# Fingerprinting date palm genotypes (*Phoenix dactylifera* L.) using Inter Simple Sequence Repeat (ISSR) markers

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**ABSTRACT**

Identification of date palm genotypes is important for farmers and date palm industries. Correct identification is usually dependent on fruit analysis, however the generation time is long and genotype identification requires phenotypic data that may be variable due to environmental conditions. Traditionally, date palm is propagated by off shoots, but currently it is also propagated by tissue culture which may result in mutations and genetic instability. Ten date palm genotypes propagated by off shoots were analyzed using Inter Simple Sequence Repeat markers (ISSRs) to identify, fingerprint, and study the genetic relationship among them. Genotypes were clearly identified using eight of the twelve primers tested. Total number of 428 bands with fragment sizes between 400 and 5000 bp were identified. The primer [(AG)10T] produced the largest number of bands (92), while primer [(CT)10T] revealed the highest degree of polymorphism (100%) among genotypes. These primers were also used to compare four genotypes that were propagated by off shoots to those propagated by tissue culture. Genotype Medgoul showed the highest similarity (0.93) followed by Hayyani (0.92), Zehdi (0.86) and Bream (0.82). Method of propagation did not show clear separation among the genotypes tested. The similarity ranged from 0.82 to 0.93 according to the method of propagation.

## INTRODUCTION

Date palm (*Phoenix dactylifera* L.) belongs to the Arecaceae family, which comprises 200 genera and more than 2500 species (Diaz et al 2003). It is a diploid (2n=36) dioecious perennial monocotyledon fruit trees with long generation time (Al-Bekr 2002). Date palm has traditionally been vegetativelly propagated from off shoots; tissue culture is another way to propagate date palm (Ben-Abdallah 2000) but mutation often appears during callus formation (Pierik 1987), which means this technique is genetically unstable.

Correct identification of date palm trees is usually possible if the fruits are produced. However, identification is difficult because of the long generation time and because it requires a large set of phenotypic data that are often hard to assess and sometime variable due to environmental conditions (Sedra et al 1993 and 1996). Since the certification of varieties based upon phenotypes characterization, the recognition of the genotypes by farmers has been a real problem. The genetic identification of genotypes is important for certified plant materials, but requires fast and reliable techniques (Bianchi et al 2002).

The last few years have revealed a widespread use of molecular markers for fingerprinting, selection, and breeding of fruit crops. Inter Simple Sequence Repeats (ISSRs), also called microsatellites, have been used as primers in polymerase chain reaction. The resultant PCR reaction amplifies of this sequence, yielding a multilocus marker system useful for fingerprinting, diversity analysis and genome mapping (Godwin et al 1997). In addition, ISSR can be targeted towards particular sequences, which are reported to be abundant in the genome and can overcome the technical difficulties of RFLP and RAPD (Rajesh et al 2002, Barth et al 2002). Since high level of polymorphism with ISSR has been observed in plants, this technique was used for fingerprinting of many fruit trees such as: oil palm (Billotte et al 2005), date palm (Zehdi et al 2004), fig (Salhi-Hannachi et al 2004), olive (Gemas et al 2004; Bandelj et al 2002), Indian cashew (Archak et al 2003).

ISSR also used in field crops like rice (Saini et al 2004); Cicer (Mehmet 2004, Rajesh et al 2002), barley (Fernandez et al 2002, Bahattin 2003), ryegrass (Ghariani et al 2003), bean (Galvan et al 2003), wheat (Ammiraju et al 2001).

This study aimed to identify different genotypes of date palm (*Phoenix dactylifera* L.) that grown in Jordan at molecular level using ISSR markers. It also aimed to show if date palm genotypes that are produced by tissue culture technique have some genetic changes comparing with the same genotypes that propagated by offshoot.

## MATERIALS AND METHODS

*Plant materials*

Fourteen date palm genotypes were used in this study; 10 genotypes represent offshoot propagation and 4 genotypes represent tissue culture propagation (TABLE 1). These genotypes were obtained from a collection maintained in the experimental orchard of the National Center for Agricultural Research and Extension (NCARE), in Jordan. For each genotype, samples (about 50 gm) from the tips of young leaves were collected, labeled, and kept in ice box; the samples were then stored at -70 °C until using.

*DNA extraction*

Genomic DNA from each sample was extracted according to CTAB method as described by Jubrael et al. (2005). RNA that can interfere with PCR was removed by digesting each sample with 4µl of Dnase-free Rnase (10 mg/ml) at 37°C for 20 min (Sambrook et al 1989).

