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CHARACTERIZATION, SEX IDENTIFICATION AND GENE EXPRESSION FOR FRUIT QUALITY GENES IN DATE PALM (*PHOENIX DACTYLIFERA* L.)

Amira H.M. Hassan, Mona H. Hussein, El-sayed G. Ibrahim and Salah EL Din S. EL Assal

Department of Genetics, Faculty of Agriculture, Cairo University, Egypt.

Abstract

Date palm (*Phoenix dactylifera* L.) is an important and oldest fruit tree. Determination of genetic relationships between date palm (*Phoenix dactylifera* L.) cultivars is very useful for characterization of date palm germplasm, breeding programs, conservation purposes and genetic improvement. In this work the PCR-based markers (ISSR-SRAP) have been used as tools to determine the relationships between date palm cultivars that are difficult to distinguish morphologically. Results revealed that the total polymorphism detected by the ISSR assay (59.02%) with total polymorphic bands (36) was higher than that observed for SRAP (51.85%) with total polymorphic bands (56). On the other hand, we have attempted to determine the sex-specific DNA markers for some date palm cultivars and selected progeny using molecular technique (RAPD and ISSR) to detect the sex in date palm seedlings at early stage. The using both RAPD and ISSR analyses gave one positive specific markers for female and eight for male in RAPD analysis in addition to five positive specific markers for female and three for male in ISSR analysis and the level of polymorphism between cultivars was 77.01% and 80.45% as revealed by RAPD or ISSR, respectively. Moreover, the gene expression investigations aimed to evaluate the gene expression for the three genes, Phosphofructokinase, pectin (pectate) lyase and Xyloglucan (Xyloglucosyl transferase) for fruit quality of date palms (Barhee and Madjool) in Khalal stage. The expression levels of these genes in treatments T1, T2 and T3 in Majhool *cv.* were higher than the expression level of these genes in the same treatments of Barhee *cv.* The treatment T2 gave high expression (1.70) in Majhool *cv.* compared with control for Phosphofructokinase gene but the expression of Xyloglucan gene has been observed with higher expression (1.69 and 1.73 respectively) in treatments (T1 and T2), in Majhool *cv.* compared with the reference gene.

Key words: Date Palm, RAPD, ISSR and SRAP DNA markers, Gender identification

Introduction

The genus Phoenix, which includes the date Palm (*Phoenix dactylifera* L., Arecaceae or palmae family) ($2n$ = 36) is a long living dioecious monocotyledon (Srivashtav, 2013). The date palm fruit produced largely in the hot arid regions of South West Asia and North Africa. The major date producers in the world are located in the Middle East and North Africa (Zaid, 2002). The total world production 8, 166.014 million tons of dates and Egypt was ranked the first in the world among date producing countries, with a production volume that amounted to about 1.6 million metric tons of dates that year (Fao Stat, 2017).

The date palm contains 18 pairs of chromosomes (2n = 36), where the XX/XY system is used to determine its sex. Its genome has recently been sequenced, showing a length of ~671 Mbp and including ~41, 600 genes (Al-Mssallem *et al.,* 2013). Morphological characterization of the date palm cultivars is practically difficult and Biochemical studies are not enough to detect DNA polymorphism within the palm genome (Al-Jibouri and Adham, 1990; Yusuf *et al.,* 2015). It is also a hard task to follow segregating genes among palm generations through breeding for many reasons. First, palm tree is dioecious and it depends on random cross pollination. Secondly, it usually takes from 8-10 years for the tree to reach maturity and reveal its physio-socio-economic productivity. Inter Simple Sequence Repeats (ISSR) and Sequence Related Amplified Polymorphic (SRAP) have been shown to be very powerful PCR–based techniques in molecular analysis of date palm, genotyping and marker-assisted

selection in breeding programs (LI and Quiros, 2001; Elshibli and Korpelainen, 2009 and Said *et al.,* 2012). In the date palm, a major problem for farmers is to identify the sex of saplings at an early stage so In recent years, there have been serious efforts to understand the genetic basis of sex determination in plants and to develop methods to identify sex at an early stage by using molecular marker tools. (Younis *et al.,* 2008). RAPD and ISSR markers system have been used for sex identification in dioecious plants (Milewicz and Sawicki, 2011).

Quantitative real time PCR (qPCR), which is a commonly used technique to study mRNA abundances, has become a powerful tool for quantifying gene expression. However, for accurate quantification of gene expression, proper reference genes for normalization must be used (Sinha *et al.,* 2015). A number of housekeeping genes such as ß Actin and Tubulin have been used as reference genes for normalization of target gene expression in different plant species (Wang *et al.,* 2015).

Therefore, this research aimed

1- Study the genetic variation between males and females date palm cultivars under study by molecular markers (ISSR-SRAP).

2- Study the sex identification in date palm at early stages (seedling stage) using molecular markers (RAPD and ISSR).

3- Study the gene expression for three genes (Phosphofructokinase, pectin (pectate) lyase and Xyloglucan: Xyloglucosyl transferase) for fruit quality of date palms (Barhee and Madjool) in Khalal stage of date palm development stages.

Materials and Methods

This study was carried out in the private farm in desert region at Monifia Governorate on Barhee and Madjool date palm *cv.* of about 10 years old and the six male types as males date palm *cv.* (Fard, Ghnamiy, Dayri and Sapad) and local date palm seeding males Sewy and Barhee (an individual of Sewy and Barhee satellite seedling as suggested by Mason, 1927) and biotechnology lab of the Horticulture Research Institute, Agricultural Research Center (ARC), Giza, Egypt and Genetic Engineering Center of the Faculty of Agriculture, Cairo University, Giza, Egypt.

The present study was conducted through two growing seasons 2015 and 2016 to study the genetic variation between males and females date palm cultivars under study by molecular markers (ISSR-SRAP) and Studying the sex identification in date palm at seedling stage after crossing between males and females date palms using molecular markers (RAPD and ISSR).

Molecular markers to identification for males and females used in this study

Genomic DNA was isolated from leaves of the females and males date palms of by using CTAB method, described in Rogers and Bendich (1985).

a-ISSR analysis conditions

The ISSR analysis was performed as described by Sabir *et al.,* (2014). ISSR primers (were synthesized by Invitrogen by Thermo Fisher Scientific, UK) were screened using 8 different primers. The sequence of ISSR primers used in this work is shown in table 1. The PCRs were programed for one cycle at 94°C for 5 minutes followed by 5 cycles 94°C for 30 sec., 35°C for 1 min., 72°C for 30 sec. and followed by 35 cycles 94°C for 30 sec., 50°C for 30 sec., 72°C for 30 sec. and 72°C for 5 minutss and the PCR products were separated on 1% agaros gels.

b-SRAP analysis conditions

The SRAP analysis was performed as described by LI and Quiros (2001). SRAP primers (were synthesized by Invitrogen by Thermo Fisher Scientific, UK) were screened using 15 different combinations of 3 forward and five reverse primers. The sequence of SRAP primers used in this work is shown in table 2. The PCRs were

Table 1: Name and sequences of ISSR primers used in this study.

No	Name	Sequence
1	HB9	$(GT)_6GG$
2	HB10	$(GA)_6CC$
3	HB11	$(GT)_6CC$
4	HB12	$(CAC)_3GC$
5	HB13	$(GAG)_3GC$
6	814	$(CT)_8TG$
7	844A	$(CT)_8AC$
8	844B	$(CT)_8GC$

Table 2: Name and sequences of SRAP primers used in this study.

No	Name	Sequence
Forward primers		
1	me2	52 -TGAGTCCAAACCGGAGC-32
2	me3	52 -TGAGTCCAAACCGGAAT-32
3	me4	52 -TGAGTCCAAACCGGACC-32 .
Revers primers		
1	em1	52 -GACTGCGTACGAATTAAT-3
2	em2	52 -GACTGCGTACGAATTTGC-32
3	em3	52 -GACTGCGTACGAATTGAC-32
4	em4	52 -GACTGCGTACGAATTTGA-32
5	em5	52 -GACTGCGTACGAATTAAC-32

programed at 94°C for 5 minutes followed by 5 cycles 94°C for 30 sec., 35°C for 1 min., 72°C for 30 sec. and followed by 35 cycles 94°C for 30 sec., 50°C for 30 sec., 72°C for 30 sec. and 72°C for 5 minutes and The PCR products were separated on 1% agaros gels.

c-Band scoring and cluster analysis: The ISSR and the SRAP gel images were scanned using the gel Doc 2000 Bio-Rad system and analyzed with Quantity One Software v 4.0.1 (Bio-Rad Laboratories, Hercules, Co. USA). The bands were sized and then binary coded by 1 or 0 for the presence or absence in each genotype. Data were than computed with the SPSS-16 program to produce a genetic distance matrix which assesses the similarity between two populations on the basis of the number of generated bands using Dice similarity coefficient (Dice, 1945).

Sex identification at early stages for progeny of date palms by molecular markers

The seeds were soaked for both Barhee and Madjhool females pollinated with different males date palm *cv.* (Fard, Ghnamiy, Dayri and Sabbad) and local date palm seeding males Sewy and Barhee (an individual of Sewy and Barhee satellite seedling as suggested by Mason, 1927) grown in Egypt for a period of 10 days with the change of water every period and after that the seeds were transferred to a cloth to complete the germination process. After germination, the germinated seeds are planted in black plastic pots containing a mixture of peatmoss and sand at a ratio of 2:1 by weight under the greenhouse conditions to form small plants (plantlets) to use in molecular analysis.

Total genomic DNA was isolated from young and fresh leave samples from each cultivar. The bulked DNA extraction was performed using DNeasy plant Mini Kit and following the manual instructions (Qiagen, Germany).

a. RAPD -PCR analysis conditions

Only five primers (were synthesized by Invitrogen by Thermo Fisher Scientific, UK) succeeded to generate reproducible polymorphic DNA products table 3. The

Table 3: List of the used RAPD and ISSR primer names and their nucleotide sequences.

