to identify date palm cultivars (Rhouma, *et al.,* 2011).

Consequently, to understand the genetic relationship among and within date palm varieties, RFLPs, RAPDs, ISSRs, SSRs and AFLPs markers have been used widely and efficiently to analyze the genetic diversity within and among date palm cultivars in many middle east countries such as Egypt (Saker *et al.*, 2006; Hemeida *et al.*, 2010), Oman (Al-Ruqaish *et al.,* 2008), Morocco (Sedra *et al*., 1998), Suadi Arabia (Al-Khalifah and Askari, 2003), Tunisia (Zehdi *et al.*, 2004 a, b) and Sudan (Elshibli and Korpelainen, 2009). These studies have permitted to identify markers suitable for identification of date palm varieties.

The objective of this work is therefore to analyze the genetic diversity of common cultivars of New Valley governorate (El-Kharga and Dakhleh) based on both morphological variability and molecular markers.

2. Material and Methods

New Valley Governorate is one of the governorates of Egypt. It is located in the southwestern part of the country, in the Libyan Desert section of the Sahara - between the Nile, northern Sudan, and southeastern Libya. The New Valley Governorate is Egypt's largest governorate and one of the biggest on the African continent (Fig. 1).

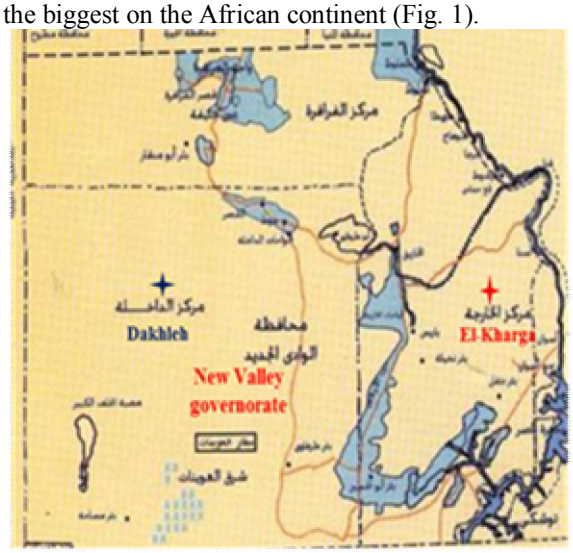

Figure 1. Distribution area of date palms in New Valley governorate

2.1. Plant material

The plant material consisted of three date palm cultivars and one individual unknown female were collecting from New Valley Governorate (El-Kharga and Dakhleh, Fig. 1). The cultivars used were: Siwi, Tamr, Hegazi and one individual unknown female from El-Kharga city (called Faleg or Meghel and has high quality and desirable traits). Cultivars were chosen for their good fruit quality and are the most common genotypes in the main plantations areas.

2.2. Measurement of the morphological traits

Different traits were measured in all collected samples of each variety and the average of each trait per variety was calculated (Hemeida *et al*., 2008). The morphological traits were Trunk (diameter, tree crown appearance); Frond (leaves); Leaf base; Leaflet (pinna); Spine; Sheath fiber; Fruit stalk (Peduncle) and Spikelet. Furthermore, thirteen different fruits traits were measured in all collected samples of each variety. These traits were: Varietal kind (texure); Khalal stage (color; shape and sweetness); Commercial stages/ variety; Earliness; Size; Average weight; Volume/cm^3; Density W/V ratio; Fruit quality index; Fruit skin (Epicarp) and Fruit cap (Perianth).

2.3. Total genomic DNA extraction

Genomic DNA was extracted through DNA isolation kit (Gene JET^{TM}, plant genomic DNA purification mini kit, Fermentas). To purify DNA from polyphenol, lysis Buffer was supplemented with polyvinylpyrrolidone (PVP) at 2% (W/V) final concentration. DNA was quantified by Gene quant spectrophotometer at absorbance of 260/280nm. The quality was further checked on 1% agarose gel.

2.4. RAPDs amplification

RAPD analysis was carried out using eighteen oligonucleotide primers (Table 1) that were selected from the Operon Kit (Operon Technologies Inc., Alabameda, CA). RAPD analysis was performed according to the method of Hemeida *et al*., (2010).

2.5. SSRs amplification

A set of 16 date palm specific SSRs primer pairs developed by Billotte *et al*., (2004) was tested (Table 1). SSRs reactions were carried out in 25µl containing 50 ng of DNA; 0.5µM of each primer and 12.5 µl of Maximo *Taq* DNA Polymerase 2X-preMix (Gene ON, Germany). PCR amplification was performed in a Biometra *T1* gradient thermalcycler for 35 cycles after initial denaturation for 5 min at 94°C. Each cycle consisted of denaturation at 94°C for 1 min; annealing at 55°C for 1 min; extension at 72°C for 1 min and final extension at 72°C for 10 min.

Amplification products for RAPDs and SSRs were separated on 1.2% agarose gels; stained with ethidium bromide; visualized with ultraviolet light and photographed. DNA fragment lengths were determined by comparisons with the 100 pb DNA ladder run on each gel.

2.6. AFLP amplification

AFLP procedure was applied according to Vos *et al.* (1995) with few modifications. Genomic DNA (500ng) was digested with two restriction enzymes (*Eco*RI and *Mse*I) for 14–16 h at room

temperature and ligated to double-stranded *Eco*RI and *Mse*I adapters. The ligates were pre-amplified with pre-selective primers using 1 cycle of 2 min at 72°C and 40 cycles, each consisting of 20 sec at 94°C; 30 sec at 56°C and 2 min at 72°C. A final cycle was performed for 30 min at 60°C. For the selectively amplifications, four sets of AFLP *Eco*RI and *Mse*I primers (Table 1) were used. For each reaction, 15µl super-hot start Master Mix; 1µl *Eco*RI primer and 1µl *Mse*I primer were mixed. The PCR program consisted of an initial warm at 94ºC for 2 min then one cycle at 94ºC for 20 sec; 66ºC for 30 sec and 72ºC for 2 min, followed by 10 subsequent cycles each at 1ºC and finally 25 cycles at 94ºC for 20 sec; 56ºC for 30 sec and 72ºC for 2 min. A final cycle was performed at 60ºC for 30 min. The PCR products were separated on 6% polyacrylamide gels; stained with silver staining and photographed. DNA fragment lengths were determined by comparisons with 100 pb DNA ladders run on each gel.

