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DNA FINGERPRINTING OF SOME PAKISTANI DATE PALM (*PHOENIX DACTYLIFERA* L.) CULTIVARS USING ISSR MARKERS

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Abstract

Date palm is one of the oldest cultivated and economically important fruit trees. First time Inter Simple Sequence Repeats (ISSR) markers were used with twenty five economically important date palm cultivars of Pakistan for DNA fingerprinting analysis. Samples were collected from four provinces of Pakistan i.e., Sindh, Punjab, Khyber Pakhtoonkhwa and Balochistan. The study was carried out using seven ISSR markers. The twenty five date palm cultivars showed variation at the DNA level. The ISSR primers showed high polymorphism (84%) in the studied date palm cultivars. Dice's similarity index was in range from 0.608 to 0.980 and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) divided twenty five date palm cultivars into two main clusters and sub-clusters. However ISSR markers efficiently discriminated for assessing genetic diversity among commercial Pakistani date palm cultivars.

Key words: *Phoenix dactylifera* L., ISSR markers, Genetic diversity, Phylogenetic relationship, Pakistan.

Introduction

The date palm (*Phoenix dactylifera* L.) is one of the major (Taain, 2013) and oldest cultivated plants by human being and has been used as food for 6000 years (Al-Shahib & Marshall, 2003). Date Palm is a long lived dioecious, monocotyledon (Zehdi *et al.*, 2002), perennial plant, belongs to order Arecales and family Arecaceae (Palmae) (Perveen *et al.*, 2012; Khanam *et al.*, 2012; Al-Dous *et al.*, 2011). Arecaceae is a large family having 200-210 genera and 2800 - 3000 species distributed mainly in the tropical and sub-tropical regions of the world (Marwat *et al.*, 2012; Jassim & Naji, 2007. Genetically date palms is a diploid (2n = 2x = 36) (Chabane *et al.*, 2010; Haider & Nabulsi, 2012; Mirbahar *et al*., 2014a) and the predicted genome size is estimated to be approximately between 550 to 650 Mbp long (Bodian *et al.*, 2012).

Date palm is a tree of economic importance (Abdalla, 2010), almost its every part is used either food or as industrial products. It plays an important role in the rural communities and economy of many developing countries (Mirbahar, *et al.*, 2014b). Pakistan is considered fifth largest dates producing country in the world having a production of 622,000 tons year^{-1} which is 9% of the world's total production of dates (Marwat *et al.*, 2012). The date palm holds a very significant position on the agricultural horizon of Sindh (Marwat *et al.*, 2012). Genetic markers are an efficient tool for the identification of cultivars and estimation of relatedness (Mehmood *et al*., 2010; Rabbani *et al*., 2010). A number of DNA markers are used to study genetic diversity of date palm and other crops such as; Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Random Amplified Microsatellite Polymorphism (RAMPO), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLPs) and Simple Sequence Repeats (SSR) (Hamza *et al.*, 2012; Shinwari *et al*., 2011; Zeb *et al*., 2011). Inter simple sequence repeats (ISSR) is a PCR-based technique which imply the amplification of inter-tandem repeats of short DNA sequences. Inter sequence repeats are highly polymorphic (Nookaraju & Agrawal, 2012), powerful, fast, easy, reproducible and cheap technique to assess genetic similarity/diversity or to classify closely related crop plants (Blair *et al.*, 1999; Turi *et al*., 2012) including fruit trees. It can detect polymorphism without prior knowledge of DNA sequence in the region between two microsatellites (Khanam *et al.*, 2012). In this technique a single primer is used to target multiple genomic loci (Hussein *et al.*, 2005). Date palm has a number of cultivars growing commercially in Pakistan using ISSR markers. The aim of present study is to check genetic diversity/similarity among some commercially important date palm cultivars of Pakistan.

Materials and Methods

Plant material: Twenty five date palm cultivars used in this study are listed in Table 1. Leaf samples from each cultivar were collected from four provinces of Pakistan i.e., Sindh (ten samples from Khairpur), Punjab (three from Multan and Muzafargarh), Khyber Pakhtunkhwa (two from D.I. Khan) and Balochistan (eight samples from Turbat).

Plant DNA extraction: One and half gram of fresh leaf sample of date palm was ground to fine powder using liquid nitrogen. The powder was dissolved in modified CTAB buffer (NaCl 1.4 M, CTAB 2% w/v, Tris Hcl 200 mM pH 8.0, EDTA 20 mM pH8.0, 2-mercaptoethanol 2%, PVP) (Doyle & Doyle, 1990). The DNA was visualized using 0.8% agarose gel electrophoresis in 1xTBE buffer and stained with ethidium bromide 0.5µg/ml. Optical Density (O.D.) of extracted DNA was taken on 260nm and 280nm using spectrophotometer (JENWAY, Model Genowa, Serial No. 1489).

Table 1. Total number of bands calculated from 25 date palm cultivars with seven ISSR primers.