No	RAPD Primer code	Sequence	No	ISSR	Sequence Primer code
1	OP-A02	5´TGCCGACGTG 3´	1	844-B	$(CT)_8GC$
2	OP-A10	5´GTCATCGCAG 3´	2	809	$(AG)_7GG$
3	OP-B18	5´CCACAGCAGT 3‘	3	IS-A02	$(GA)_9C$
4	OP-C19	5‘GTTGCCAGCC 3‘	4	17898-A	$(CA)_6AC$
5	OP-F01	5‘ACGGATCCTG 3‘	5	HB-11	$(GT)_6CC$

RAPD-PCR reactions were subjected to one cycle at 95ºC for 5 minutes, followed by 35 cycles at 96ºC for 30 seconds, 37ºC for 30 seconds and 72ºC for 30 seconds, then a final cycle of 72ºC for 5 minutes. The PCR products were separated on a 1.5% agarose gels and fragments sizes were estimated with 100bp ladder markers (1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100bp).

b. ISSR-PCR analysis

ISSR-PCR reactions were conducted using five primers (were synthesized by Invitrogen by Thermo Fisher Scientific, UK). The PCRs were programmed for one cycle at 94ºC for 4 min. followed by 45 cycles of 1 min. at 94ºC, 1 min. at 57ºC and 2 min at 72ºC the reaction was finally stored at 72ºC for 10 min. The PCR products were separated on a 1.5% agarose gels and fragments sizes were estimated with the 100bp ladder marker table 3.

c- Data analysis

The RAPD and ISSR gel images were scanned using the gel Doc 2000 Bio-Rad system and analyzed with Quantity One Software v 4.0.1 (Bio-Rad Laboratories, Hercules, Co. USA). The bands were sized and then binary coded by 1 or 0 for the presence or absence in each genotype. Data were than computed with the SPSS-16 program to produce a genetic distance matrix which assesses the similarity between two populations on the basis of the number of generated bands using Dice similarity coefficient (Dice, 1945).

Gene expression analysis for three genes (Phosphofructokinase, pectin(pectate) lyase and Xyloglucan: Xyloglucosyl transferase) for fruit quality of date palms (Barhee and Madjool) in Khalal stage of date palm development stages

For gene expression analysis, samples from fruits of date palm produced from crossing between males of date palm *cv.* (Ghnamiy, Sapad and local date palm seeding males (Sewy and Barhee) and females of date palms (Barhee and Madjool date palm *cv.*). The samples of fruits collected in Khalal stage of date palm fruits development and stored in liquid nitrogen and transported them to the laboratory (CURP Biotechnology, Cairo, Egypt) on dry ice and stored inside 80 C° freezers until use.

a. RNA extraction

Nucleic acids were extracted in 100 mM TRIS-HC1 pH 8.0, 1.5 M NaC1, 20raM EDTA, 20ram DTT (dithiothreitol) and 2% CTAB (hexadecyltrimethyl ammonium bromide). Date palm fruit tissues were finely

ground in liquid nitrogen and added frozen to the extraction buffer at a ratio of 1 : 10 (w/v). The solution was gently mixed at room temperature for 15 min, extracted with chloroform and centrifuged at 10000 g for 30 rain. Polysaccharides were precipitated from the aqueous phase by the slow addition of 0.33 vol. ethanol followed by another extraction with chloroform. The aqueous phase was saved and used for RNA isolation.

For isolating RNA the aqueous phase was made to 3.0 M LiC1, 1% v/v mercaptoethanol and placed at -20°C overnight. The precipitated RNA was collected by centrifugation at 10000 g for 30 rain, solubilized in TE, extracted with an equal volume of phenol-chloroform (1 : 1), reprecipitated with 2 vol. ethanol, 0.3 M sodium acetate and resuspended in sterile H_20 to a final concentration of 1 mg/ml. Storage of the RNA was at -80°C. Poly(A) + RNA was isolated by oligo (dT)-cellulose chromotography (Maniatis *et al.,* 1982).

b. Gel electrophoresis:

It was evaluated on 1% agarose gel stained with 10% ethidium bromide and observed using ultraviolet light.

c. cDNA synthesis

RNA samples were treated with DNase I to remove to remove trace amounts of DNA according to manufacturer recommendations. Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (No.#K1621) (https://assets.thermofisher.com/TFSAssets/LSG/manuals/MAN0012715_RevertAid_FirstStrand_cDNA_Syn_K1621_UG.pdf) was used to synthesize cDNA a according to manufacturer recommendations.

In a sterile, nuclease- free tube add the - 5 µg templet RNA, 1 µL Oligo (dT) primer and add Water, nuclease-free to 12 µL, Reaction was centrifuged briefly and incubated at 65°C for 5 min on ice. Followed by addition of 4 µL of 5X reaction buffer, 1 µL RiboLock RNase Inhibitor (20 U/µL), 2 µL of 10 mM dNTP Mix and 1 µL of Revert Aid M-MuLV RT (200 U/µL) and the total volume was 20 µL. mixed solutions was incubated for 60 min at 42°C and terminated the reaction by heating at 70°C for 5 min. The reverse transcription reaction product can be directly used in PCR applications or stored at -20°C for less than one week. For longer storage, -70°C is recommended.

d. Real time- PCR

Real time polymerase reaction (RT-PCR) of the samples were performed at the CURP Biotechnology Center, Faculty of Agriculture, Cairo University, Giza, Egypt using Fluorescent dye SYBR Green (Simply Green qPCR Master Mix, Rox)(https://www.genedirex.com/wp-content/uploads/2019/06/SQ101-0100.pdf) by qPCR (BioRAD catalog no. 1855200). Four qPCR primers were chosen that are associated with fruit quality in date palm according to date palm genome database (https://www.ncbi.nlm.nih.gov/). The nuclotied sequnces of the primers used in this study are presented in table 4.

Primers were designed by Gene Runner software for using Phosphofructokinase, pectin (pectate) lyase and Xyloglucan: Xyloglucosyl transferase from https://www.ncbi.nlm.nih.gov/ table 5. The β Actin most commonly used as reference gene was selected based on gene expression studies in different plant species (Sinha *et al.,* 2015). (https://www.ncbi.nlm.nih.gov)

e. Statistical analysis:

The following equations have been used for gene expression

ΔCt = Ct (gene of interest) - Ct (housekeeping gene)

The ΔCt where used to calculate $\Delta\Delta Ct$ according to the following equation

$\Delta\Delta Ct = \Delta Ct$ (gene of interest) - ΔCt (housekeeping gene)

And from these knowledge the fold gene expression change calculated by as $2^{-\Delta\Delta Ct}$

Results and Discussion

Molecular markers and genetic diversity between males and females of this study

a- ISSR analysis and polymorphism

Table 4: The qRT-PCR primers used in this study.

Gene	No. of isoforms	Function	Forward primer sequence	Reverse primer sequence
Phosphofructo-kinase (Predicted)	15	Starch and sucrose metabolism	5'CCTCGCCACCC TCACCAACCCC-3'	5'CGGCGGCGGC GGAGGAGA-3'
pectin(pectate) lyase(Predicted)	20	Fruit Ripening	5'GGGAGGGGGG CCAAGGTGGC-3'	5'CGGGGACCTACG GACCATGCCC-3'
Xyloglucan:Xyloglucosyl transferase(Predicted)	5	Fruit Ripening	5'GGCAATGTCAGC GGCCAACCGT-3'	5'CCGCGAAGATG ACATGCTGCGG-3'
β Actin	-	Housekeeping gene	5'TCAATGTGCCT GCCATGTATGT-3'	5'GCGGCCGCTA GCATAGAG- 3'

Table 5: The accession number of genes and isoforms used in this study.

Gene	No. of isoforms	Accession numbers
Phosph-ofructo-kinase	15	XM_017841435.2, XM_026802388.1, XM_026806207.1, XM_026808209.1, XM_008780812.3, XM_008780974.3, XM_008783617.2, XM_008795862.3, XM_008795864.3, XM_008795865.3, XM_008796996.2, XM_008797271.3, XM_008800379.3, XM_008801540.3, XM_008804395.3,
pectin (pectate) lyase	20	XM_008778688.2, XM_008778689.2, XM_008785547.1, XM_008788538.2, XM_008795282.2, XM_008796160.2, XM_008797801.2, XM_008802341.3, XM_008802342.3, XM_008806223.3, XM_008806227.2, XM_008806228.2, XM_008807435.1, XM_008814183.3, XM_017842743.1, XM_017846687.2, XM_026801736.1, XM_026807979.1, XM_026807980.1, XR_001879538.2,
Xyloglucan: Xyloglucosyl transferase	4	XM_008801217.2, XM_017844494.2, XM_026807288.1, XM_008780219.2,
β Actin	-	XM_008778129

The ISSR technique involves amplification of DNA segments present at an amplifiable distance between two identical microsatellite repeats oriented in opposite directions. The technique uses microsatellites, usually 16-25 bp long, as primers in a single primer PCR reaction. This targets multiple genomic loci and usually yields dominant markers. (Zehdi *et al.,* 2004b).

In this work, the genetic diversity between Barhee and Madjool as female date palms and the six male types as males date palms (Fard, Ghnamiy, Dayri and Sapad) and local date palm seeding males Sewy and Barhee (an individual of Sewy and Barhee satellite seedling as suggested by Mason, 1927) were investigated using 8 ISSR primers. Fig. 1. illustrates the ISSR profiles amplified with the primers.

As illustrated in table 6 the ISSR profiles amplified by the different primers comprised multiple bands ranging from 3 to 13 bands in Barhee and Madjool date palm *cv.* and the six male types. The total number of DNA fragments amplified by the eight primers was 61 with an average of 7.63 bands/primer. The number of polymorphic bands ranged from 1 to 12 per primer with an average of 4.50 bands/primer and the monomorphic bands ranged from 1 to 6 with an average of 3.13 bands/primer. Primer 844B amplified the highest number of bands (13) and The highest polymorphism percentage between cultivars was 92.31% by the primer 844B. While, the lowest number of polymorphic bands was revealed by the primer HB8 (1). Therefore, the different primers expressed different levels of polymorphism, ranging from 3.33% with the primer HB8 to 92.31% with the primer 844B and the total polymorphism percentage was 59.02% for this cultivars. The size of amplified fragments varied with the different primers, ranging from 1157 to 157 bp Fig. 1.

