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Research Article
Efficiency of RAPD and ISSR Markers for the Detection of Polymorphisms and Genetic Relationships in Date Palm

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Abstract
Background and Objective: Several DNA markers have been used to detect the genetic variation among date palm cultivars, selecting of molecular markers for their specific purposes is the main challenge for researchers, the aim of this study was to evaluate the efficiency of RAPD and ISSR markers in assessing genetic diversity of date palm. **Methodology:** The DNA of 18 female date palms, comprising Khalas cultivar representing the diversity of date palm genotypes in Qatar was assessed using 29 ISSR primers and 8 RAPD primers, the data were then computed with the PowerMarker software to detect the major allele frequency, number of alleles, gene diversity and Polymorphism Information Content (PIC) value. **Results:** Twenty nine ISSR primers produced a total of 132 DNA fragments of which 72 fragments (54.5%) were reproducible polymorphic amplified fragments, while 8 RAPD primers produced a total of 32 DNA fragments, of which 22 fragments (68.75%) were polymorphic across all the genotypes. The most effective ISSR and RAPD primers were BT14 and OPC-11 respectively, which generated 7 bands all of which were 100% polymorphic. The RAPD marker exposed 22% gene diversity and 18% (PIC), while ISSR exposed 14 and 12% gene diversity and Polymorphism Information Content (PIC) respectively. **Conclusion:** The molecular markers obtained by the ISSR technique revealed a remarkable molecular discrimination among date palm compared to the RAPD, while RAPD provides perfect outcomes for date palms genetic diversity.

Key words: *Phoenix dactylifera* L., molecular marker, polymorphism information content, genetic diversity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Dates (*Phoenix dactylifera* L.) are dioecious perennial, monocotyledon fruit trees that belong to the family of Arecaceae and its heterogeneous genetic form makes its progeny strongly heterogeneous and variable[1]. Date palm has been domesticated for at least 5000 years in the Middle East region as the most important fruit crop and represents a big source of income in oases. Date palm trees are critical to agriculture in many hot and arid regions and dates are the most important agricultural product of many countries. These plantations help to create favorable conditions for improving secondary crop cultures like barley, alfalfa and cloves as forage[2].

There are more than 2000 date varieties with differences in color, flavor, shape, size and ripening time[3]. The most common characteristics that are used to identify different cultivars of date palm are the morphology of leaves, spines and fruit, which are mainly based on the characterization of introduced date palm cultivars[4]. However, morphological traits are often unreliable or imprecise indicators of plant genotype because they are influenced by environmental conditions and vary with the developmental stage of plants.

The identification and evaluation of genetic diversity between cultivars on the basis of morphological markers is not possible until the onset of fruiting, which takes three to five years. Furthermore, characterizing varieties requires a large set of phenotypic data that is difficult to access statistically and shows variations because of environmental factor effects[5].

Genetic fingerprinting using molecular markers has many important applications, including germplasm conservation, assessment of seed purity and verification of labeling and the identity of plants in production and marketing[6]. In recent years DNA fingerprinting marker technology has become increasingly important for discriminating between closely related cultivars. Several marker systems have been used to study the genetic diversity of date palm. Randomly amplified polymorphic DNA (RAPD) fingerprints have been used to identify date palms in Pakistan[7], Tunisia[8], Saudi Arabia[9] and Egypt[10]. In addition, ISSR[11] and microsatellite markers have been used to assess the genetic diversity and relationships of date palm varieties in Sudan[12], Morocco[13], Oman[14], Nigeria[15] and Qatar[16].

The aim of this study was to evaluate the efficiency of RAPD and ISSR markers in assessing genetic diversity of date palm cultivars and makes a comparison between those two DNA markers.

MATERIALS AND METHODS

Plant materials: Young leaves from mature, randomly sampled trees from 18 female date palms, comprising Khalas cultivar and representing the diversity of date palm genotypes in Qatar were collected from different locations as shown in Table 1. The samples were stored at -80°C for DNA extraction.