2.7. Data analysis

Phoretix electrophoresis gel image analysis, ID software was used for scanogram tracing of fragments size (bp). Data matrices were entered into the NTSYS (Numerical Taxonomic and Multivariate Analysis System) program, version 2.1, Applied Biostatistics Inc. (Rohlf, 2000). Similarity coefficients were used to construct dendrograms using the UPGMA (Unweighted Pair Group Method with Arithmetic average) and the SAHN (Sequential Agglomerative Hierarchical Nested clustering) routing in the NTSYS software.

3. Results and Discussion

3.1. Phenotypic variability

Different morphological traits for Date palm cultivars collected from different locations in the New Valley Governorate are demonstrated in Table (2). The values of these traits varied form female to another. For all cultivars and Faleg female (unknown female), the trunk diameters varied from cultivar to another. The highest value was 115.3±3.1 cm for Siwi cultivar, while the lowest value was 75.4±6.2 cm for Tamr cultivar. The other values were intermediate. In addition for all cultivars, tree crown appearances were opened, while, it was closed for Tamr cultivar.

The length of frond (leaves) was long (>425cm) for Tamr and Siwi cultivars, whereas, it was short (<325cm) for Hegazi cultivar and medium (325-425cm) for Faleg female. The shape was straight for all cultivars. The highest values of leaf base thickness and breadth were 10.1±1.09cm and 21.1±1.2cm for Tamr and Siwi cultivars, respectively; in the contrary, the lowest values were 5.9±0.4cm and 10.6±1.8cm for Faleg female and Hegazi cultivar, respectively. The color of dorsal surfaces was light brown for all cultivars, except Hegazi cultivar it was dark brown.

Moreover, the numbers of leaflet (Pinna) varied from cultivar to another. The highest number was 190.1±6.1 for Tamr cultivar and the lowest value was 169.6±4.8 for Hegazi cultivar. For all cultivars, the arrangement on the Midrib was double. The highest area covered on the Midrib was 75.3% for Tamr cultivar and the lowest value was 57.3% for Faleg female. Furthermore, leaflet lengths was short (<60cm) for all cultivars. At the same time, breadth of leaflet (Pinna) was narrow (<38mm) for all cultivars. The color of Midrib surfaces was light green for all cultivars, except Hegazi cultivar it was dark green.

As shown in Table (2), the number of spines was large (more than 30) for Faleg female and Hegazi cultivar, except Siwi was average (20 - 30) and it was few in Tamr cultivars (<20). The area covered on midrib was medium (15-25%) for Siwi and Tamr cultivars, while, was long (>25%) for Faleg female and Hegazi cultivar. Similar, spine thickness was thick hard for Siwi and Tamr cultivars, while, Faleg female and Hegazi cultivar was thin hard. Furthermore, spine length for Siwi cultivar and Faleg female were short (<10cm) and it was medium (10-15cm) for Tamr and Hegazi cultivars. Moreover, spine base was thick long for Siwi and Tamr cultivars, while, Faleg female and Hegazi cultivar were flat base. The arrangement on the midrib was double for all cultivars, while it was single for Tamr cultivar.

Table (2) revealed that the texture of sheath fiber was wide netting for all cultivars, except the Hegazi cultivar it was close netting. Furthermore, the color was dark, while the rest of cultivars were light. For fruit stalk (Peduncle), the data showed that length was short (<90cm) for Faleg female and Siwi cultivar has long fruit stalk (>150 cm). In contrast, the other cultivars (Tam and Hegazi) were intermediate in their Peduncle length (90-150cm). In addition, the orange fruit stalk was observed only in Siwi cultivar and the yellow color was specific for the rest of cultivars. The Spikelet length was 38.1±2.3cm for Hegazi cultivar and 90.7±5.2cm for Siwi cultivar. Also, variable numbers of Spikelet were observed. The highest number was 79.3±2.1 for Siwi cultivar and the lowest value was 38.2±3.2 for Tamr cultivar. The other cultivars showed intermediate length and number.

For all cultivars understudy, the varietal kind (texture) of fruits was soft for Faleg female and Hegazi cultivar, while, it was semidry for Siwi cultivar, and was dry for Tamr cultivar. Furthermore, the color of fruits in the khalal stage was yellow, except Hegazi cultivar was red. The Khalal shape was Cylindrical for all tested samples. In addition, Siwi and Tamr cultivars had fiberus and flavoun in the khalal sweetness stage, while, the Hegazi cultivar was sweet fruits.

For commercial stage/variety, khalal and rutab stages are the commercial stage for Siwi cultivar and Faleg female, while, Tamr and rutab stage for Tamr and Hegazi cultivars, respectively. Considering fruit maturation, Siwi cultivar and Faleg female were early earliness, in contrast Tamr cultivar was late and Hegazi cultivar was medium. The average of fruit size varied from one cultivar to another. The highest value of fruit length was 50.7±5.1mm for Hegazi cultivar and the lowest values were 31.5±5.6mm for Tamr cultivar. The highest value of fruit width was 25.2±4.1mm for Faleg female and the lowest value was 10.2±2.1mm for Siwi cultivar. The highest value of date's number in 500gm was 62.9±4.1gm for Tamr cultivar, while the lowest value was 29.3±3.1gm for Siwi cultivar (Table 2).

Table 1. The nucleotide sequences of primers used for RAPDs, SSRs and AFLPs amplification