Table 6: Polymorphism percentages among the 8 date palm cultivars by ISSR markers.

	Primers	Band size range (bp)	Generated Bands	Monomorphic bands	Polymorphic bands	Polymorphism %
1	HB9	242-526	3	2	1	33.33
2	HB10	276-723	3	3	0	00.00
3	HB11	172-640	9	3	6	66.67
4	HB12	211-1157	11	6	5	45.46
5	HB13	251-100	5	5	0	00.00
6	814	157-1107	11	3	8	72.73
7	844A	190-565	6	2	4	66.67
8	844B	284-928	13	1	12	92.31
Total			61	25	36	59.02
Average			7.63	3.13	4.50	

In this respect, Haider *et al.,* (2012) detected the genetic polymorphism in 23 date palm cultivars in Syria representing 18 female and five male cultivars was assessed using ISSR markers. Results revealed that the average polymorphism detected by the ISSR (50.6%). Sabir *et al.,* (2014) used ISSR molecular marker to detect relationships among 10 date cultivars from Saudi Arabia. Thirteen ISSR primers were examined. The level of polymorphism among cultivars for ISSRs ranged from 20% to 100% with an average of 85%. AJWA cultivar had the highest number of cultivar-specific ISSR markers, whereas DEK, PER, SUK-Q, SHA and MOS-H cultivars had the lowest. The highest pairwise similarity indices for ISSRs, were 84% between DEK (female) and PER (female). The lowest similarity indices were 65% between TAB (female) and SUK-Q (male). Khalifa *et al.,* (2016) applied the ISSR markers on top quality eight commercial cultivars in Libya (Umfetity, Bekrary, Alhamraya, Sufeer Genab, Alsaeedy Show, Farag Barameel, Majhool Alheelo and Alkhadraya). DNA variations were explored using eleven ISSR. All markers used generated polymorphic bands among the different cultivars that can be used as molecular markers for their differentiation. The results

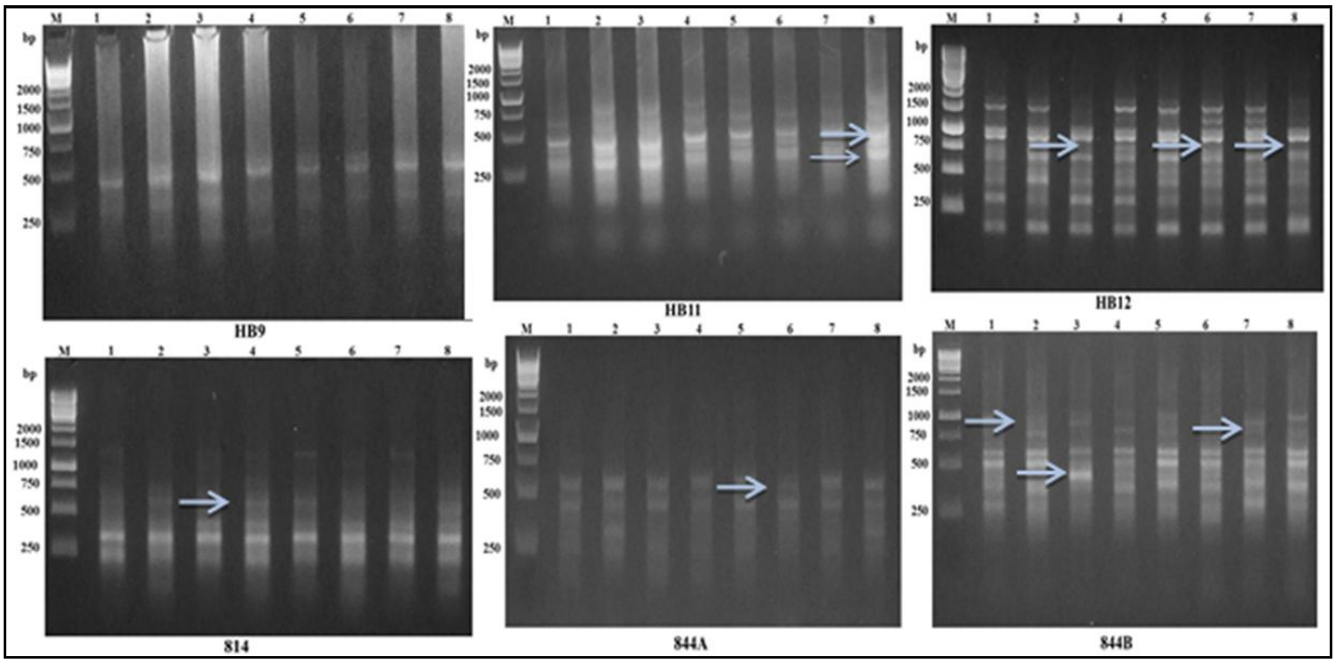

Fig. 1: ISSR profiles using the 8 primers with eight date palm cultivars. 1: Barhee; 2: Majhool; 3: Fard; 4: Ghnamiy; 5: Dayri; 6: Sapad; 7: Barhee Satellite seedling and 8: Sewy Satellite seedling. M represents the 1Kb DNA molecular marker.

indicate that ISSR system can efficiently identify and differentiate between the selected cultivars.

Genetic relationships and cluster analysis of the 8 date palm cultivars revealed by ISSR analysis

To determine the genetic relationships between the 8 date palm cultivars, the scoring data (1 for presence and 0 for the absence) resulting from the eight tested primers were used to compute the similarity matrices according to Nei and Li (1979) coefficients. These similarity matrices were used to generate a dendrogram using UPGMA method. As shown in table 7 the genetic similarity ranged from 0.144 to 1.000. The highest genetic similarity revealed by the ISSR analysis (1.000) was between Fard and Sapad cultivars. While, the lowest genetic similarity (0.144) was between Barhee cultivar and Dayri male date palm, while the genetic similarity (0.00) was between Ghnamiy and Dayri male date palms.

Based on Nei and Li's coefficients of genetic similarity between the studied date palms, dendrograms using the UPGMA method Fig. 2 and table 7 were constructed. The dendrogram Fig. 2 grouped into two main clusters. The first cluster the Fard male date palm and the second culster divedid to 2sub culsters one included Majhool date palm and the second included the Barhee date palm, Fard, Ghnamiy, Dayri Sapad and Sewy satellite seedling and Barhee satellite seedling as male

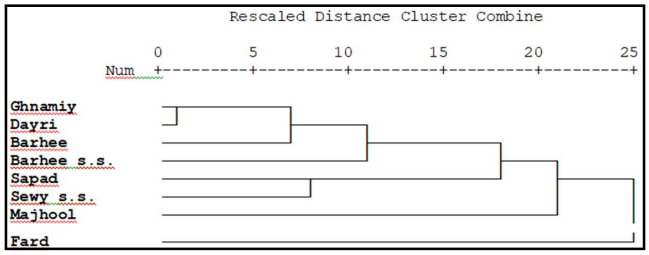

Fig. 2: A dendrogram for 8 date palm cultivars based on ISSR data.

Table 7: Genetic similarity matrix 8 date palm cultivars based on ISSR data.

	Barhee	Mejhool	Fard	Ghnamiy	Dayri	Sapad	Barhee Satellite seedling	Sewy Satelliteseedling
Barhee	1.000							
Mejhool	0.653	1.000						
Fard	0.266	0.582	1.000					
Ghnamiy	0.182	0.266	0.465	1.000				
Dayri	0.144	0.465	0.559	0.000	1.000			
Sapad	0.709	0.535	1.000	0.300	0.144	1.000		
Barhee Satellite seedling	0.388	0.582	0.801	0.233	0.197	0.266	1.000	
Sewy Satellite seedling	0.888	0.602	0.814	0.490	0.349	0.182	0.349	1.000

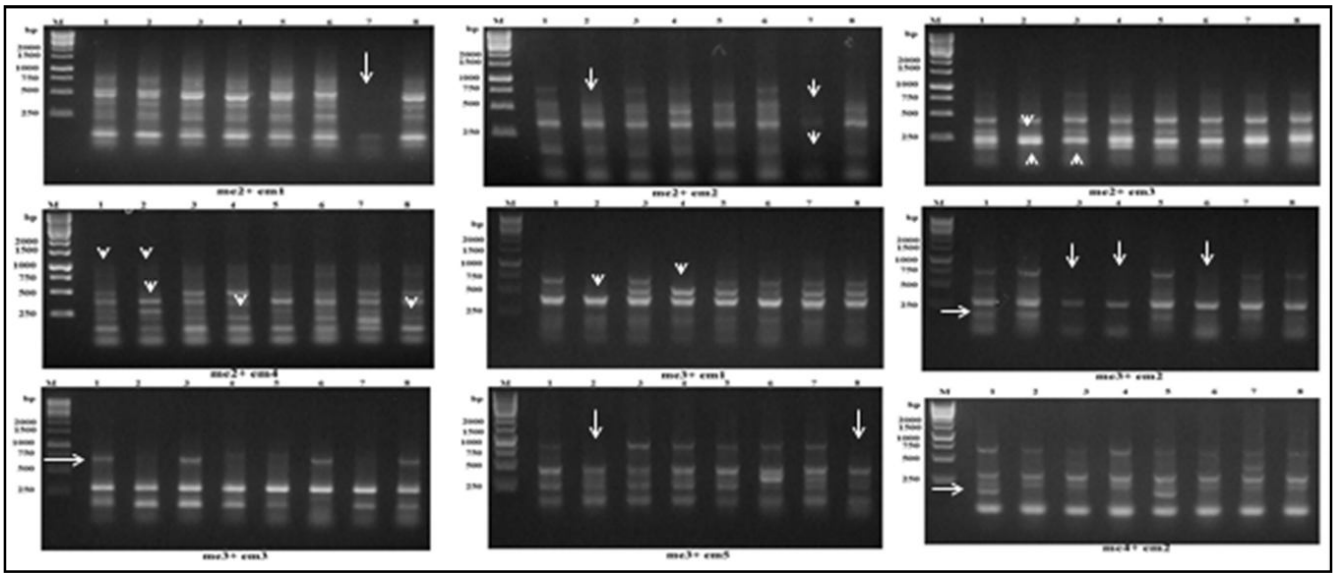

Fig. 3: SRAP profiles using the 8 primers with eight date palm cultivars. 1: Barhee; 2: Majhool; 3: Fard; 4: Ghnamiy; 5: Dayri; 6: Sapad; 7: Barhee Satellite seedling and 8: Sewy Satellite seedling. M represents the 1Kb DNA molecular marker.