DNA extraction: The frozen young leaf tissues were first cleaned carefully with sterile distilled water 3 times. One gram of leaf sample was weighed and was cut into small pieces and ground into fine powder using liquid nitrogen. The DNeasy Plant Maxi kit protocol (QIAGEN) was used to extract DNA by following the manual instructions of the kit (DNeasy Plant Handbook). The obtained DNA was quantified and qualified using a nanodrop spectrophotometer. For further estimation of the DNA quantity 2 µL was loaded on 0.85% agarose gel at 100 V for 30 min. The gels mixture was stained with ethidium bromide and visualized under UV light.

ISSR and RAPD amplification: Thirty ISSR and 15 RAPD primers were custom synthesized from integrated DNA Technologies (IDT), Inc., USA. These primers were screened using Polymerase Chain Reaction (PCR) in a total reaction mixture of 20 µL containing 2 µL (20-30 ng) of total genomic DNA, 10 µL of AmpliTaq Gold 360 mastermix (Applied Biosystems), 1 µL (5 pmol μL^{-1}) of primers each and 7 µL of nuclease free water. Amplification was carried out in a Veriti 96 well fast thermal cycler (Applied biosystems) under the following conditions: Initial de-naturation 95°C for 10 min, 35 cycles (denaturation 95°C for 30 sec, annealing temperature depending on primer for 30 sec, extension 72°C for 1 min) and final extension 72°C for 10 min.

The amplified DNA fragments (µL) and 2 µL of loading dye (making a total volume of 7 µL) were loaded on to the gel

Table 1: Eighteen samples representing date palm of Khalas cultivars (*Phoenix dactylifera* L.) from different location in Qatar state

Farm No.	Variety name	Collection area	Label	Farm No.	Variety name	Collection area	Label
618	Khalas	North	KHL-N-1	1145	Khalas	East	KHL-E-3
643	Khalas	North	KHL-N-2	323	Khalas	East	KHL-E-4
C1	Khalas	North	KHL-C-1	RF	Khalas	East	KHL-E-5
C2	Khalas	North	KHL-C-2	280	Khalas	West	KHL-W-1
C3	Khalas	North	KHL-C-3	1085	Khalas	West	KHL-W-2
C4	Khalas	North	KHL-C-4	834	Khalas	South	KHL-S-1
C5	Khalas	North	KHL-C-5	562	Khalas	South	KHL-S-2
586	Khalas	East	KHL-E-1	1038	Khalas	South	KHL-S-3
648	Khalas	East	KHL-E-2	TCE	Khalas	Tissue culture	KHL-T-1

using the 1.5% agarose at 30 V for 180 min in 1X TAE buffer (30 mM), the gels were stained with ethidium bromide and visualized on a UV transilluminator and documented using gel documentation system Al-phImager EC by alpha view software V.3.0.0.0.

The ISSR and RAPD bands were precisely measured by the gel documentation system software and scored for each genotype. Each reproducible polymorphic DNA band at a particular position on the gel was treated as a separate character and scored as present (1) or absent (0) to generate a binary data matrix.

Data analysis: Data were analyzed with the PowerMarker software[17] version 3.0 to determine the major allele frequency, number of alleles, gene diversity and Polymorphic Information Content (PIC) value (which is commonly used in genetics as a measure of polymorphism) for a marker locus used in linkage.The phylogenetic relationship among the genotypes was drawn by past software[18] version 1.91 on the basis of Hamming similarity index with 100 bootstraps.

RESULTS

Of the 30 ISSR and 15 RAPD primers tested for their ability to generate bands patterns in date palm genotypes, 29 ISSR (Table 2) and 8 RAPD primers (Table 3) successfully produced clear bands in most of the studied genotypes. One ISSR primer (BT3 $(ACTG)_4$) and 7 RAPD primers (A12, D10, OPBO4, OPA12, OPA19, OPC-06 and OPC-15) did not amplify clear bands in the genetic materials, even when different PCR conditions were used.