*Polymerase Chain Reaction (PCR)*

The method described by Zehdi et al. (2004) was followed with some modifications in the annealing temperature. 25µl reaction mixture was used For PCR amplifications; it contained 25 ng of genomic DNA, 0.2 mM of primer, 1X PCR buffer [100 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl2, 0.01% Difco Gelatin], 1.5 unit of Taq DNA polymerase, 0.2 mM of each dNTPs and one drop of mineral oil to prevent evaporation during PCR cycles.

DNA amplification was performed in the amplification thermocycler (MJ Research PTC-200, USA) according to the following conditions: an initial denaturation step of 5 min at 94°C followed by 35 cycles of 30 sec at 94°C, 90 sec at the proper annealing temperature (TABLE 2); 90 sec at 72°C followed by 5 min at 72°C for final extension.
A negative sample with water instead of the genomic DNA was added to check the possibility of contamination in the amplification reactions. Amplifications were performed twice to insure accuracy. Amplification products were separated on 1.4% agarose gel according to (Sambrook et al 1989). The molecular size of amplification products were estimated using a 100 bp and 1 Kb DNA ladder.

*Primers and Data scoring*
Twelve ISSR primers were tested in this study. The sequences and annealing temperatures for theses primers are listed in (TABLE 2). For each primer, the gel was analyzed by scoring the presence or absence of ISSR bands. The presence of an amplified fragment was scored as 1, while the absence was scored as zero. All unclear bands were ignored.

**TABLE 1**

**Name and source of propagation of date palm genotypes that used in this study**

| Code | Genotypes | Source of propagation |
|---|---|---|
| 1 | Khestawi | Off shoot |
| 2 | Hayyani | Off shoot |
| 3 | Medgoul | Off shoot |
| 4 | Barhi | Off shoot |
| 5 | Deglet Nour | Off shoot |
| 6 | Zehdi | Off shoot |
| 7 | Khalas | Off shoot |
| 8 | Zaglool | Off shoot |
| 9 | Usta Umran | Off shoot |
| 10 | Bream | Off shoot |
| 11 | Medgoul* | Tissue culture |
| 12 | Bream* | Tissue culture |
| 13 | Zehdi* | Tissue culture |
| 14 | Hayyani* | Tissue culture |

*Date palm genotypes propagated by tissue culture

*Statistical Analysis*

Using Nei similarity index (Nei and Li 1979), data generated from ISSR analysis were analyzed according the equation: similarity index = 2Nab/ (Na + Nb) where: Nab= number of amplified fragments scored with the same molecular weight shared between genotypes a and b; Na= number of amplified fragments scored in genotype a; Nb= number of amplified fragments scored in genotype b. Dendrograms were constructed based on the similarity matrix data by applying unweighted pair group method with arithmetic average (UPGMA) cluster analysis using the NTSYSpc program version 2.1 by Rohlf (Exeter, Software, New York).

## RESULTS

The amplification products of eight primers (1, 2, 3, 8, 9, 10, 11, and 12) were selected out of the twelve used in this study; the primers generated polymorphic amplification fragments that were obvious and highly reproducible. The other four were ignored because either they did not produce any markers or they produced monomorphic ones. The eight primers successfully amplified the DNA of all the genotypes; the fragment size ranged between 400 with genotype 'Medgoul' (FIGURE 1 as an example) to 5000 bp

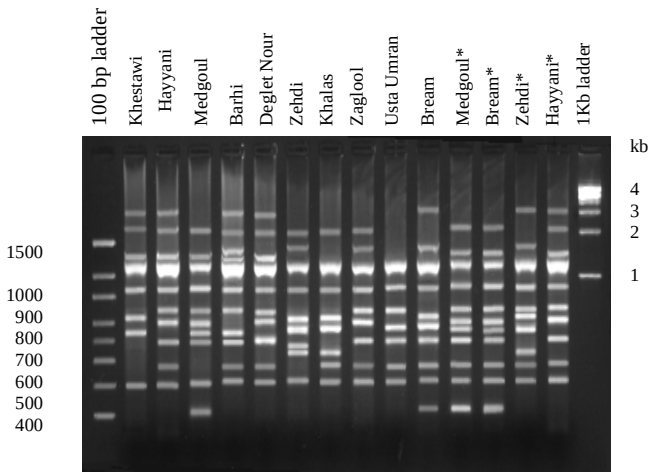


FIGURE 1 ISSR patterns of fourteen date palm genotypes with primer No. 9.