date palms.

b- SRAP analysis

In this work, the variability of date palm genome were studied by applying SRAP technique. Fifty combinations of SRAP primer pairs were used for the Barhee and Madjool as female date palms and the six male types as males date palms (Fard, Ghnamiy, Dayri and Sapad) and local date palm seeding males Sewy and Barhee (an individual of Sewy and Barhee satellite seedling as suggested by Mason, 1927) generating 108 bands in total with an average 7.2 out of which 56 bands were polymorphic with an average 3.7 and all the tested primers produced the polymorphic bands. The total number of amplified amplicons among tested primers ranged from 4 by the combination me3x em3 to 10 fragments by the combination me 2x em1 with size range 100-925 bp Fig. 3 and table 8. The combination me 2x em1 exhibited the highest polymorphism percentage (80.0%) while the combination me 4x em5 gave the lowest polymorphism (25.0%). The total of polymorphism% generated with all primers used was 51.85%.

Table 8: Polymorphism percentages among the 8 date palm cultivars by SRAP markers.

No	Primers	Band size range (bp)	Gene-rated Bands	Monom-orphic bands	Polym-orphic bands	Poly-morp-hism %
1	me 2x em1	124-697	10	2	8	80.00
2	me2 x em2	103-682	7	2	5	71.43
3	me2 x em3	181-475	7	5	2	28.57
4	me 2x em4	171-837	8	4	4	50.00
5	me 2x em5	200-721	8	5	3	37.50
6	me 3x em1	153-449	6	3	3	50.00
7	me 3x em2	141-690	8	2	6	75.00
8	me 3x em3	166-549	4	2	2	50.00
9	me 3x em4	194-498	5	3	2	40.00
10	me 3x em5	171-795	7	3	4	57.14
11	me 4x em1	100-744	6	4	2	33.33
12	me 4x em2	118-597	6	4	2	33.33
13	me 4x em3	146-414	9	3	6	66.67
14	me 4x em4	178-357	9	4	5	55.56
15	me 4x em5	174-925	8	6	2	25.00
Total			108	52	56	51.85
Average			7.2	3.5	3.7	

In this regards, Khalifa *et al.,* (2016) studied the molecular analysis of commercial date palm cultivars in Lybia using nine combinations of SRAP markers. All markers used generated polymorphic bands among the different cultivars that can be used as molecular markers for their differentiation. The results indicate that SRAP systems can efficiently identify and differentiate between the selected cultivars.

Genetic relationships and cluster analysis of the 8 date palm cultivars revealed by SRAP analysis

The genetic relationships between the 8 date palm cultivars resulting from the fifty combination of tested primers were used to compute the similarity matrices according to Nei and Li (1979) coefficients. These similarity matrices were used to generate a dendrogram using UPGMA method. As shown in table 9 the genetic similarity ranged from 0.021 to 1.000. The highest genetic similarity revealed by the SRAP analysis (1.000) was between Barhee as female date palm and Barhee satellite

Table 9: Genetic similarity matrix 8 date palm cultivars based on SRAP data.

	Barhee	Mejhool	Fard	Ghnamiy	Dayri	Sapad	Barhee Satellite seedling	Sewy Satelliteseedling
Barhee	1.000							
Mejhool	0.500	1.000						
Fard	0.596	0.734	1.000					
Ghnamiy	0.472	0.708	0.000	1.000				
Dayri	0.041	0.528	0.339	0.405	1.000			
Sapad	0.287	0.500	0.021	0.277	0.133	1.000		
Barhee Satellite seedling	1.000	0.744	0.529	0.813	0.528	0.700	1.000	
Sewy Satellite seedling	0.421	0.543	0.256	0.126	0.174	0.235	0.642	1.000

seedling as male date palm. While, the lowest genetic similarity (0.021) was between Fard cultivar and Sapad male date palms while, the genetic similarity (0.00) was between Fard and Ghnamiy male date palms.

Based on Nei and Li's coefficients of genetic similarity between the studied date palms, dendrograms using the UPGMA method Fig. 4 and table 9 were constructed. The dendrogram Fig. 4 grouped into two main clusters. The first cluster the contained Barhee satellite seedling as male date palm and the second cluster divided to 2sub clusters one included Majhool date palm and the second included the Fard, Ghnamiy, Sapad, Dayri, Sewy satellite seedling as male date palms and Barhee as female date palm.

c- Combined identification based on ISSR and SRAP analyses:

Varieties distribution on the consensus tree according to the banding patterns of ISSR differed from SRAP that based on banding patterns, which may be due to that each technique, amplified different parts of the genome. So, it is better to use the combination of the banding patterns of the two techniques to use more segments of the genome that will increase the validity of the consensus tree.

The genetic similarities and phylogenetic relationships between Barhee and Madjool as female date palms and the six male types as males date palms (Fard, Ghnamiy, Dayri and Sapad) and local date palm seeding males Sewy and Barhee genotypes based on a combined data of ISSR and SRAP-PCR markers table 10 and Fig. 5 were determined using UPGMA computer program. The highest similarity values were recorded (1.0) between Barhee as female and Dayri as male genotypes, while the lowest similarity value (0.136) was recorded between Majhool as female and Sapad as male date palm

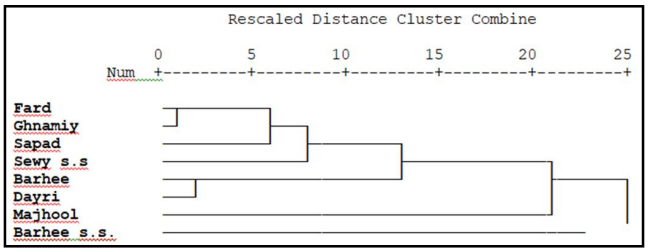

Fig. 4: A dendrogram for 8 date palm cultivars based on SRAP data.

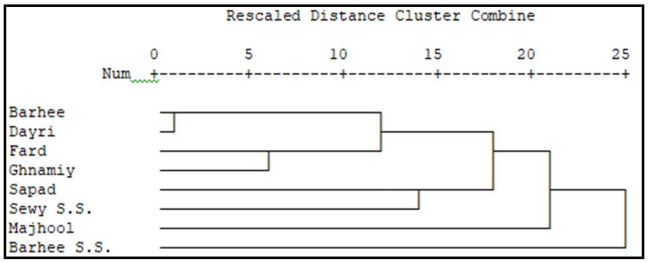

Fig. 5: A dendrogram for 8 date palm cultivars based on combination between ISSR and SRAP data.

Table 10: Genetic similarity matrix 8 date palm cultivars based on combination between ISSR and SRAP data.

	Barhee	Mejhool	Fard	Ghnamiy	Dayri	Sapad	Barhee Satellite seedling	Sewy Satelliteseedling
Barhee	1.000							
Mejhool	0.318	1.000						
Fard	0.545	0.455	0.000					
Ghnamiy	0.682	0.500	0.818	1.000				
Dayri	1.000	0.364	0.409	0.909	1.000			
Sapad	0.364	0.364	0.318	0.636	0.682	1000		
Barhee Satellite seedling	0.227	0.136	0.000	0.227	0.273	0.545	1.000	
Sewy Satellite seedling	0.182	0.273	0.227	0.636	0.591	0.591	0.182	1.000

genotypes and there was no similarity between Fard and Barhee Satellite seedling genotypes.

The dendrogram based on ISSR and SRAP-PCR markers Fig. 5 grouped into two main clusters. The first cluster contanied the Barhee Satellite seedling male date palm and the second culster divedid to 2sub culsters one included Majhool date palm and the secoond included the Barhee date palm, Dayri, Fard, Ghnamiy, Sapad and Sewy satellite seedling as male date palms.

Sex identification at early stages for progeny of date palms by molecular markers

In this work, the using both RAPD and ISSR markers for sex determination analysis of Palm germplasm was studied at seedling stage or early stage to identify the gender of the selected small plants of date palm resulting from crossing between Barhee and Madjhool females with different males date palm *cv.* (Fard, Ghnamiy, Dayri and Sabbad) and local date palm seeding males Sewy and Barhee (an individual of Sewy and Barhee satellite seedling as suggested by Mason, 1927).

a- RAPD analysis and polymorphism

At the polymorphism level, a high level of polymorphism was generated by using the 5 RAPD primers Fig. 6 and table 11. The total number of DNA fragments amplified by the five primers was 87 with an average of 17.4 bands/primer ranged from 12-22 bands. The number of polymorphic bands ranged from 8 to 20 per primer with an average of 13.4 bands/primer and the monomorphic bands ranged from 2 to 7 with an average of 4 bands/primer. Primers OP-B18 and OP-C19 amplified the highest number of bands (22) and The highest polymorphism percentage between cultivars was 90.91% by the primer OP-B18 but the lowest polymorphism percentage was 66.67% by the primer OP-A10 and the total polymorphism percentage was 77.01%. While, the highest and lowest number of polymorphic bands was revealed by the primers OP-B18 and OP-A10 (20, 8) respectively. The size of amplified fragments varied with the different primers, ranging from 1404 to 170 bp.

Genotype identification of positive and negative cultivar specific markers of the 10 date palm cultivars (Male, female, progeny from Barhee and progeny from Majhool) detected in RAPD analysis

Table 11: Polymorphism percentages among the 10 date palm cultivars (Male, female, progeny from Barhee and progeny from Majhool) by RAPD markers.