Amplification	Primer code	Sequence (5`-3`)	Primer code	Sequence (5`-3`)
RAPDs	OPA-01	CAG GCC CTT C	OPC-01	TTC GAG CCA G
	OPA-02	TGC CGA GCT G	OPC-02	GTG AGG CGT C
	OPA-03	AGT CAG CCA C	OPC-03	GGG GGT CTT T
	OPA-05	AGG GGT CTT G	OPC-12	TGT CAT CCC C
	OPA-10	GTG ATC GCA G	OPC-16	CAC CAT CCA G
	OPA-15	TTC CGA ACC C	OPD-01	ACC GCG AAG G
	OPB-01	GTT TCG CTC C	OPD-04	TCT GGT GAG G
	OPB-02	TGA TCC CTG G	OPD-11	AGC GCC ATT G
	OPB-03	CAT CCC CCT G	OPR-01	GGT GCG GGA A
SSRs	mPdCIR010	F: ACC CGG ACG TGA GGT G R: CGT CGA TCT CCT CCT TTG TCT C		
	mPdCIR015	F: AGC TGG CTC CTC CCT TCT TA R: GCT CGG TTG GAC TTG TTC T		
	mPdCIR016	F: AGC GGG AAA TGA AAA GGT AT R: ATG AAA ACG TGC CAA ATG TC		
	mPdCIR025	F: GCA CGA GAA GGC TTA TAG T R: CCC CTC ATT AGG ATT CTA C		
	mPdCIR032	F: CAA ATC TTG CCG TGA G R: GGT GTG GAG TAA TCA TGT AGT AG		
	mPdCIR035	F: ACA AAC GGC GAT GGG ATT AC R: CCG CAG CTC ACC TCT TCT AT		
	mPdCIR044	F: ATGCGGACTACACTATTCTAC R: GGTGATTGACTTTCTTTGAG		
	mPdCIR048	F: CGAGACCTACCTTCAACAAA R:CCACCAACCAAATCAAACAC		
	mPdCIR050	F: CTGCCATTTCTTCTGAC R:CACCATGCACAAAAATG		
	mPdCIR057	F: AAGCAGCAGCCCTTCCGTAG R: GTTCTCACTCGCCCAAAAATAC		
	mPdCIR063	F: CTTTTATGTGGTCTGAGAGA R: TCTCTGATCTTGGGTTCTGT		
	mPdCIR070	F: CAAGACCCAAGGCTAAC R: GGAGGTGGCTTTGTAGTAT		
	mPdCIR078	F: TGGATTTCCATTGTGAG R: CCCGAAGAGACGCTATT		
	mPdCIR085	F: GAGAGAGGGTGGTGTTATT R: TTCATCCAGAACCACAGTA		
	mPdCIR090	F: GCAGTCAGTCCCTCATA R: TGCTTGTAGCCCTTCAG		
	mPdCIR093	F: CCATTTATCATTCCCTCTCTTG R: CTTGGTAGCTGCGTTTCTTG		
AFLPs	Pre-amplification Primers			
	*Eco*RI + 1-A		5'-GACTGCGTACCAATTC + A-3'	
	*Mse*I + 1-C		5'-GATGAGTCCTGAGTAA + C-3'	
	Primer combinations used in selective			
	*Eco*RI-ACT & *Mse*I-CAT (A)		5'-GAC TGC GTA CCA ATT CAC T-3' 5'-GAT GAG TCC TGA GTA ACA T-3'	
	*Eco*RI-AAG & *Mse*I-CTG (B)		5'-GAC TGC GTA CCA ATT CAA G-3' 5'-GAT GAG TCC TGA GTA ACT G-3'	
	*Eco*RI-AAC & *Mse*I-CTC (C)		5'-GAC TGC GTA CCA ATT CAA C-3' 5'-GAT GAG TCC TGA GTA ACT C-3'	
	*Eco*RI-ACA & *Mse*I-CAA (D)		5'-GAC TGC GTA CCA ATT CAC A-3' 5'-GAT GAG TCC TGA GTA ACA A-3'	

Table 2. Vegetative characters for the Date palm cultivars collected from New Valley Governorate

Characters			Date palm cultivars			
			Siwi	Faleg female	Tamr	Hijazi
			A. Tree			
1. Trunk	Diameter (cm)		115±3.1	80±4.7	75.4±6.2	87.5±5.4
	Tree crown appearance		Opened	Opened	Closed	Opened
2. Frond (Leaves)	Length	Short <325cm	-----	-----	-----	310.4±3.2
		Medium 325-425cm	-----	390.8±4.5	-----	-----
		Long >425cm	510.3±8.8	-----	490.3±7.9	-----
	Shape		Straight	Straight	Straight	Straight
3. Leaf base	Thickness (cm)		7.3±0.9	5.9±0.4	10.1±1.09	7.4±0.6
	Breadth (cm)		21.1±1.2	11.2±2.1	18.4±4.9	10.6±1.8
	Color of the dorsal surface		Light Brown	Light Brown	Light Brown	Dark Brown
4. Leaflet (Pinna)	Number/Front		177.3±6.6	188.78±5.1	190.1±6.1	169.6±4.8
	Arrangement on the Midrib		Double	Double	Double	Double
	Area covered on the Midrib (%)		67	57.3	75.3	71.7
	Length	Short <60cm	47.2±3.9	57.3±3.1	47.9±5.1	50.4±5.1
		Medium 60-75cm	-----	-----	-----	-----
		Long >75cm	-----	-----	-----	-----
	Breadth	Narrow <38 mm	35.1±3.2	30.4±5.4	20.1±2.4	30.3±3.4
		Medium 38- 44 mm	-----	-----	-----	-----
		Wide >44 mm	-----	-----	-----	-----
	Color		Light green	Light green	Light green	Dark green
5. Spine	Number	Few < 20	-----	-----	18.9±2.9	-----
		Average 20 - 30	23.3±2.9	-----	-----	-----
		Large More than 30	-----	57.3±3.5	-----	35.5±3.8
	Area covered on the Midrib	Short <15%	-----	-----	-----	-----
		Medium 15-25%	19.2	-----	15.4	-----
		Long >25%	-----	31.3	-----	26.5
	Thickness		Thick hard	Thin hard	Thick hard	Thin hard
	Length	Short <10cm	7.2±1.5	9.8±1.8	-----	-----
		Medium 10-15cm	-----	-----	13.7±2.5	14.5±2.6
		Long >15cm	-----	-----	-----	-----
	Spine base		Thick long	Flat	Thick long	Flat
	Arrangement on the midrib		Double	Double	Single	Double
6. Sheath fiber	Texture		Wide netting	Wide netting	Wide netting	Close netting
	Color		Light	Light	Light	Dark
7. Fruit stalk (Peduncle)	Length	Short <90cm	-----	√	-----	-----
		Medium 90-150cm	-----	-----	√	√
		Long >150cm	√	-----	-----	-----
	Color		Orange	Yellow	Yellow	Yellow
8. Spikelet	Length (cm)		90.7±5.2	72.4±7.1	45.4±3.2	38.1±2.3
	Number		79.3±3.9	45.8±3.9	38.2±3.2	42.4±2.1
	Arrangement on the peduncle		Zigzag	Zigzag	Zigzag	Zigzag
			B. The Fruits			
1. Varietal Kind (texture)			Semidry	Soft	Dry	Soft
2. Khalal stage color			Yellow	Yellow	Yellow	Red
3. Khalal shape			Cylinderical	Cylinderical	Cylinderical	Cylinderical
4. Khalal sweetness			Fiberus and flavoun	Sweet	Fiberus and flavoun	Sweet
5. Commercial stages/ varieties			Khalal and Rotab	Khalal and Rotab	Tamr only	Rotab only
6. Earliness			Early	Early	Late	Medium
7. Size	Length (mm)		40.4±3.9	45.4±4.9	31.5±5.6	50.7±5.1
	Width (mm)		10.2±2.1	25.2±4.1	19.1±3.2	20.1±2.4
	No. of dates in 500 gm	Small >100	-----	-----	-----	-----
		Medium 80-100	-----	-----	-----	-----
		Large<80	29.3±3.1	40.9±2.1	62.9±4.1	32.9±1.1
8. Average weight (gm)			17.3±2.2	12.5±1.2	8.2±1.9	5.8±1.4
9. Volume / cm^3			13.3±1.4	14.3±1.5	10.2±0.9	20.1±3.2
10. Density W/V Ratio			1.28	0.93	0.8	1.25
11. Fruit quality index		length/width	3.96	1.80	1.65	2.52
12. Fruit Skin (Epicarp)			Skin attached	Thin smooth	Skin attached	Skin loose
13. Fruit Cap (Perianth)	Height over surface		1 mm	1 mm	1-2 mm	1-2 mm
	The Edge		Wide and Circle	Wide and Circle	Wide and Circle	Narrow and Small