with all genotypes. For all genotypes, the total number of markers ranged from 35 to 92 and the number of the scored markers with different size ranged from 5 to 16 with primer No. 10 and 9 respectively.
The polymorphism ranged from 42.9% with primer No. 3 to 100% with primer No. 12 (TABLE 3). With primer No. 2, each of the two genotypes 'Hayyani' and 'Medgoul' produced a unique banding pattern; with primer No.3, the genotypes 'Khestawi', 'Barhi', 'Zehdi', 'Khalas' 'Zaglool' and 'Bream' shared the same banding pattern. With primer No. 9, each genotype produced a unique banding pattern (FIGURE 1).
The amplification products obtained with the eight primers were analyzed pair wise to compare the genetic relationship among genotypes. Pair wise comparison of all ISSR profiles revealed the similarity matrix that is presented in FIGURE 2.

**TABLE 2**

**ISSR primers sequences and their annealing temperatures that used in this study**

| Primer No. | Sequence 5'----------3' | Annealing temperature (°C) |
|---|---|---|
| 1 | (TGGA)5 | 55 |
| 2 | (GACA)4 | 50 |
| 3 | (ACTG)4 | 50 |
| 4 | (GACAG)4 | 55 |
| 5 | (AG)10 | 55 |
| 6 | (AGG)6 | 62 |
| 7 | (AG)10 G | 62 |
| 8 | (AG)10 C | 58 |
| 9 | (AG)10 T | 62 |
| 10 | (CT)10 A | 57 |
| 11 | (CT)10 G | 58 |
| 12 | (CT)10 T | 57 |

The highest similarity (0.89) between the ten genotypes that were produced by off shoot was found between 'Khalas' and 'Khestawi' and the lowest similarity (0.62) was found between 'Zehdi' and 'Medgoul'. The similarity between the four genotypes that were propagated by off shoot and the same four genotypes propagated by tissue culture was varied. Genotypes 'Medgoul' and 'Medgoul*' had the highest (0.93) similarity, genotypes 'Hayyani' and 'Hayyani*' had (0.92) similarity, while lower similarity (0.86) existed between 'Zehdi' 'Zehdi*' followed by (0.82) between 'Bream' and 'Bream*'.

The dendrogram, which was constructed depending on the similarity values of the ten genotypes propagated by off shoot, recognized two main clusters (FIGURE 3). The first one included six genotypes 'Khestawi', 'Khalas', 'Barhi', 'Zehdi', 'Usta Umran' and 'Zaglool', with 0.775 similarity. The second cluster included four genotypes, 'Hayyani', 'Medgoul', 'Deglet Nour' and 'Bream' with 0.775 similarities also. The dendrogram, which was constructed depending on the similarity values of the four genotypes that propagated by off shoot and the same four genotypes that propagated by tissue culture, showed two main clusters (FIGURE 4). The first cluster includes 'Hayyani', 'Hayyani*', 'Medgoul', 'Medgoul*', 'Bream' and 'Bream*' with 0.775 similarity, while the second cluster includes 'Zehdi' and 'Zehdi*' with 0.925 similarity..

Genotype 'Bream*' clustered with genotypes 'Medgoul' and 'Medgoul*' with 0.925 similarity The amplification products that obtained with the eight primers were analyzed pair wise to compare the genetic relationship among genotypes. Genotypes ‘Zehdi’ and 'Medgoul', which revealed the lowest similarity value, have differences in fruit size and shape. Genotypes 'Khalas' and 'Khestawi', which revealed the highest similarity value, have differences in fruit color but nearly have the same fruit size. Genotypes 'Zehdi' and 'Khalas' also revealed a high similarity value (0.83) and showed the same fruit color and size. In comparison between the genotypes that produced by off shoot and the same genotypes that produced by tissue culture, genotypes 'Medgoul' and 'Medgoul*' and 'Hayyani' and 'Hayyani*' showed a high similarity (0.93 and 0.92 respectively), 'Zehdi' and 'Zehdi*' revealed 0.86 similarity and 'Bream' and 'Bream*' revealed 0.82 similarity (FIGURE 2). None of the four pairs showed similarities equal to or above 95%. This means that there are some variations between genotypes that propagated by off shoot and those produced by tissue culture; but this dissimilarity did not show clear separation among them.