	Primers	Band size range (bp)	Gene-rated Bands	Monom-orphic bands	Polym-orphic bands	Poly-mor-phism %
1	OP-A02	170-1156	14	4	10	71.43
2	OP-A10	224-1421	12	4	8	66.67
3	OP-B18	186-1106	22	2	20	90.91
4	OP-C19	253-1386	22	3	19	86.36
5	OP-F01	210-1404	17	7	10	85.83
6	Total		87	20	67	77.01
7	Average		17.4	4	13.4	

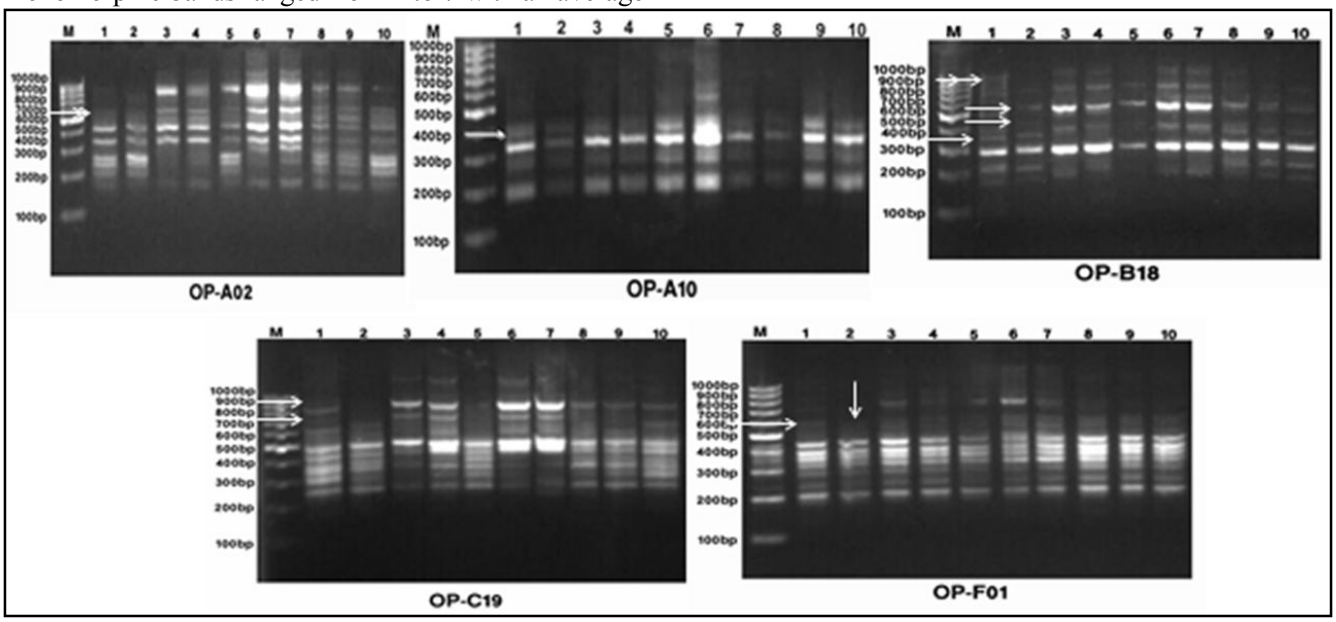

Fig. 6: RAPD profiles using the 5 primers with ten date palm cultivars. 1: Barhee Satellite seedling; 2: Barhee; 3: Progeny from Barhee 1; 4: Progeny from Barhee 2; 5: Progeny from Barhee 3; 6: Progeny from Barhee 4; 7: Progeny from Majhool1; 8: Progeny from Majhool 2; 9: Progeny from Majhool 3; 10: Progeny from Majhool 4. M represents the 1Kb DNA molecular marker.

Table 12: List of positive and negative cultivar specific markers of the 10date palm cultivars (Male , female , progeny from Barhee and progeny from Majhool) detected by RAPD analysis.

Primer	Number and MW in bp of cultivar specific marker										
	Barhee Satellite seedling	Barhee	Prog. from Bar. 1	Prog. from Bar. 2	Prog. from Bar. 3	Prog. from Bar. 4	Prog. from Maj. 1	Prog. from Maj. 2	Prog. from Maj. 3	Prog. from Maj. 4	Total
OPA02		-	-	1(273)	-	-	-	-	-	-	1
OP-A10	1(386)	-	-	-	1(451)	2(624)(570)	-	-	1(331)	-	5
OP-B18	4(1016)(879)(499)(398)	1(467)	1(199)	-	-	-	-	1(222)	-	-	7
OP-C19	2(882)(748)	-	-	-	1(452)	-	1(253)	-	-	1(590)	5
OP-F01	1(575)	-	-	-	-	1(642)	-	-	-	-	2
Total	8	1	1	1	2	3	1	1	1	1	20

The genotype specific unique bands of RAPD marker with approximate size are shown in table 12, 5 primers were able to generate unique bands that could differentiate the studied date palm genotypes. The total number of generate unique bands ranged from 1 to 7 bands. The maximum number of unique markers was identified in Barhee Satellite seedling as male date palm with 8 bands but the minimum number of unique markers was 1 band in Barhee as female, progeny from Barhee 1, 2 and progeny from Majhool 1, 2, 3, 4. Primer OP-18 generated the highest number of cultivar-specific markers (7), while primer OP-A02 generated the lowest (1).

In conclusion, all RAPD primers used in this study it allowed sufficient distinction between the male and female date palms and offspring. The RAPD markers can be used in subsequent experiments to detect molecular markers for genes with male and female identification in palm cultivars. Based on results, only four primers OP-A10, OP-B18, OP-C19 and OP- F01 yielded a clear and characteristic amplification products, approximately (386), (1016, 879, 499 and 398), (882, 784) and (575) bp in size, respectively only in male and not in female Fig. 6 and table 15. While primer OP-B18 yielded a clear and characteristic amplification marker, approximately 467 bp long, only in Female and not in male Fig. 6 and table 15.

These results are in agreement with that of Younis *et al.,* (2008) who reported that the using RAPD anylsis for identification the gender in date palm gave three positive specific markers for females by primers A10-490, A12-750, D10-800 and two for males by primers A12-370, D10-675 and the level of polymorphism across cultivars was 70% as revealed by RAPD. Dhawan *et al.,* (2013) reported that using of, genomic DNA from 45 individual plants (25 female and 20 male) belonging to different varieties of date palm was subjected to PCR amplification using 100 random amplified polymorphic DNA (RAPD) and 104 intersimple sequence repeat

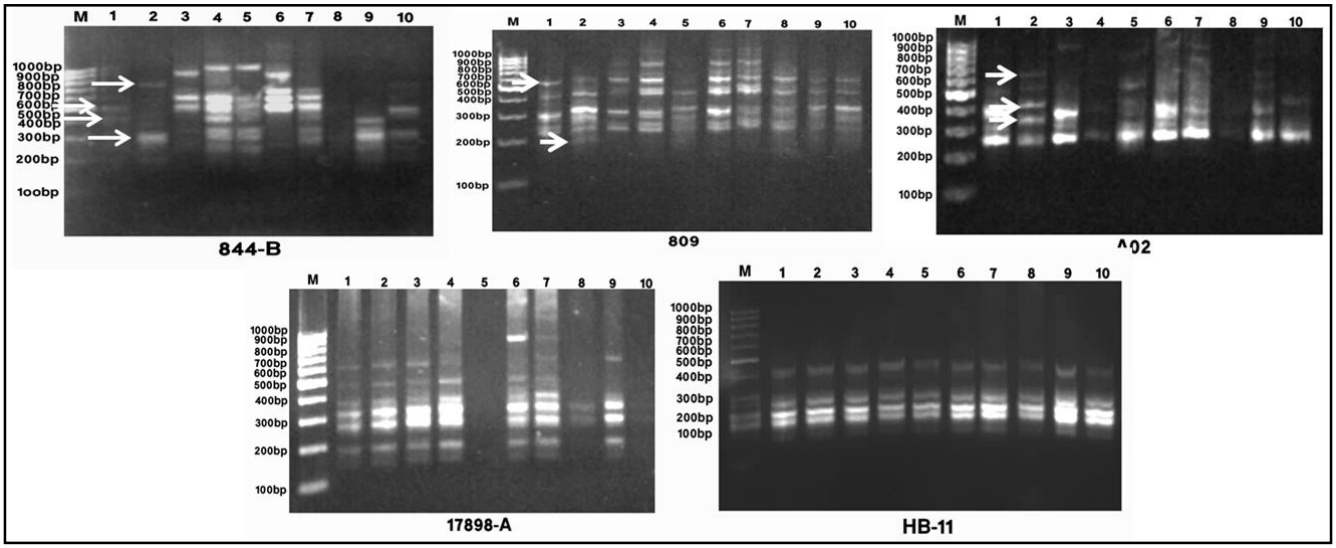

Fig. 7: ISSR profiles using the 5 primers with ten date palm cultivars. 1: Barhee Satellite seedling; 2: Barhee; 3: Progeny from Barhee 1; 4: Progeny from Barhee 2; 5: Progeny from Barhee 3; 6: Progeny from Barhee 4; 7: Progeny from Majhool 1; 8: Progeny from Majhool 2; 9: Progeny from Majhool 3; 10: Progeny from Majhool 4. M represents the 1Kb DNA molecular marker.

Table 13: Polymorphism percentages among the 10 date palm cultivars(Male , female , progeny from Barhee and progeny from Majhool) by ISSR markers.

No	Pri-mers	Band size range (bp)	Gene-rated Bands	Monom-orphic bands	Polym-orphic bands	Polym-orphism %
1	844-B	182- 1589	34	0	34	100.00
2	809	167- 1048	20	1	13	95.00
3	IS-A02	227 - 1700	16	1	15	93.75
4	17898-A	181-406	10	3	7	70.00
5	HB-11	192 - 462	7	6	1	14.29
6	Total		87	12	70	80.45
7	Average		17.4	2.4	14.0	

(ISSR) primers. Only one RAPD primer, OPA-02, amplified a fragment of ~1.0 kb in all the individual samples of male genotypes, whereas this fragment was absent in all the female genotypes. Mohammed and Mohamed (2019) reported that the using of six RAPD primers were examined for their validation in sex determination of date palm genotypes. PCR amplification was performed using these primers. Four RAPD primers OPA02, OPJ-09, RD A02 and RD A21 were amplified male specific band with size of 1000, 1100, 1000 and 1400 pb respectively. The specific bands were observed clearly among all male genotypes and absent in female samples.

b- ISSR analysis and polymorphism

ISSR is a class of molecular markers based on inter-tandem repeats of short DNA sequences. These inter repeats are highly polymorphic, even among closely related genotypes, due to the lack of functional constraints in these nonfunctioning regions (Younis *et al.,* 2008).