Furthermore, Siwi cultivar recorded the highest value of average weights of fruits (17.3±5.1gm); density W/V ratio (1.28) and fruit quality index (3.96). In contrast, for the same fruit characters, Hegazi and Tamr cultivars illustrated the lowest value, respectively. In addition, the highest value of volume/cm^3 was 20.1±3.2 for Hegazi cultivar and the lowest value was 10.2±0.9 for Tamr cultivar. Regarding the fruit skin (Epicarp), Table (2) shows a skin attached for Siwi and Tamr cultivars and it was thin smooth for Faleg female, while was skin loose for Hegazi cultivar. In addition, for all cultivars, the Edge was wild and circle, except Hegazi cultivar it was narrow and small. The height over surface ranged from 1mm (Siwi cultivar and Faleg female) to 1-2mm in (Tamr and Hegazi cultivars).

Multivariate of quantitative traits has been used previously to measure genetic relationships between Date palm cultivars and unknown female. Since these traits were affected by environment, so, data were taken for two successive years and the averages were calculated. In addition, variations in horticultural traits were observed by Hemeida *et al.* (2010) and Hamza *et al.,* (2011). They generally attributed these variations to some genetic factors. The overall partitioning of genetic diversity based on fruit traits suggests that the surveyed date palm cultivars represent a complex gene pool within which historical movement of germplasm, recent introductions and human selection are shaping the genetic structure. Elshibli and Korpelainen (2009) indicated that hundreds of date palm cultivars and strains were recognized and selected by farmers through a long history of more than 3000 years of cultivation in Sudan. The most common characters used to identify cultivars are tree and fruit morphology as well as softness characters of fruits, which are detectable only at tree maturity.

On another point of view, Hamza *et al.,* (2009) showed that the morphological studies of date palm have always been considered difficult to undertake because they require a large set of phenotypic data and because they are varied due to the environment effect. The majority of the phenotypic date palm studies are aimed at studying the spectrum genetic variation but they cannot allow definitive discrimination between cultivars, fruit, quality and plant behavior. However, our study agrees with the recommendation of Ferchichi & Hamza (2008) and Hamza *et al*. (2011). Where, it was indicated that future studies should be considering the possible relations of other important phenotypic markers related to the tolerance towards oases stress. This should be backed up by others studies such as molecular ones to provide reliable tools for measuring genetic divergence.

3.2. RAPDs amplification

The eighteen random primers were used to differentiate through RAPD analysis among the three date palm (*Phoenix dactylifera* L.) cultivars (Siwi, Tamr, Hegazi) and Faleg female (unknown female) were collecting from New Valley Governorate (El-Kharga and Dakhleh). As shown in Figure (2) and Table (3), the number of the amplified fragments per cultivar varied from 107 fragments for Hegazi cultivar to 138 fragments for Siwi cultivar giving a total of 512 fragments. Two hundred and sixteen of the 512 fragments were polymorphic across the four date palm cultivars. The percentages of the polymorphism ranged from 46% (64 fragments) were recorded for Siwi cultivar to 31% (33 fragments) for Hegazi cultivar. In the meantime, there were specific DNA-markers for each date palm genome. The numbers of these unique markers ranged from 9 fragments for Hegazi cultivar to 12 fragments for Faleg female and giving a total of 42 unique markers for all cultivars under study.

It could be concluded that RAPD analysis is an efficient tool for the identification and characterization of date palm genomes, which agreed with the findings of Adawy *et al.* (2004) who identified some Upper Egypt Date palm cultivars using the same molecular DNA techniques and demonstrated different levels of inter-cultivar polymorphisms and specific DNA-markers. Moreover, Adawy *et al.* (2005) reported that such unique bands could be used as DNA markers for cultivar identification. These results are also in harmony with those of Abou Gabal *et al.* (2006) and Hemeida *et al.* (2010) that scored high level of polymorphism using RAPD analysis. Moreover, Rania *et al.*, (2008) revealed the power of RAPD in distinguishing among palm cultivars grown in the same location. Also, indicated that the RAPD markers can be used in subsequent experiments to detect molecular markers for genes with female identification in palm cultivars.

3.3. SSRs amplification

Figure (3) and Table (3) illustrates different SSRs profiles. The number of the amplified fragments per cultivar varied between 19 fragments for Tamr and Hegazi cultivars and 23 fragments for Faleg female with a total of 83 fragments. From these amplified fragments, 38 fragments with 46% were polymorphic.

Moreover, the SSR profiles exhibited different allele per locus in the sampling, with homozygous and heterozygous individuals clearly identifiable. A total of 28 alleles with a mean of 1.75 alleles per locus were scored, however, the number of alleles varied from one to three (Table 4). The number of alleles per locus detected in this study was lower than those scored by Zehdi *et al.* (2004a) who recognized 7.14 alleles per locus when examining 46

Tunisian date palm accessions using 14 microsatellite loci. On the other hand, Elshibli and Korpelainen (2007) identified 21.4 alleles per locus, which is more than the number of alleles per locus detected in this study. This may be a result of using a greater number of different date palm accessions (68 Sudan and Morocco). In addition, the primers successfully produced clear amplified SSRs fragments with sizes ranging from 111 bp with primer mPdCIR090 to 446 bp with primer mPd-CIR044. Among the sixteen SSRs primers tested for their ability to generate expected amplified SSRs fragment patterns, five primers successfully produced clear single fragment in the studied genotypes. Similarly to the results of Elmeer and Mattat (2012) where the fragment sizes ranged from 118 to 302 bp.