Twenty nine ISSR primers used in this study provided a total of 132 bands with an average of 4.6 bands per primer, 72 of which were polymorphic (54.5% polymorphism). The percentage of polymorphism produced by each primer differed from one primer to another. The maximum value of polymorphism was 100%, produced by ten primers (BT01, BT04, BT05, BT08, BT09, BT10, BT11, BT13, BT14 and BT27), while 6 primers (BT02, BT18, BT19, BT21, BT25 and BT30) scored as monomorphic (Fig. 1), the other 13 primers produced various percentage of polymorphism ranged from 13% in BT15 to 83% in BT12.

Table 2: Code, melting temperature (Tm), sequence and types of the amplified DNA bands of the 29 DNA ISSR primers used for identifying the date palms

ISSR primers	Tm	Sequence	No. of bands	Polymorphic	Monomorphic	Polymorphic loci (%)
BT01	51.4	(AC)8T	4	4	0	100
BT02	60.6	(ACC)6	3	0	3	0
BT04	51.1	(AG)10C	1	1	0	100
BT05	52.8	(AG)10T	5	5	0	100
BT06	47	(AG)8T	4	3	1	75
BT07	57.5	(AGG)6	5	2	3	40
BT08	43.6	(ATG)6	2	2	0	100
T09	43.6	(CA)6AC	1	1	0	100
BT10	46.2	(CA)6GG	4	4	0	100
BT11	44.7	(CA)6GT	3	3	0	100
BT12	50.3	(CA)8A	6	5	1	83
BT13	44.7	(CAC)3GC	5	5	0	100
BT14	50.9	(CT)10A	7	7	0	100
BT15	52.4	(CT)10G	8	1	7	13
BT16	51.6	(CT)10T	6	1	5	17
BT17	46.7	(CT)8AC	4	2	2	50
T18	50.5	(CT)8GC	5	0	5	0
BT19	47.6	(CT)8TG	2	0	2	0
BT20	41.8	(CTC)3GC	7	5	2	71
BT21	42.3	(GA)6CC	2	0	2	0
BT22	41.9	(GA)6GG	4	3	1	75
BT23	46.8	(GA)8C	6	1	5	17
BT24	47.4	(GACA)4	3	1	2	33
BT25	41	(GAG)3GC	6	0	6	0
BT26	47.5	(GGAT)4	5	3	2	60
BT27	46.2	(GT)6CC	6	6	0	100
BT28	46.6	(GT)6GG	5	3	2	60
BT29	44	(GTG)3GC	8	4	4	50
BT30	53.6	(TC)10C	5	0	5	0
Total			132	72	60	54.5%
Average			4.6	2.5	2.1	

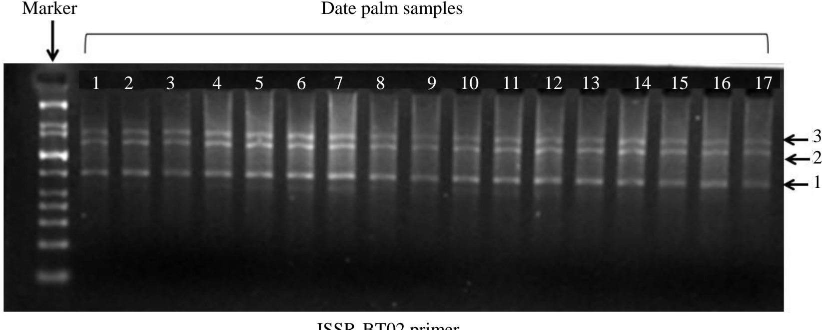

Fig. 1: ISSR monomorphism banding patterns in Khalas date palm cultivar using ISSR-BT02 primer