In the dendrogram of the ten date palm genotypes that propagated by off shoot (FIGURE 3), UPGMA separates the genotypes into two main groups. The first group includes six genotypes 'Khestawi', 'Khalas', 'Barhi', 'Zehdi', 'Usta Umran' and 'Zaglool'.

**TABLE 3**

**Number of different size markers, number of monomorphic markers, % of monomorphic markers, and % of polymorphic markers generated by each of the eight selected primers with the ten genotypes propagated by off shoot.**

| Primer No. | Total No. of bands | No. of bands/ genotype* | No. of different size markers | No. of monomorphic markers | % of monomorphic markers | % of polymorphic markers |
|---|---|---|---|---|---|---|
| **1** | 56 | 5.6 | 8 | 2 | 25% | 75% |
| **2** | 61 | 6.1 | 10 | 2 | 20% | 80% |
| **3** | 48 | 4.8 | 7 | 4 | 57.1% | 42.9% |
| **8** | 53 | 5.3 | 8 | 4 | 50% | 50% |
| **9** | 92 | 9.2 | 16 | 3 | 18.8% | 81.2% |
| **10** | 35 | 3.5 | 5 | 1 | 20% | 80% |
| **11** | 46 | 4.6 | 8 | 2 | 25% | 75% |
| **12** | 37 | 3.7 | 6 | 0 | 0% | 100% |
| **Total** | **428** | - | **68** | **18** | - | - |

* Calculated by dividing the total number of bands produced by each primer on the total number (10) of genotypes

## DISCUSSION

The PCR amplification products allowed the distinguishing of each genotype and the study of the genetic relationship among them. The total number of markers scored for the eight primers were 428 (TABLE 3). The relatively large number of polymorphic markers obtained with these primers is consistent with the findings of Zehdi et al (2004), which indicate that date palm genotypes are highly polymorphic. The result of this study revealed that each genotype has a certain amplification products, which allow us to distinguish the genotype at early stage. Since date palm has a long generation time, this will reduce the required time for distinguishing the genotypes depending on late phenotypic characters. All amplification pattern obtained with these eight primers were clear, but do not have the same intensity under UV light. Yang and Quiros (1993) have suggested that band intensity may reflect differences in the copy number of the amplified sequence. These bands could be useful polymorphic markers for all date palm genotypes tested in this study. The recommended primers for fingerprinting the ten date palm genotypes are summarized in TABLE 4; these keys could be used for date palm identification and for distinguishing closely related genotypes.

These genotypes have approximately the same fruit size, shape and color (yellow) except 'Khestawi' and 'Zaglool' which are red. These results are in agreement with that previously reported by Sedra et al. (1998) and Jubrael et al. (2005). The second cluster includes four genotypes; 'Hayyani', 'Medgoul', 'Deglet Nour' and 'Bream'; these genotypes have different fruit color, shape and size. In (FIGURE 4), the dendrogram separated the four genotypes that propagated by off shoot and by tissue into two groups. The first group include 'Hayyani', 'Hayyani*', 'Bream', 'Bream*', 'Medgoul' and 'Medgoul*'. The second group include 'Zehdi' and 'Zehdi*'. Each of the four genotypes that propagated by tissue culture clustered with the same genotype that propagated by off shoot with high similarity ('Medgoul' and 'Medgoul*' 0.94, 'Hayyani' and 'Hayyani*' 0.93, 'Zehdi' and 'Zehdi*' 0.92,). These results indicated that methods of propagation did not show clear effects on the genetic variations between genotypes. Genotype 'Bream*' clustered with 'Medgoul' and 'Medgoul*' with similarity 0.925; these two genotypes could be originated from the same ancestry.

## CONCLUSIONS

The eight primers that chosen in this study were successfully amplified the DNA of all date palm genotypes. The present study confirmed the usefulness of ISSR markers as a powerful tool for date palm identification. These primers could be used

**TABLE 4**

**Recommended primers for identification of the ten date palm genotypes**

| Code | Genotypes | Recommended Primer(s) |
|---|---|---|
| **1** | Khestawi | 1 and 8 |
| **2** | Hayyani | 2, 3 and 10 |
| **3** | Medgou | 8, 9 and 11 |
| **4** | Barhi | 1 and 3 |
| **5** | Deglet Nour | 8, 10 and 11 |
| **6** | Zehdi | 9 and 11 |
| **7** | Khalas | 9 and12 |
| **8** | Zaglool | 9 and 11 |
| **9** | Usta Umran | 2 and 10 |
| **10** | Bream | 1, 3 and 10 |

in studying the genetic relationships and could be used for fingerprinting the date palm genotypes that grown in Jordan.
Also, the data obtained can be used for establishing a genetic database for date palm genotypes that grown in the region.