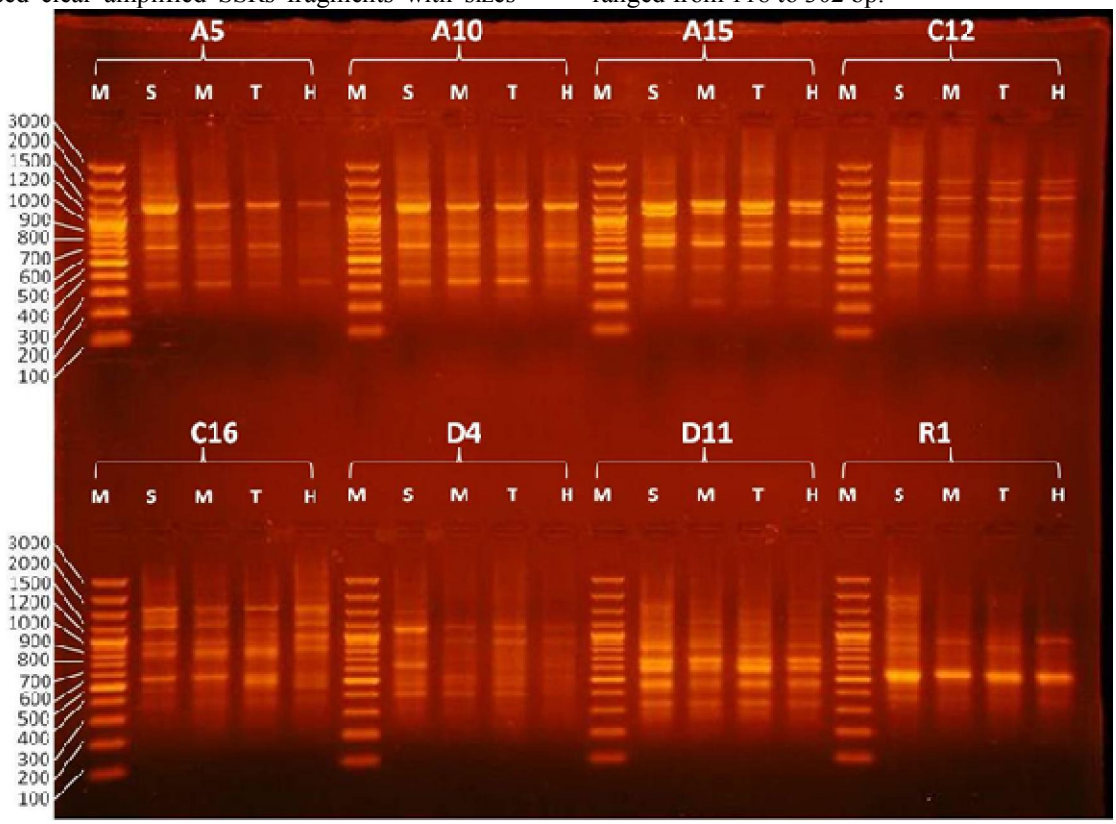

Figure 2. An example of DNA polymorphism of the three date palm cultivars and one unknown female using eight RAPD primers. Siwi: S, Tamr: T, Hegazi: H and Faleg: M

Table 3. Total numbers of fragments and specific markers for the different molecular analyses of the four date palm cultivars

Cultivars		Analysis type		
		RAPDs	**SSRs**	**AFLPs**
Siwi	**TF**	138	22	71
	Sm	10	---	8
	PF (%)	*64 (46%)*	*12 (55%)*	*44 (62%)*
Tamr	**TF**	134	19	65
	Sm	11	---	3
	PF (%)	*60 (45%)*	*6 (32%)*	*38 (59%)*
Hegazi	**TF**	107	19	57
	Sm	9	---	2
	PF (%)	*33 (31%)*	*6 (32%)*	30 (53%)
Faleg	**TF**	133	23	53
	Sm	12	---	7
	PF (%)	*59 (44%)*	*14 (61%)*	26 (49%)
Total	**TF**	**512**	**83**	**246**
	Sm	**42**	**---**	**20**
	PF (%)	***216 (42%)***	***38 (46%)***	**138 (56%)**

TF: total number of fragments; Sm: number of specific markers; PF (%): Polymorphic fragments and Percentages of polymorphism

Table 4. Summary of microsatellite allele data revealed by 16 microsatellite loci in three date palm cultivars and one unknown female (Faleg)

	Primer code	Allelic range (bp)	Major allele frequenecy	Allele no.
1.	mPdCIR010	148-163	0.75	2
2.	mPdCIR015	132-161	0.50	3
3.	mPdCIR016	150-162	0.75	2
4.	mPdCIR025	197-215	0.50	2
5.	mPdCIR032	279	1.00	1
6.	mPdCIR035	175-191	0.75	2
7.	mPdCIR044	281-446	1.00	2
8.	mPdCIR048	185-248	1.00	2
9.	mPdCIR050	194	1.00	1
10.	mPdCIR057	230	1.00	1
11.	mPdCIR063	136-158	0.75	2
12.	mPdCIR070	200	1.00	1
13.	mPdCIR078	169-241	1.00	2
14.	mPdCIR085	163	1.00	1
15.	mPdCIR090	111-163	1.00	2
16.	mPdCIR093	161-266	1.00	2
Total				28