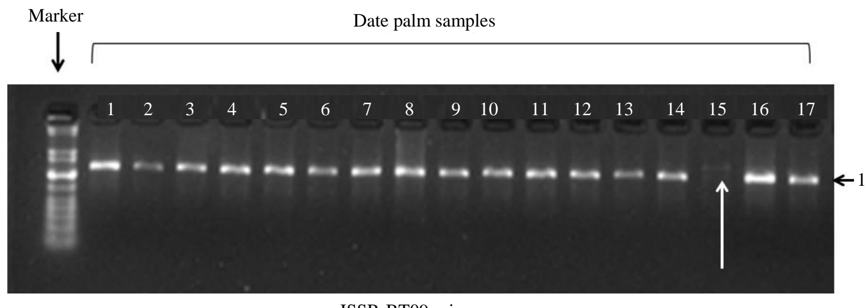

Fig. 2: ISSR polymorphism banding patterns in Khalas date palm cultivar using ISSR-BT09 primer

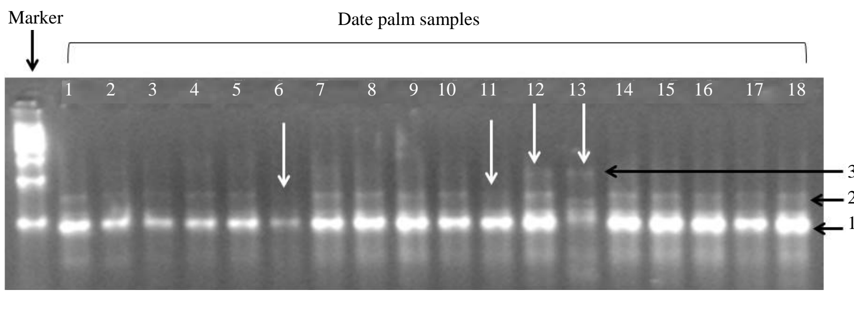

Fig. 3: RAPD polymorphism banding patterns in Khalas date palm cultivar using RAPD-OPC02 primer

Table 3: Code and types of the amplified DNA bands of the 8 DNA RAPD primers used for identifying the date palms

RAPD primer	Sequence	No. of bands	Polymorphic	Monomorphic	Polymorphic (%)
OPC11	GTG ATC GCA G	7	7	0	100
OPDO7	TTG GCA CGG G	4	1	3	25
OPA10	GTG ATC GCA G	2	2	0	100
OPA13	CAG CAC CCA C	4	1	3	25
OPC10	TGT CTG GGT G	2	2	0	100
OPD20	ACC CGG TCA C	6	5	1	83.33
OPDO5	TGA GCG GAC A	4	2	2	50
OPC02	GTG AGG CGT C	3	2	1	75
Total		32	22	10	68.75
Average		4	2.75	1.25	68.75

The ISSR profiles of the amplified products of each primer are shown in Table 2. A maximum number of eight bands were amplified with primer BT15 and BT29 and a minimum of one band with primer BT04 and BT09 (Fig. 2).

Eight RAPD primers used in this study provided a total of 32 bands, 22 of which were polymorphic (68.75% polymorphism). The total numbers of 32 amplified DNA bands were generated across the studied genotypes with an average of 4 bands per primer. The RAPD profiles of the amplified products of each primer are shown in Table 2. The maximum value of polymorphism was 100%, produced by 3 primers (OPC11, OPA10 and OPC10), while no primers scored as monomorphic, the other five primers produced various percentage of polymorphism ranged from 25% in OPD07 and OPA13, reach to 75% in primer OPC02 (Fig. 3) and 83% in primer OPD20. A maximum number of seven bands were amplified with primer OPC11 and a minimum of two bands were amplified with primers OPA10 and OPC10.

Similarity coefficient matrices were used to generate a dendrogram of Khalas cultivar based on Hamming analysis with 100 bootstrap. The ISSR technique recognized high similarity value (99.97%) between KHL-S1 and KHL-S2, while RAPD technique recorded 100% between KHL-N2 and KHL-N1 and also between KHL-S2 and KHL-C5. Lower similarity value 64% in ISSR and 42% in RAPD technique recorded between KHL-T1 and KHL-E1 (Fig. 4).