At the polymorphism level, a high level of polymorphism was generated by using the 5 ISSR primers Fig. 7 and table 13. The total number of DNA fragments amplified by the five primers was 87 with an average of 17.4 bands/primer ranged from 7-43 bands. The number of polymorphic bands ranged from 1 to 34 per primer with an average of 14.0 bands/primer and the monomorphic bands ranged from 1 to 6 with an average of 2.4 bands/primer. Primer 844-B amplified the highest number of bands (34) and The highest polymorphism percentage between cultivars was 100.00% by this the primer but the lowest polymorphism percentage was 14.29% by the primer HB-11 and the total polymorphism percentage was 80.75%. While, the highest and lowest number of polymorphic bands was revealed by the primers 844-B and HB-11 (34, 1) respectively. The size of amplified fragments varied with the different primers, ranging from 1700 to 167 bp.

Genotype identification of positive and negative cultivar specific markers of the 10 date palm cultivars (Male, female, progeny from Barhee and progeny from Majhool) detected in ISSR analysis.

The genotype specific unique bands of ISSR marker with approximate size are shown in table 14, 3 ISSR primers from 5 ISSR primers were able to generate unique bands that could differentiate the studied date palm genotypes. The total number of generate unique bands ranged from 1 to 6 bands. the maximum number of unique markers was identified in Barhee cultivar as female date palm with 5 bands but the minimum number of unique markers was 1 band in progeny from Majhool 2, 3. Primer 809 generated the highest number of cultivar-specific markers (6), while primer 17898-A and primer HB-11 generated the lowest (1).

In conclusion, the 3 ISSR primers from 5 ISSR primers used in this study it allowed sufficient distinction between the male and female date palms and offspring. The ISSR markers can be used in subsequent experiments to detect molecular markers for genes with male and female identification in palm cultivars. Based on results, only two primers 844-B and 809 yielded a clear and characteristic amplification products, approximately (404) and (576, 322) bp in size, respectively only in male and not in female Fig. 7 and table 15. While primer 844-B, 809 and IS-A02

Table 14: List of positive and negative cultivar specific markers of the 10 date palm cultivars (Male, female, progeny from Barhee and progeny from Majhool) detected by ISSR analysis.

Primer	Number and MW in bp of cultivar specific marker										
	Barhee Satellite seedling	Barhee	Prog. from Bar. 1	Prog. from Bar. 2	Prog. from Bar. 3	Prog. from Bar. 4	Prog. from Maj. 1	Prog. from Maj. 2	Prog. from Maj. 3	Prog. from Maj. 4	Total
844-B	1(404)	1(802)	-	-	-	-	-	-	-	-	2
809	2(576)(322)	2(498)(208)	-	1(528)	-	-	-	1(167)	-	-	6
IS-A02	-	2(692)(333)	-	-	-	-	-	-	-	-	2
17898-A	-	-	-	1(716)	-	-	-	-	-	-	1
HB-11	-	-	-	-	-	-	-	-	1(270)	-	1
Total	3	5	-	2	-	-	-	1	1	-	12

Table 15: Sex-specific markers in Date Palm based on RAPD and ISSR analysis.

	Male positive marker	NO	Female positive markers	NO
RAPD	OP-A10(386),OP-B18(1016,879,499,398) OP-C19 (882,748) OP-F01(575)	8	OP-B18 (467)	1
ISSR	844-B (404),809-(576,322)	3	844B(802),809(498,208),IS-A02(692,333)	5

yielded a clear and characteristic amplification marker, approximately (802), (498, 202), (692, 333) bp long, only in Female and not in male Fig 7 and table 15.

These results are in agreement with that of Mohammed and Mohamed (2019) who reported that using of Eight primers, six RAPD and two ISSR primers were examined for their validation in sex determination of date palm genotypes. Four RAPD primers OPA02, OPJ-09, RD A02 and RD A21 were amplified male specific band with size of 1000, 1100, 1000 and 1400 pb respectively, while ISSR markers could not.

These results are in agreement with that of Younis *et al.,* (2008) who reported that the using ISSR anylsis for identification the gender in date palm gave five positive specific markers for males by primers HB10-1010, HB9-340, HB12-375, 814-590 and 844A-920 and the level of polymorphism across cultivars was 87% as revealed by ISSR. Al-Ameri *et al.,* (2016) reported that screening of two hundred Inter ISSR primers among male and female Date palm plants to identify sex-specific marker, only two primers (IS A02 and IS A71) were found to be associated with sex. The primer IS A02 produced a unique band of size 390 bp and was found in all female plants, while it was absent in all male plants. Contrary to this, the primer IS A71 produced a unique band of size 380 bp and was clearly found in all male plants and it was absent in all the female plants. These markers are efficient, highly reliable and reproducible for sex identification at the early stage of seedling.

Gene expression analysis for three genes (Phosphofructokinase, pectin (pectate) lyase and Xyloglucan: Xyloglucosyl transferase) for fruit quality of date palms (Barhee and Madjool) in Khalal stage of date palm development stages

Fruits or dates are the most economically valuable product of date palm, we made an effort to identify differentially expressed genes involved in fruit development and ripening to provide a starting point for further investigation. Studding the quality genes of date palm in the Khalal stage after pollination with different males of date palms (Ghnamiy, Sapad and local date palm seeding males Sewy and Barhee) because The Khalal stage is a critical period for the fruit to begin maturing and shows many metabolic activities, such as carbohydrate, amino acid, lipid and cofactors/vitamins metabolisms. The genes of starch/ sucrose/galactose metabolisms and glycolysis/ gluconeogenesis, which peak at this stage (Yin *et al.,* 2012).

Tissues from date palms Barhee and Majhool fruits separated for RNA extraction, cDNA synthesis and real time PCR investigation. The amplification data showed that the peaks exceed the threshold line. The results of the Phosphofructokinase, pectin (pectate) lyase and Xyloglucan: Xyloglucosyl transferase gene expression of the fruits of Barhee and Majhool date palms in the Khalal stage from development stages of date palm fruits shown in table 16.

The data were analyzed to determine the highest time for gene expression by determine the fold of the gene expression through using Ct value (the cycle threshold = number of cycles required for the fluorescent signal to cross the threshold of the background level) of housekeeping gene (β Actin) and Ct of the target gene (Phosphofructokinase) to get:

ΔCt = Ct (gene of interest) - Ct (housekeeping gene)

The Ct levels are inversely proportional to the amount of target nucleic acid in the sample (*i.e.* the lower the Ct level, the greater the amount of target nucleic acid in the sample).

The ΔCt where used to calculate ΔΔCt according to the following equation ΔΔCt = ΔCt (gene of interest) - ΔCt (housekeeping gene) And from these knowledge the

Table 16: Differences in delta Cycle Threshold (ΔCt) of genes studied in Barhee and Majhool date palms in Khalal stage after pollination with the four males of date palms.

Treatments	ΔCt Phosphofr-uctokinase	ΔCt Pectin lyase	ΔCt Xylog-lucan
Barhee x Ghnamiy	2.96	-2.04	10.62
Barhee x Sapad	5.80	-1.59	12.76
Barhee x Barhee Satellite seedling	3.68	-0.31	13.32
Barhee x Sewy Satellite seedling	4.60	2.08	12.83
Mejhool x Ghnamiy	2.36	2.06	9.86
Mejhool x Sapad	2.63	2.23	9.09
Mejhool x Barhee Satellite seedling	1.59	3.61	9.07
Mejhool x Sewy Satellite seedling	4.74	2.31	10.36

fold gene expression change calculated by as $2^{-\Delta\Delta Ct}$ table 16.

a. The Phosphofructokinase gene expression analysis

In the current study, the fold change values in which the quantitative levels of mRNA of genes of interest in relative to the reference gene, have been used to compare the response of the female date palms Barhee and Majhool pollinated with four males date palms (Ghnamiy, Sapad and local date palm seeding males Sewy and Barhee) in the Khalal stage from the stages of the date palm development fruits.

Treatments (Mejhool x Sapad (T1), Mejhool x Barhee S.S. (T2) and Mejhool x Sewy S.S. (T3)) in Majhool *cv.* gave higher expression for Phosphofructokinase gene compared with treatments for Barhee *cv.* (Barhee x Sapad (T1), Barhee x Barhee S.S. (T2) and Barhee x Sewy

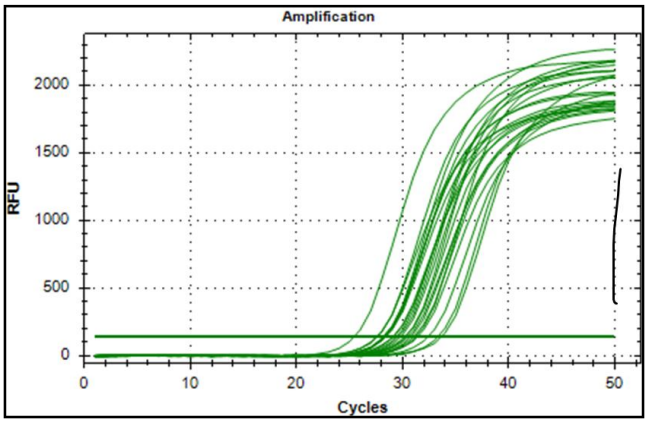

Fig. 8: Amplification plot of Phosphofructokinase gene in fruit samples results from crossing between Barhee and Majhool as female date palms and Ghnamiy , Sapd, Barhee satellite seedling and Sewy satellite seedling as male date palms in Khalal satge.