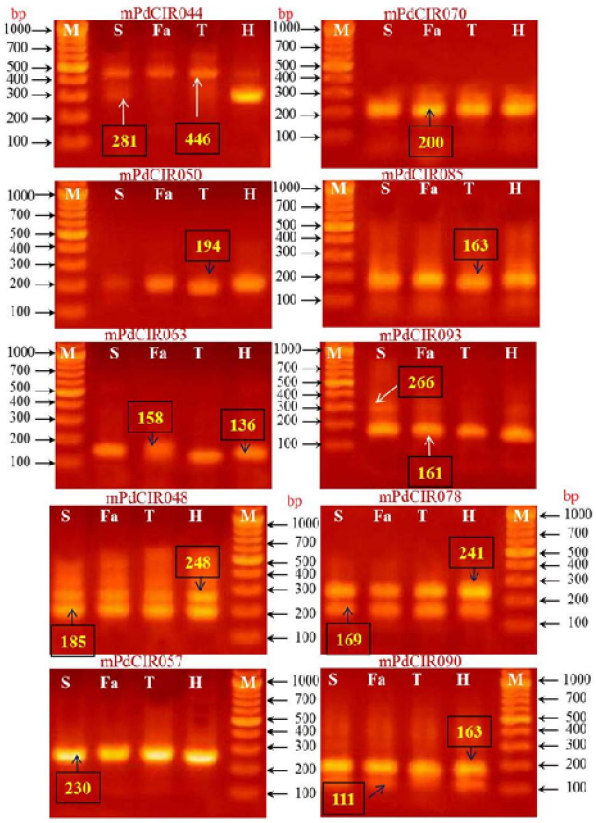

Figure 3. An example of DNA polymorphism of the three date palm cultivars and the unknown female using ten microsatellites primers. Siwi: S, Tamr: T, Hegazi: H and Faleg: M

Table (5) represents 28 loci hetero and homozygous allele (base pairs) markers that identify date palm cultivar and one unknown female. Five primers (mPdCIR033, mPdCIR050, mPdCIR057, mPdCIR070 and mPdCIR085) could not distinguish between female samples whereas the remaining 11 microsatellite primers identified 22 loci. Using these loci, 36% of those loci were heterozygous alleles, which is in agreement with the finding of Al-Dous *et al.* (2011) who scanned 3.5 million SNP genotypes in the female genomes of date palm to identify polymorphisms. The primer mPdCIR035 detected only one heterozygous allele even as the remaining three alleles were homozygous, in the contrary, the primer mPdCIR090. Moreover, the heterozygous allele sized 281/190 exhibited by primer mPdCIR044 and 161/266 exhibited by primer mPdCIR093, respectively, were repeated twice in the date palm tree tested. The primer mPdCIR048 and primer mPdCIR078 were presented monomorphic microsatellite. Furthermore, the homozygous allele sized 148/148 exhibited by primer mPdCIR010 and 162/162 exhibited by primer mPdCIR016, respectively, were repeated one time in the tested samples.

3.4. AFLPs amplification

The AFLP fingerprinting of the four Date palm genotypes tested using four primer combinations, *Eco*RI-ACT/*Mse*I-CAT; *Eco*RI-AAG/*Mse*I-CTG; *Eco*RI-AAC/*Mse*I-CTC and *Eco*RI-ACA/*Mse*I-CAA. The AFLPs generated from the four primer combinations is shown in Figures 2 & 3. With four primer combinations the AFLP analysis

yielded a total of 246 AFLP loci ranging in length from 98 to 2022 bp among the four Date palm genotypes. Of these loci 138 (= 56%) were polymorphic and 108 monomorphic (=44%; Table 3). The highest number of amplified DNA fragments was revealed by Siwi cultivar (71 fragments), while the lowest value was exposed by Faleg genome (53 fragments). Moreover, the highest number of these was illustrated by Siwi cultivar (44 fragments) with percentage 62% polymorphic fragments. In contrast, the lowest values were showed for Faleg genome (26 fragments) with 49% polymorphic fragments (Table 3). Furthermore, for the four genomes, 20 specific DNA-markers were screened. The highest number of these markers indicated for Siwi cultivar (8 markers) and the lowest value was specified for Hgazi cultivar (2 markers, Table 3).

Similarly, using the same molecular DNA technologies, Adawy *et al.* (2005) demonstrated different levels of inter-cultivar polymorphisms and specific DNA-markers among some Delta and Upper Egypt Date palm cultivars. This feature may be attributed to the limited number of AFLP selective primers used in the present analyses; the amplification of different parts of the genomes (Amel *et al.*, 2005) or/and the reliability of each technique to react with the nine Date palm genomes (Garcia *et al.*, 2004). AFLP being able to detect a large number of polymorphic bands in a single lane rather than high levels of polymorphism at each *locus* such as is the case for ISSR methods (Hemeida *et al.*, 2010).

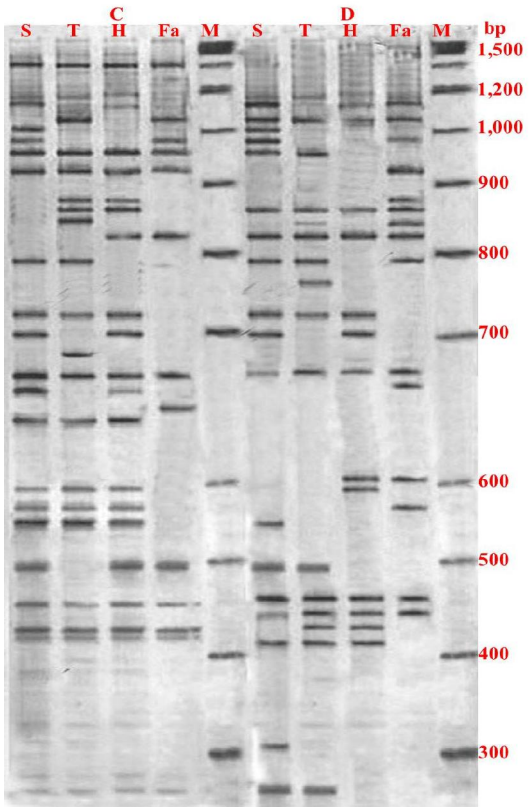

Figure 4. An example of photographs showing AFLPs products of the four different cultivars of Date palm using four selective primers. Siwi: S, Tamr: T, Hegazi: H and Faleg: Me. and M: DNA marker. C: primer combination *Eco*RI-AAC/*Mse*I-CTC and D: primer combination *Eco*RI-ACA/*Mse*I-CAA

Table 5. Twenty eight loci hetero and homozygous allele (base pairs) markers that identify date palm cultivar and one unknown female