The mean gene diversity with ISSR marker was 0.14, the highest value was 50%, while the mean gene diversity with RAPD marker was 0.22 with the same high value as ISSR technique. The Polymorphism Information Content (PIC) value was 0.12 with ISSR marker, reached to 0.38 in primer BT6, BT8, BT17, BT24 and BT26, while in RAPD technique the average of PIC was 0.18 reached up to 0.40 in primer OP11.

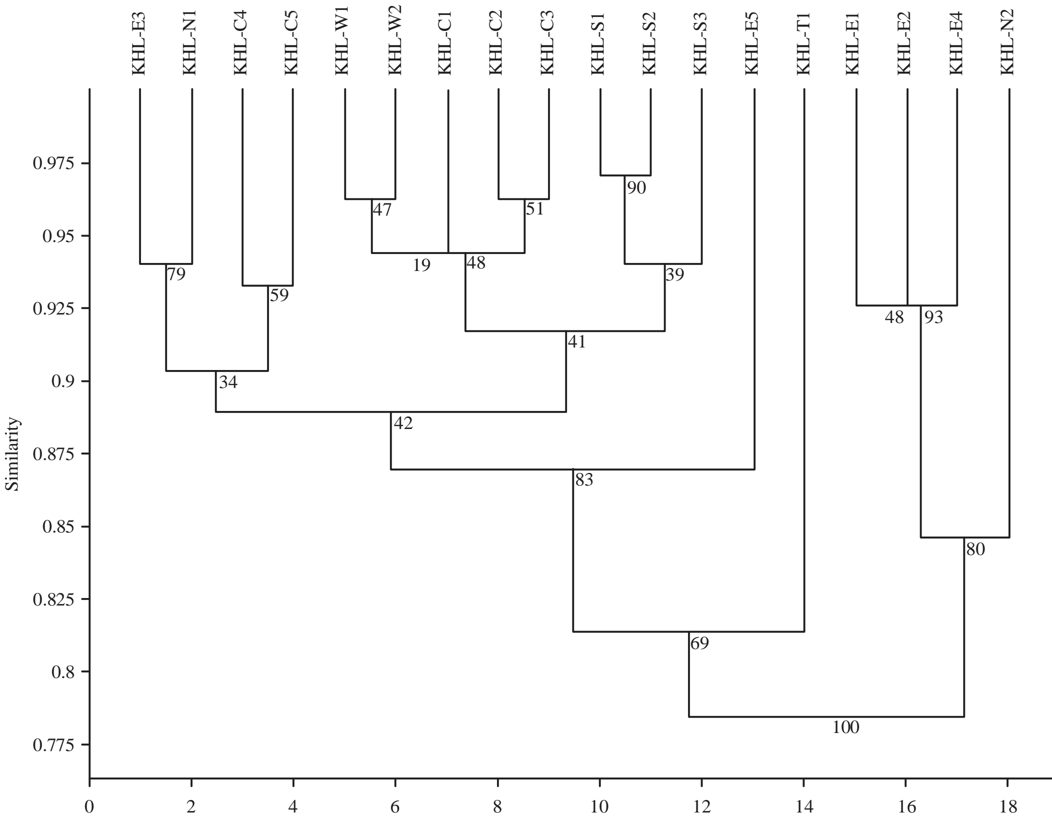

Fig. 4: Dendrogram of Khalas cultivar based on ISSR technique according to hamming similarity coefficient with 100 bootstrap

DISCUSSION

Techniques using molecular genetic markers have an important potential for the detection of genetic differences among species[19]. However, the date palm biodiversity knowledge is becoming an urgent priority to guide the use of this diversity in improvement programs of date palm, however, previous results indicated the efficiency of RAPD and ISSR marker systems for representing genetic relationships among date palm genotypes[20].