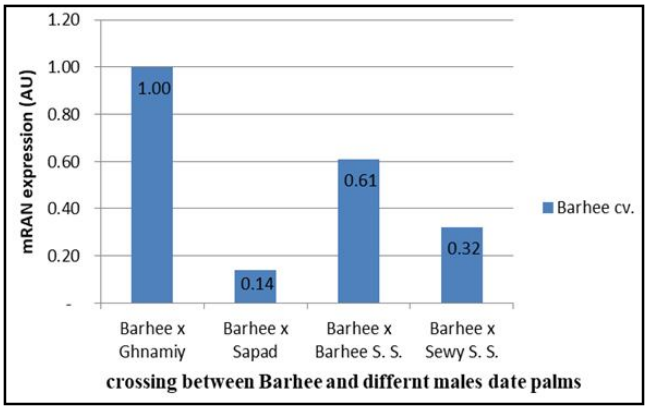

Fig. 9: Expression of Phosphofructokinase gene in fruit samples results from crossing between Barhee as female date palm and Ghnamiy , Sapd, Barhee satellite seedling and Sewy satellite seedling as male date palms in Khalal satge.

Table 17: The fold change of the Phosphofructokinase gene in Barhee and Majhool date palms.

Samples of Barhee	β Actin	Phospho.	ΔCt	Avg.	ΔΔCt	Fold change	Samples of Majhool	β Actin	Phospho.	ΔCt	Avg.	ΔΔCt	Fold change
Barhee x Ghnamiy(c)	25.47	27.49	2.03	2.69	0	1	Mejhool x Ghnamiy(c)	27.08	32.35	5.27	2.36	0	1
Barhee x Ghnamiy(c)	27.10	30.28	3.18				Mejhool x Ghnamiy(c)	26.98	29.37	2.39			
Barhee x Ghnamiy(c)	25.40	29.09	3.68				Mejhool x Ghnamiy(c)	25.95	25.39	-0.57			
Barhee x Sapad (T1)	25.32	31.04	5.73	5.80	2.84	014	Mejhool x Sapad(T1)	28.75	34.94	6.20	2.63	0.26	0.83
Barhee x Sapad(T1)	24.63	30.70	6.07				Mejhool x Sapad(T1)	27.52	27.59	0.07			
Barhee x Sapad(T1)	25.75	31.37	5.61				Mejhool x Sapad(T1)	27.92	29.53	1.61			
Barhee x Barhee S.S. (T2)	25.44	29.12	3.67	3.68	0.71	0.61	Mejhool x Barhee S.S. (T2)	28.57	31.20	2.63	1.59	-0.77	1.70
Barhee x Barhee S.S. (T2)	25.25	28.36	3.11				Mejhool x Barhee S.S. (T2)	27.13	28.19	1.06			
Barhee x Barhee S.S. (T2)	24.01	28.27	4.26				Mejhool x Barhee S.S. (T2)	27.21	28.30	1.09			
Barhee x Sewy S.S. (T3)	25.39	28.13	2.75	4.60	1.64	0.32	Mejhool x Sewy S.S. (T3)	29.05	33.62	4.57	4.74	2.37	0.19
Barhee x Sewy S.S. (T3)	22.17	29.77	7.60				Mejhool x Sewy S.S. (T3)	27.12	30.16	3.04			
Barhee x Sewy S.S. (T3)	27.01	30.47	3.46				Mejhool x Sewy S.S. (T3)	26.69	33.29	6.60			

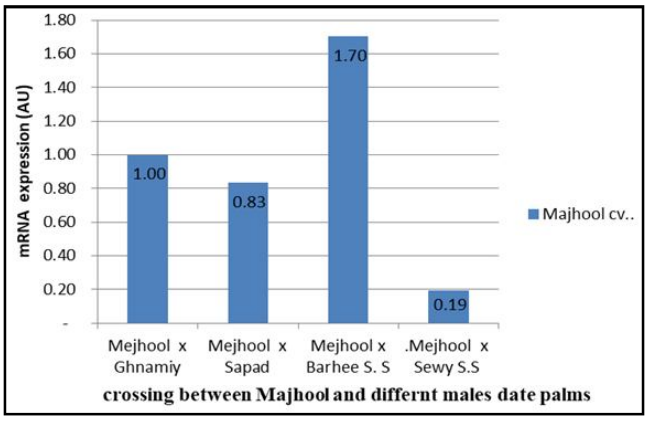

Fig. 10: Expression of Phosphofructokinase gene in fruit samples results from crossing between Majhool as female date palm and Ghnamiy , Sapd, Barhee satellite seedling and Sewy satellite seedling as male date palms in Khalal satge.

S.S. (T3)) and the treatment T2 gave high expression (1.70) in Majhool *cv.* flowed by T1 (0.83) but the treatment T3 (0.19) gave low expression compared with control (Mejhool x Ghnamy(C)).

But in Barhee *cv.* the treatments (Barhee x Sapad (T1), Barhee x Barhee S.S. (T2) and Barhee x Sewy S.S. (T3)) gave low expression (0.14, 0.61 and 0.32 respectively) compared with control. Table 17 and Figs. 8, 9 and 10.

The Ct values from these experiments were collected and used to exterminate the fold of gene expression as described before table 16.

The genes directly associated with sugar metabolism 'carbohydrate metabolism' are much more activated at the later stages of fruiting and that their expressions peak at 120 (Khalal stage) or 135 (Rutab stage) days post pollination (DPP) in date palms. Taking the 'fructose and mannose metabolism pathway' as an example, the gene expression of this pathway increases gradually and reaches its highest level at 120 DPP (Khalal stage). This general pattern provides a possible explanation for the high fructose content in the dates (Al-Mssallem *et al.,* 2013).

b. The Pectin (pectate) lyase gene expression analysis

In the present study the treatments (Mejhool x Sapad (T1) and Barhee x Sapad (T1), in both Majhool and Barhee *cvs.* enhance the m RNA level of the pectein lyase gene but this expression was lower than control ((Mejhool x Ghnamy (C) and (Barhee x Ghnamy (C)). In Majhool *cv.* the treatments T1 and T3 gave higher expression for pectein lyase gene compared with treatments for Barhee *cv.*(0.89 and 0.84) respectively

Table 18: The fold change of the Pectin (pectate) lyase gene in Barhee and Majhool date palms.

Samples of Barhee	β Actin	Pectin Lyase	ΔCt	Avg.	ΔΔCt	Fold change	Samples of Majhool	β Actin	Pectin Lyase	ΔCt	Avg.	ΔΔCt	Fold change
Barhee x Ghnamiy(c)	25.47	22.40	-3.07	-2.02	0	1	Mejhool x Ghnamiy(c)	27.08	35.17	8.09	2.06	0	1
Barhee x Ghnamiy(c)	27.10	25.29	-1.80				Mejhool x Ghnamiy(c)	26.98	26.67	-0.31			
Barhee x Ghnamiy(c)	25.40	24.23	-1.17				Mejhool x Ghnamiy(c)	25.95	24.34	-1.61			
Barhee x Sapad (T1)	25.32	21.95	-3.36	-1.59	0.43	0.74	Mejhool x Sapad(T1)	28.75	-		2.23	0.17	0.89
Barhee x Sapad(T1)	24.63	22.34	-2.29				Mejhool x Sapad(T1)	27.52	27.25	-0.27			
Barhee x Sapad(T1)	25.75	26.64	0.89				Mejhool x Sapad(T1)	27.92	32.64	4.72			
Barhee x Barhee S.S. (T2)	25.44	25.18	-0.26	-0.31	1.70	0.31	Mejhool x Barhee S.S. (T2)	28.57	29.09	0.52	3.61	1.56	0.34
Barhee x Barhee S.S. (T2)	25.25	25.56	0.31				Mejhool x Barhee S.S. (T2)	27.13	30.56	3.43			
Barhee x Barhee S.S. (T2)	24.01	23.02	-0.99				Mejhool x Barhee S.S. (T2)	27.21	34.10	6.89			
Barhee x Sewy S.S. (T3)	25.39	24.45	-0.94	2.80	4.10	0.06	Mejhool x Sewy S.S. (T3)	29.05	36.53	7.48	2.31	0.26	0.84
Barhee x Sewy S.S. (T3)	22.17	26.45	4.28				Mejhool x Sewy S.S. (T3)	27.12	28.91	1.79			
Barhee x Sewy S.S. (T3)	27.01	29.91	2.90				Mejhool x Sewy S.S. (T3)	26.69	24.37	-2.32			

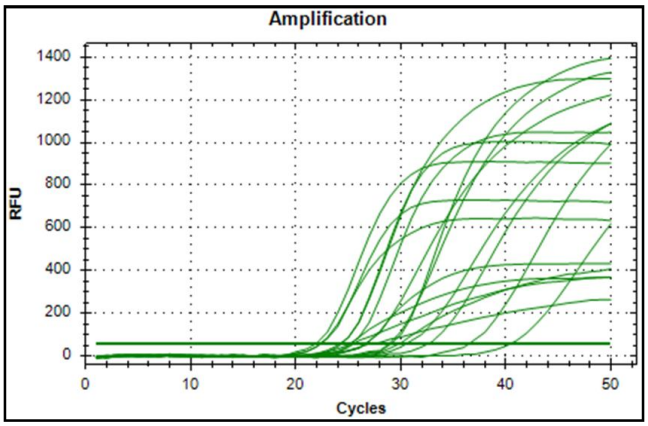

Fig. 11: Amplification plot of Pectin (pectate) lyase gene in fruit samples results from crossing between Barhee and Majhool as female date palms and Ghnamiy , Sapd, Barhee satellite seedling and Sewy satellite seedling as male date palms in Khalal stage.