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| 1 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 0.70 | | | | | | | | | | | | | |
| 3 | 0.72 | 0.74 | | | | | | | | | | | | |
| 4 | 0.81 | 0.83 | 0.79 | | | | | | | | | | | |
| 5 | 0.71 | 0.79 | 0.77 | 0.81 | | | | | | | | | | |
| 6 | 0.76 | 0.63 | 0.63 | 0.82 | 0.65 | | | | | | | | | |
| 7 | 0.89 | 0.70 | 0.66 | 0.82 | 0.71 | 0.83 | | | | | | | | |
| 8 | 0.72 | 0.73 | 0.69 | 0.82 | 0.77 | 0.80 | 0.80 | | | | | | | |
| 9 | 0.78 | 0.72 | 0.70 | 0.77 | 0.73 | 0.65 | 0.75 | 0.72 | | | | | | |
| 10 | 0.74 | 0.68 | 0.77 | 0.81 | 0.79 | 0.74 | 0.77 | 0.74 | 0.76 | | | | | |
| 11 | 0.68 | 0.70 | 0.93 | 0.78 | 0.76 | 0.62 | 0.65 | 0.71 | 0.76 | 0.81 | | | | |
| 12 | 0.65 | 0.71 | 0.89 | 0.76 | 0.75 | 0.63 | 0.66 | 0.75 | 0.68 | 0.82 | 0.95 | | | |
| 13 | 0.73 | 0.73 | 0.63 | 0.86 | 0.74 | 0.86 | 0.80 | 0.80 | 0.72 | 0.82 | 0.62 | 0.66 | | |
| 14 | 0.71 | 0.92 | 0.75 | 0.87 | 0.82 | 0.71 | 0.74 | 0.80 | 0.73 | 0.74 | 0.77 | 0.75 | 0.80 | |

FIGURE 2 Similarity coefficient matrix for pairwise comparison of 14 date palm genotypes. 1: Khestawi, 2: Hayyani, 3: Medgoul, 4: Barhi, 5: Deglet Nour, 6: Zehdi, 7: Khalas, 8: Zagloo, 9: Usta Umran, 10: Bream, 11: Medgoul*, 12: Bream*, 13: Zehdi*, 14: Hayyani*

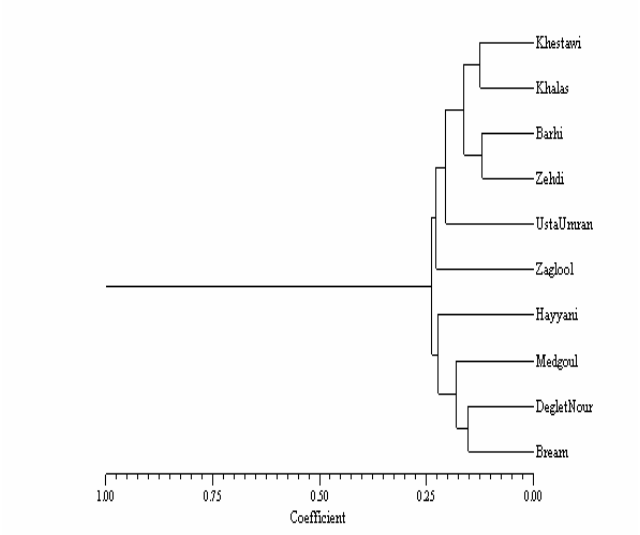


FIGURE 3 Dendrogram of 10 date palm genotypes generated by UPGMA cluster analysis of the dissimilarity values.

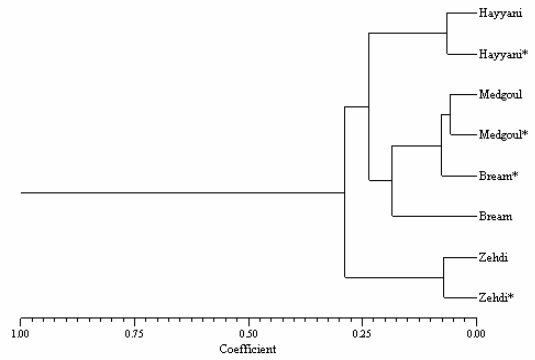


FIGURE 4 Dendrogram of 4 date palm genotypes propagated by two method (off shoot and tissue culture*) generated by UPGMA cluster analysis of the dissimilarity values

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