Results revealed that the overall polymorphism among date palm genotypes identified by RAPD markers (68.75%) was higher than that observed for the ISSR markers (56.7%) These results broadly concur with those detected by Haider *et al.*[21] which was observed average polymorphism by the RAPD assay (58.5%) higher than ISSR (50.6%), furthermore RAPD markers detected 39.77% as compared to 23.07% of ISSR markers[22], suggesting that RAPD markers are very effective for assessing the molecular polymorphism of date palm tree crop[9,23]. This may be explained by the fact that RAPD is less specific than ISSR because it uses shorter primers that need lower annealing temperature which makes annealing of primers more random[21], in additon that 6 primers were identified the monomorphic pattern among date palm genotypes by ISSR, while no monomorhic primers scored in RAPD technique, emphasizing that RAPD markers were efficient to determine the genetic relationships in date palm which was agreed with the finding of Haider *et al.*[21].

Hamming genetic similarity coefficient using ISSR technique recognized high similarity value (99.97%) between KHL-S1 and KHL-S2, also RAPD technique recognized high similarity value 100%, which was recorded between KHL-N2 and KHL-N1. For the reason that they are from the same location (South or North area) in addition to close farms. The highest similarity indices for ISSRs in this study is more than Sabir *et al.*[24], which were using different cultivar while this study treaty with only one cultivar, so the variation is only within cultivars.

The lower similarity value (64%) in ISSR and was 42% in RAPD, recorded between KHL-T1 and KHL-E1 may be due to that KHL-T1 was generated from tissue culture, which was firstly from a different area and secondly may subject to somaclonal variation. This is in agreement with the detecting of genetic variation by ISSR in three cultivars of date palm regenerated through cell culture[25]. The identification and characterization of species became possible through

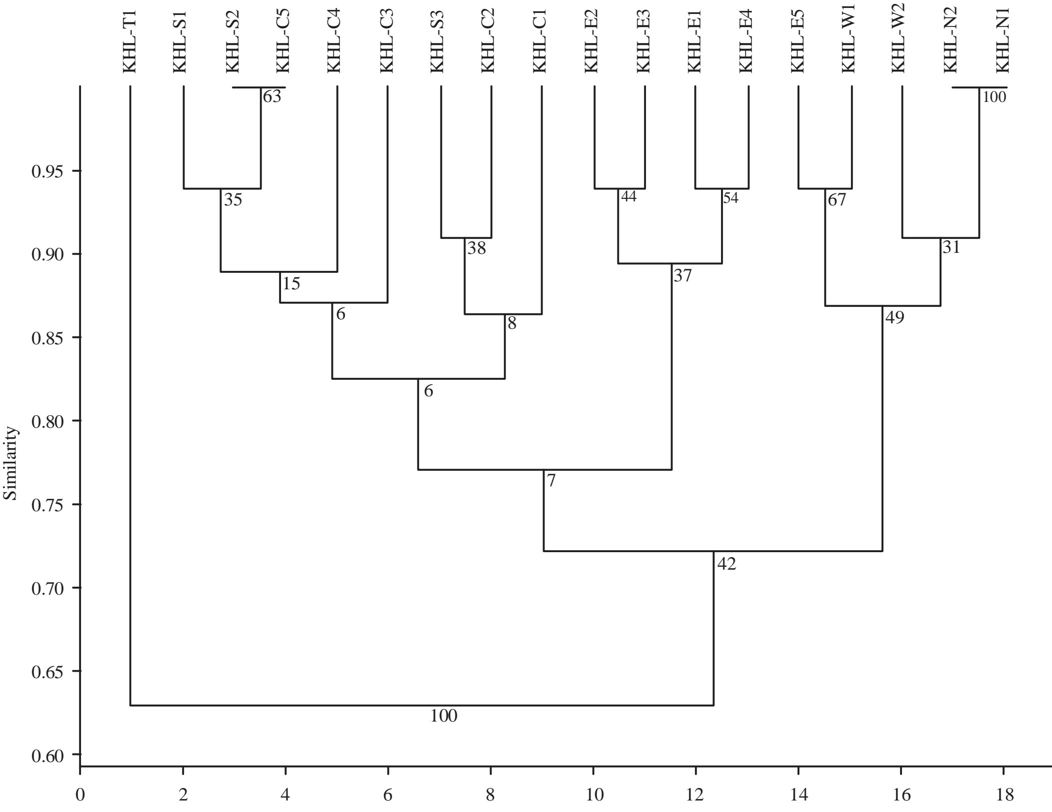

Fig. 5: Dendrogram of Khalas cultivar based on RAPD technique according to hamming similarity coefficient with 100 bootstrap

fingerprinting of each species and cultivars since DNA is a source of informative polymorphism[26].