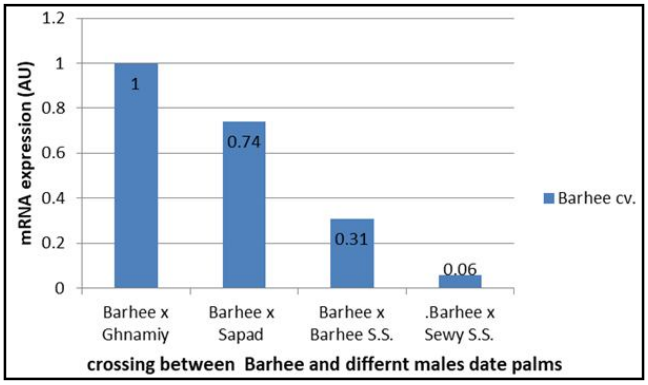

Fig. 12: Expression of Pectin (pectate) lyase gene in fruit samples results from crossing between Barhee as female date palm and Ghnamiy , Sapd, Barhee satellite seedling and Sewy satellite seedling as male date palms in Khalal satge.

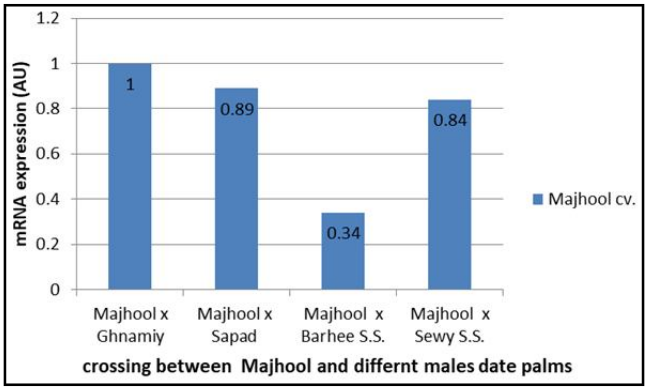

Fig. 13: Expression of Pectin (pectate) lyase gene in fruit samples results from crossing between Majhool as female date palm and Ghnamiy , Sapd, Barhee satellite seedling and Sewy satellite seedling as male date palms in Khalal satge.

table 18 and Figs. 11, 12 and 13.

The Ct values from these experiments were collected and used to exterminate the fold of gene expression as

Table 19: The fold change of the Xyloglucan(Xyloglucosyl transferase) gene in Barhee and Majhool date palms.

Samples of Barhee	β Actin	Xyloglu-can	ΔCt	Avg.	ΔΔCt	Fold change	Samples of Majhool	β Actin	Xyloglu-can	ΔCt	Avg.	ΔΔCt	Fold change
Barhee x Ghnamiy(c)	25.47	37.31	11.85	10.62	0	1	Mejhool x Ghnamiy(c)	27.08	36.71	9.63	9.86	0	1
Barhee x Ghnamiy(c)	27.10	36.50	9.40				Mejhool x Ghnamiy(c)	26.98	36.26	9.28			
Barhee x Ghnamiy(c)	25.40	-	-				Mejhool x Ghnamiy(c)	25.95	36.62	10.66			
Barhee x Sapad (T1)	25.32	37.63	12.32	12.76	2.14	0.23	Mejhool x Sapad(T1)	28.75	37.06	8.32	9.10	-0.76	1.69
Barhee x Sapad(T1)	24.63	38.09	13.46				Mejhool x Sapad(T1)	27.52	35.11	7.60			
Barhee x Sapad(T1)	25.75	38.27	12.51				Mejhool x Sapad(T1)	27.92	39.31	11.39			
Barhee x Barhee S.S. (T2)	25.44	37.03	11.58	13.32	2.69	0.15	Mejhool x Barhee S.S. (T2)	28.57	35.98	7.41	9.07	-0.79	1.73
Barhee x Barhee S.S. (T2)	25.25	40.95	15.70				Mejhool x Barhee S.S. (T2)	27.13	36.32	9.18			
Barhee x Barhee S.S. (T2)	24.01	36.68	12.67				Mejhool x Barhee S.S. (T2)	27.21	37.82	10.61			
Barhee x Sewy S.S. (T3)	25.39	36.75	11.36	12.83	2.20	0.22	Mejhool x Sewy S.S. (T3)	29.05	40.72	11.66	10.36	0.50	0.71
Barhee x Sewy S.S. (T3)	22.17	39.21	17.04				Mejhool x Sewy S.S. (T3)	27.12	36.17	9.05			
Barhee x Sewy S.S. (T3)	27.01	37.09	10.08				Mejhool x Sewy S.S. (T3)	26.69	37.05	10.36			

described before table 16.

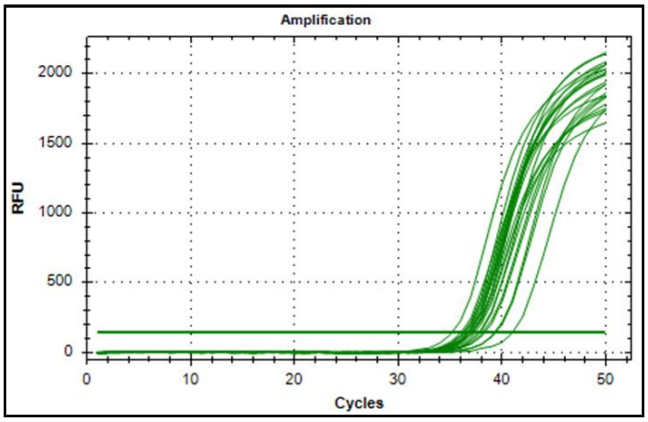

Fig. 14: Amplification plot of Xyloglucan (Xyloglucosyl transferase) gene in fruit samples results from crossing between Barhee and Majhool as female date palms and Ghnamiy, Sapd, Barhee satellite seedling and Sewy satellite seedling as male date palms in Khalal stage.

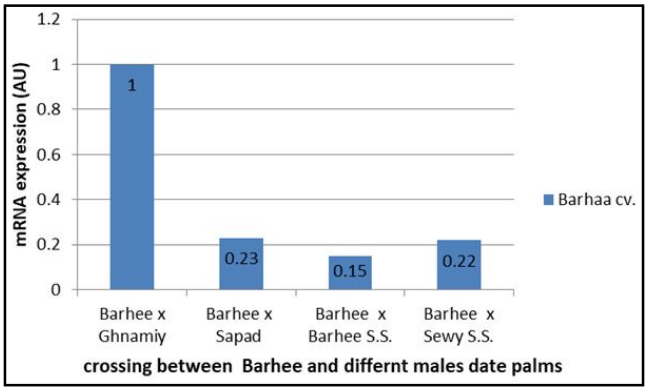

Fig. 15: Expression of Xyloglucan (Xyloglucosyl transferase) gene in fruit samples results from crossing between Barhee as female date palm and Ghnamiy, Sapd, Barhee satellite seedling and Sewy satellite seedling as male date palms in Khalal satge.

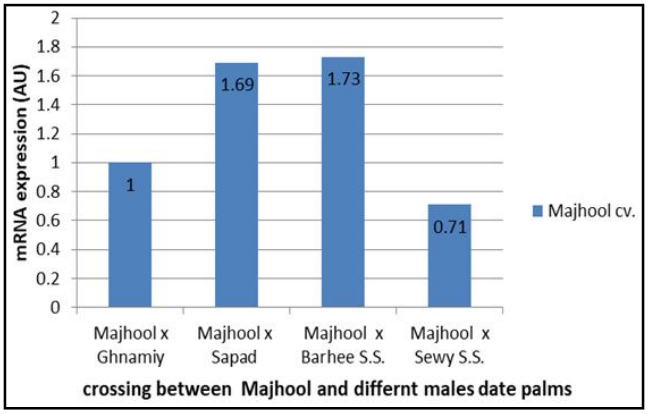

Fig. 16: Expression of Xyloglucan (Xyloglucosyl transferase) gene in fruit samples results from crossing between Majhool as female date palm and Ghnamiy, Sapd, Barhee satellite seedling and Sewy satellite seedling as male date palms in Khalal satge.

Pectein lyase gene is implicated in cell wall degradation and fruit softening and points to geographic selection for differing fruit ripening characteristics (Jimenez-Bermudez *et al.,* 2002).

Dates can be classified as being dry, semi-dry or soft when ripe. To examine whether fruit texture has a geographic association consistent with the selective sweep at the pectin lyase locus, Hazzouri *et al.,* (2015) published data on the fruit characteristics of 107 date varieties and Find that North African varieties are more variable in texture, with ~52% of varieties producing soft dates and ~31% dry. In contrast, a sample of Middle Eastern and South Asian date varieties indicated that varieties producing soft dates account for ~77% of the sample while dry date-producing varieties account for ~7%. This predominance of soft dates in Middle Eastern/ South Asian varieties is statistically significant (Fisher's exact test, Po0.0026) and is consistent with selection at the pectin lyase locus in Middle Eastern/South Asian varieties associated with cultural selection for fruit texture.

c. The Xyloglucan (Xyloglucosyl transferase) gene expression analysis

In our study the expression of Xyloglucan (Xyloglucosyl transferase) gene has been observed with higher expression (1.69 and 1.73 respectively) in treatments (Mejhool x Sapad (T1), Mejhool x Barhee S.S. (T2), in Majhool *cv.* compared with treatments for Barhee *cv.* T1, T2 and T3 and the treatment T2 gave high expression (1.73) in Majhool *cv.* flowed by T1(1.69) but the treatment T3(0.71) gave low expression compared with control (Mejhool x Ghnamy(C)). table 19 and Figs. 14, 15 and 16.

The Ct values from these experiments were collected and used to exterminate the fold of gene expression as described before table 16.

Xyloglucan is the most abundant hemicellulose in the primary cell walls of plants, where it coats and cross-links adjacent cellulose microfibrils through non-covalent associations. (Rose and Bennett 1999). xyloglucan degradation is a central factor in models of wall modification that occurs during transient wall loosening in expanding cells or in terminal wall degradation during fruit ripening and organ abscission. The pattern of xyloglucan degrading enzyme activity in ripening fruits is apparently complex (Rose *et al.,* 2003).

According to transcriptome data, Pang *et al.,* (2020) identified the softening-related enzyme xyloglucosyl transferase (XET). After Triacontanol (plant growth regulator) treatment, 16 genes of XET were up-regulated

and three genes were down-regulated. The results showed that Triacontanol promoted the activities of XET.

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