	Siwi	Faleg	Tamr	Hegazi
mPdCIR010	163/163	163/163	163/163	148/148
mPdCIR015	142/161	142/142	132/132	132/161
mPdCIR016	150/150	150/150	150/150	162/162
mPdCIR025	215/215	197/197	215/215	197/197
mPdCIR032	279/279	279/279	279/279	279/279
mPdCIR035	191/191	191/191	175/191	175/175
mPdCIR044	281/446	446/446	446/446	281/446
mPdCIR048	185/248	185/248	185/248	185/248
mPdCIR050	194/194	194/194	194/194	194/194
mPdCIR057	230/230	230/230	230/230	230/230
mPdCIR063	158/158	158/158	136/136	136/136
mPdCIR070	200/200	200/200	200/200	200/200
mPdCIR078	169/241	169/241	169/241	169/241
mPdCIR085	163/163	163/163	163/163	163/163
mPdCIR090	111/163	111/163	163/163	111/163
mPdCIR093	161/266	161/161	161/161	161/266

Table 6. Similarity indices (%) among the three date palm cultivars and one unknown female (Faleg, known as Meghel) using eighteen RAPD; sixteen SSRs and four AFLP primers

	Siwi	Faleg	Tamr	Hegazi
Siwi	1.00			
Faleg	0.44	1.00		
Tamr	0.51	0.43	1.00	
Hegazi	0.43	0.35	0.49	1.00

3.5. Phylogenetic relationships and genetic distance

Genetic relationships among the three date palm (*Phoenix dactylifera* L.) cultivars (Siwi, Tamr, Hegazi) and one unknown female (Faleg) were collecting from New Valley Governorate (El-Kharga and Dakhleh) were presenting as a dendrogram (Table 6 and Figure 5) using UPGMA method. The genetic similarity estimates ranged from 51% to 35%. The highest genetic similarity 51% was observed between Siwi and Tamr cultivars, this was followed by 49% between Hegazi and Tamr cultivars, while the lowest genetic similarity (35%) was detected between Hegazi cultivar and Faleg genome.

The dendrogram constructed based on the data from eighteen RAPDs; sixteen SSRs and four AFLPs primers were developed by using the NTSYS-pc program. The dendrogram confirmed that the Faleg genome does not cluster with any other cultivar tested and is easily distinguishable, while the cultivars Siwi and Tamr were the most genetically similar among the studied cultivars, with Hegazi cultivar come next. The dendrogram illustrated two essential clusters. The first one contained two groups. The first group included Siwi and Tamr cultivars with similarity 0.51. The second group contained Hegazi cultivar. The second cluster included Faleg genome.

This result reflects the similarity between unknown female trees and female cultivars, but this data is not sufficient to identify unknown Date palm females. Identification of unknown Date palm females exactly needs more advanced molecular studies (Trifi *et al.,* 2000). There may be reason to view with caution systematic conclusions based on RAPDs and SSRs analysis alone (Saker *et al*., 2006). On the other hand, the possibility of carrying out compatibility analysis with unlimited numbers of primers, each detecting variation at several regions in the genome, provides an advantage over other techniques. Even if some primers amplify identical regions of the genome or if the technique itself is noisy, it should be possible to build up quickly a consensus from patterns of inter-population variation. The three applied techniques amplify different parts of the genomes (Elshibli and Korpelainen, 2009).

Concerning the data of the three DNA-markers, high genetic diversity for coding and non-coding sequences was indicated among the nine Date palm genomes. However, each analytical technique exhibited a different level of polymorphism and unique markers. Comparing with RAPDs; SSRs and AFLPs analysis were the most effective method for assessing the genetic diversity and the unique DNA-markers across the nine Date palm genomes.

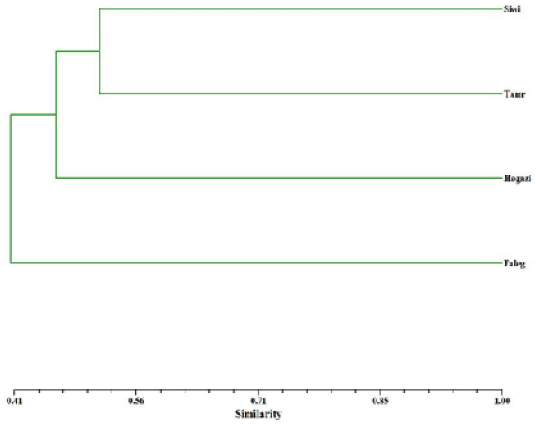

Figure 5. Dendrogram of the three date palm cultivars and one unknown female (Faleg, known as Meghel) using eighteen RAPD; sixteen SSRs and four AFLP primers

Acknowledgements:

This work was carried out through the project entitled (Development of a system for commercial production and genetic improvement of some date palm cultivars in New Valley) under the program of the science and technology development fund (STDF), Ministry of Scientific Research, EGYPT.

Corresponding Author:
Prof. Dr. Alaa A. Hemeida
Genetic Engineering and Biotechnology Research Institute (GEBRI)
University of Sadat City, Sadat City, Egypt
E.mail: alaa.hemeida@gebri.usc.edu.eg

References

1. Abou Gabal, A.A.; A.A. Abdelaziz; M.M. Harhash and H.F. El-Wakil (2006). Genetic diversity among seven Date palm landraces in Siwa oasis. Egypt. J. Genet. Cytol. 35: 117-128.
2. Adawy, S.S., E.H.A. Hussien; E.M. Samer and H.A. El-Itriby (2005). Genomic diversity in date palm cultivars (*Phoenix dactylifera* L.) as reveled by AFLPs in comparison to RAPDs and ISSRs. Arab J. Biotech. 8: 99-114.
3. Adawy, S.S.; Ebtissam Hussein, A.; Saker, M.M. and Hanaiya El-Itriby, A. (2004). Intra- and Inter-varietal variation of Upper Egypt date palm cultivars (*Phoenix dactylifera* L.): I. As revealed by RAPD and ISSR markers. Proceed. Int. Conf. Genet. Eng. & Appl., Sharm El-Sheikh, South Sinai, Egypt (April, 8-11, 2004): 165-179.