The mean gene diversity with ISSR marker was 0.14, while the mean gene diversity with RAPD marker was 0.22 indicating that RAPD is superior over ISSR for discovering date palm diversity. This level of gene diversity is less than 0.66 reported for the Qatari date palm germplasm[16], which used SSR techniques allowing higher levels of diversity because of the unique mechanism that is responsible for generating SSR allelic diversity by replication slippage. Replication slippage is thought to occur more frequently than single nucleotide mutations and insertion/deletion events, which generate the polymorphisms that are detected by RAPD analysis[27].

The results presented here indicate that the majority of Khalas cultivars are may not a result of intact cloning processes. It is expected that the propagation cycles performed by farmers were disrupted by a mixture of plants derived from seeds. This may occur because propagation by seeds is much faster; seeds germinate easily and are available in large numbers and occasionally seeds germinate just under their mother trees. This suggestion can be supported also by the genetic diversity that was observed within same cultivar.

The Polymorphism Information Content (PIC) value, (which is commonly used in genetics as a measure of polymorphism for a marker locus used in linkage analysis), in this study was 0.12 with ISSR marker and 0.18 with RAPD. Earlier studies using SSR marker have described value of 0.62 from Qatari date palms[16], indicating that SSR marker is better than RAPD and ISSR in measuring of polymorphism for a marker locus.

In previous studies on *Prosopis* species from Qatar, was revealed that ISSR markers are better tools than RAPD for phylogenetic studies[28]. Likewise in this study similarity coefficient matrices used to generate a dendrogram of Khalas genotypes based on Hamming analysis proved ISSR technique is a better molecular discrimination marker among date palm of Khalas cultivar from Qatar compared to RAPD techniques (Fig. 5). The phylogenetic analysis on the basis of ISSR (Fig. 4) discriminates between KHL-N2 and KHL-N1, while a RAPD marker does not (Fig. 5). Also the ISSR technique (Fig. 4) discriminates between KHL-S2 and KHL-C5, while RAPD markers (Fig. 5) did not. However ISSR would be a better tool than RAPD for phylogenetic studies[29,30]. This study displayed the existence of genetic variation among Khalas cultivars trees,

revealing that most Khalas cultivar trees in Qatar are derived from seed propagation rather than offshoot propagation. This results is very significant to the researchers for selecting the proper techniques in date palm fingerprinting, on behalf of determining date palm diversity RAPD is superior over ISSR techniques. However, ISSR is a better than RAPD techniques for discrimination among date palm cultivar. Because RAPD and ISSR amplify different parts of the genome, the data generated from both analyses were combined in order to obtain more balanced values for genetic variation among genotypes.

CONCLUSION

The ISSR markers provide a more accurate result in differentiating among date palm cultivars compared to RAPD markers; alternatively RAPD provides more accurate results for date palms genetic diversity.

SIGNIFICANT STATEMENTS

- Improve date palm production through providing basic information on its genetic background diversity and population structure and to contribute to understanding the genetics of date palms in general
- Molecular markers of the date palm germplasm can be used to identify regions in a plant genome important for drought and salt tolerance. These plants could serve as sources for gene mining
- Useful tool in germplasm management especially with regard to characterizing the date palm accessions available in the region. Some of the constraints identified in date palm development in the Arabian Peninsula is the non-availability of a simple, accurate technology for identifying the current cultivars of the crop
- Establish biodiversity information system database for the region

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