4. Al-Dous E., George B, Al-Mahmoud M., Al-Jaber M., Wang H, Salameh Y., Al-Azwani E., Chaluvadi S, Pontaroli A., DeBarry J, Arondel V, Ohlrogge J, Saie I., Suliman-Elmeer K., Bennetzen J., Kruegger R., Malek J. (2011) De novo genome sequencing and comparative genomics of date palm (*Phoenix dactylifera*). Nat biotechnol 29:521-528.
5. Al-Khalifah, N.S. and Askari, E. (2003). Molecular phylogeny of date palm (*Phoenix dactylifera* L.) cultivars from Saudi Arabia by DNA fingerprinting. Theor. Appl. Genet. 107(7):1266-70.
6. Amel, S.H.; Khaled, Ch; Messaoud, M.; Mahmoud, M. and Mokhtar, T. (2005). Comparative analysis of genetic diversity in two Tunisian collections of Fig cultivars based on Random Amplified Polymorphic DNA and Inter Simple Sequence Repeats Fingerprints. Genetic resources and crop evolution, 52: 563-573.
7. Amy, B.; Khadidiatou, N.; Pape, M.; Alain, B.; Djibril, S. Maurice, S. and Marie-Hélène, C. (2012). Analysis of genetic diversity of date palm (*Phoenix dactylifera* L.) cultivars from Mauritania using microsatellite markers. International Journal of Science and Advanced Technology. 2: (2) 36-43.
8. Billotte, N., Marseillac, N., Brottier, P., Noyer, J.L., Jacquemoud-Collet, J.P., Moreau, C., Couvreur, T., Chevallier, M.H., Pintaud, J.C. and Risterucci, A.M. (2004). Nuclear microsatellite marker for date palm (*Phoenix dactylifera*): characterization and utility across the genus Phoenix and in other palm genera. Molecular Ecology Notes 4:256-258.
9. Elmeer K. and I. Mattat (2012). Marker-assisted sex differentiation in date palm using simple sequence repeats. Biotech. 2:241–247.
10. Elshibli , S. and Korpelainen, H. (2009). Identity of date palm ((*Phoenix dactylifera* L.) germplasm in Sudan: from morphology and chemical characters to molecular markers. Acta Hort. (ISHS) 859: 143-153.
11. Elshibli, S. and Korpelainen, H. (2007). Microsatellite markers reveal high genetic diversity in date palm (*Phoenix dactylifera* L.) germplasm from Sudan. Genetica 134:251-260.
12. Ferchichi, A. and H. Hamza. (2008). Le patrimoine génétique phoenicicole des oasis continentals tunisiennes. Institut des régions arides, Medenine.
13. Garcia, A.A.F; Benchimol, L.L.; Barbosa, A.M.M.; Geraldi, I.O.; Souza, G.G.and Souza, A.P. (2004). Comparison of RAPD, RFLP, AFLP and SSR markers for diversity studies in tropical maize inbred lines. Gent.Mol.Biol., 27: 1415-1425.
14. Hamza, H.; Giovanni G. and Ferchichi A. (2011). Microsatellite diversity among Tunisia date palm (*Phoenix dactylfera* L.) subpopulations. Pak. J. Bot., 43(2): 1257-1264.
15. Hamza, H.; Rejeli M.; Elbekkay M. and Ferchichi A. (2009). New approach for the morphological identification of date palm (*Phoenix dactylfera* L.) cultivars from Tunisia. Pak. J. Bot., 41(6): 2671-2681.
16. Hemeida, A. A.; A. A. Nower and T. M. Abd El-Rahman (2008). Identification of unknown females of Siwan date palm (*Phoenix dactylifera* L.) by morphological and molecular analysis. Egypt. J. Genet. Cytol. 37: 35-56.
17. Hemeida, A. A.; Reham M. Abd El-Azeem and Hashem M. H. (2010). Identification and genetic similarity analysis of date palm (*Phoenix dactylifera* L.) collected from different regions in Siwa oasis using morphologically traits and molecular markers. International Symposium on Biology of Rare and Endemic Plant Species. Fethiye, Turkey, 26-29 May 2010 Ankara, Turkey, p. 41.
18. Malek J.A., (2010). Next generation DNA sequencing applied to the Date palm tree (*Phoenix dactylifera*). Acta Hortic 882:249–252.
19. Rania Younis, A.; Omayma Ismail, M. and Soliman, S.S. (2008). Identification of sex-specific DNA markers for date palm (*Phoenix dactylifera* L.) using RAPD and ISSR techniques. Research Journal of Agriculture and Biological Sciences, 4(4): 278-284.
20. Rhouma C. S., Baraket G., Dakhlaoui D. S., Zehdi A. S. and Trifi M., (2011). Molecular research on the genetic diversity of Tunisian date palm (*Phoenix dactylifera* L.) using the random amplified microsatellite polymorphism (RAMPO) and amplified fragment length polymorphism (AFLP) methods. African Journal of Biotechnology Vol. 10(51), pp. 10352-10365.
21. Rohlf FJ. (2000). On the use of shape spaces to compare morphometric method. Hystrix, Italian J. Mammology (n.s.), 11(1): 8-24.
22. Saker, M.M., Adawy, S.S., Mohamed, A.A. and El-Itriby, H.A. (2006). Monitoring of cultivar identity in tissue culture-derived date palms using RAPD and AFLP analysis. Biologia Plantarum. 50: 198-204.
23. Salem, A.; Rhouma S., Zehdi S., Marrakchi M. and Trifi M., (2008). Morphological variability of Mauritanian date palm (*Phoenix dactilyfera* L) cultivars as revealed by vegetative traits. Acta Bot. Croat. 67 (1), 81-90.
24. Sedra, M.H., Lashermes, P., Trouslot, P., Combes, M.C. and Hamon, S. (1998). Identification and genetic diversity analysis of date palm (*Phoenix dactylifera* L.) varieties from Morocco using RAPD markers. Euphytica 103:75-82.
25. Trifi, M., Rhouma, A. and Marrakchi, M. (2000). Phylogenetic relationships in Tunisian date palm (*Phoenix dactylifera* L.) germplasm collection using DNA amplification fingerprinting. Agronomie 20:665-671.
26. Vos, P., R. Rogers, M. Bleeker, M. Reijans, T. Van de Lee, M. Hornes, A. Fijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau (1995). AFLP, a new technique for DNA fingerprinting. Nucleic Acids. Res. 18: 7213-7218.
27. Zehdi, S., Sakka, H., Rhouma, A., Ould, M.S.A., Marrakchi, M. and Trifi, M. (2004a). Analysis of Tunisian date palm germplasm using simple sequence repeat primers. African Journal of Biotechnology 3:215-219.
28. Zehdi, S., Trifi, M., Billotte, N., Marrackhi, M. and Pintaud, J.C. 2004b. Genetic diversity of Tunisian date palms (*Phoenix dactylifera* L.) revealed by nuclear microsatellite polymorphism. Hereditas 141:78-287.