Primers cultivar	Aseel	AsulKhurmo	AsulKurh	Dedhi	Gajar	Karblain	Kasho-wari	Khar	Kupro	Nar Aseel	Noori	Otakin	Dhakki	Gulistan	Hillawi	Seedless	SherShahiDokka	Begum Jangi	Mazawati	Shakkri	Ab-e-Dandan	Shakkar	Hussaini	Basra	Rabae	Total
HB-08	5	4	6	3	5	5	5	6	6	5	6	5	5	5	6	6	7	3	6	3	5	3	5	3	4	**122**
HB-09	3	4	4	3	4	5	5	4	5	3	5	4	3	5	3	4	4	4	5	3	3	3	4	3	3	**96**
HB-10	2	2	2	2	2	3	2	3	3	3	2	2	2	2	2	2	3	2	5	2	3	2	3	2	1	**56**
HB-11	2	2	2	3	3	3	3	1	3	1	3	1	2	3	3	2	3	1	3	3	2	2	4	3	3	**61**
HB-12	3	3	1	1	1	3	3	2	1	2	2	3	3	3	3	3	2	0	3	3	1	3	2	2	2	**55**
HB-15	6	5	6	6	6	6	5	6	4	6	4	4	4	5	6	6	6	5	6	4	5	6	6	6	4	**133**
PO-16	3	5	4	5	4	4	5	5	1	3	5	1	5	1	5	5	5	5	1	4	4	4	5	5	5	**99**
Total	**24**	**25**	**25**	**23**	**25**	**29**	**28**	**27**	**23**	**23**	**27**	**20**	**24**	**24**	**28**	**28**	**30**	**20**	**26**	**22**	**23**	**23**	**29**	**24**	**22**	**622**

Table 2. Polymorphism percentage in 25 date palm cultivars by using seven ISSR primers.

S. No.	Primer sequence names	Annealing temperature	Scored bands	Monomorphic bands	Polymorphic bands	Polymorphism %
1.	HB-085'GAGAGAGAGAGAGG3`	40°C	8	2	6	75
2.	HB-09 5' GTGTGTGTGTGTGG 3'	40°C	5	0	5	100
3.	HB-105' GAGAGAGAGAGACC3`	40°C	5	1	4	80
4.	HB-115'GTGTGTGTGTGTCC 3'	40°C	4	0	4	100
5.	HB-125' CACCACCACGC 3'	38°C	3	0	3	100
6.	HB-15 5' GTGGTGGTGGC 3'	38°C	6	3	3	50
7.	P0-16 5' CACACACACACACC 3'	40°C	5	1	4	80
Total			**36**	**7**	**29**	**83.57**
Average			**5.14**	**1**	**4.14**	**--**
Range			**3-8**	**1-3**	**3-6**	**50-100**

DNA amplification with ISSR primers: Inter Simple Sequence Repeats-PCR amplification reactions were carried out with seven ISSR primers (Table 2). Polymerase chain reactions were performed in 25 µl reaction mixture containing, PCR buffer 10mM, MgCl 25mM, dNTPs 200mM each, *Taq* polymerase 1.25 units (Fermentas), primer 10 picomole (Bio Basic Canada), 30ng genomic DNA. The PCR mixture was introduced for amplification to a 96 well Eppendorff Mastergradient cycler (Germany). The PCR temperature program was set as follow: initial denaturation at 95°C for 05 min, 35 cycles of denaturation at 95°C for 30 Sec, annealing 38°C–40°C for 01 min, extension 72°C for 02 min and final extension at 72°C for 08 min.

The amplified products were resolved on 1.5% agarose (Gene-Link) in 1x TBE (Tris Borate EDTA) buffer pH 8.0. The electrophoresis was performed in large submarine units (Thermo EC-320) at 60V for 2hrs (Thermo EC, EC-250-90). Gels were stained with ethidium bromide 0.5 µg/ml and photographed under gel documentation system (UV TechTM, UK). The size of PCR products were determined by 100bp DNA ladder (Fermentas).

Data analysis: Data was recorded from good quality gel images of each amplification reaction. Amplified fragments were scored by starting from top of the lane to its bottom. The amplified products were scored numerically as present 1 and absent 0 to generate a binary matrix. Percentage was calculated as the number of polymorphic bands amplified by the primer to that of the total number of bands produced by the same primer. Data was subjected to Dice's co-efficient similarity matrix and cluster analysis by using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc version 2.0 Rohlf, 1998). The dendrogram was constructed by using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) using Sequential Agglomerative, Hierarchical and Nested Cluster (SAHN) (Sneath & Sokal, 1973).

Results

In this study seven ISSR anchored primers used which include five di-nucleotide and two tri-nucleotide primers (Table 2). These anchored primers produced 32 polymorphic bands. The five di-nucleotide primers produced 24 polymorphic bands while tri-nucleotide primers produced 9 polymorphic bands.

Seven ISSR primers for twenty five date palm cultivars produced total 622 bands with an average of 38.8 bands per primer (Table 1). The number of distinct bands for each primer ranged from 1 to 7. Seven monomorphic bands were generated with primers HB-08, HB-10, HB-15 and PO16 (Table 2). The highest number of amplicons was generated by Sher Shahi Dokka cultivar (08 amplicons) with primer HB-08. The lowest number of amplicons (01 amplicon) was generated by the cultivars Kupro, Asul Kurh, Dedhi, Gajar, Khar, Nar Aseel, Otaqin, Gulistan, Begum Jangi, Mazawati, Abedandan and Rabae respectively with primers HB-10, HB-11, HB-12 and PO-16 (Table 1 & Fig. 1). The highest number of

amplicons was generated from primers HB15 (133 amplicons) with all the twenty-five date palm cultivars followed by the primers HB08 (122 amplicons). The lowest number of amplicons were generated with primer HB12 (55 amplicons) with all twenty-five date palm cultivars. Similarity coefficient matrix was computed to cluster the data and to draw precise relationships. The seven ISSR primers generated 84% polymorphic and 16% monomorphic bands. All the seven ISSR primers generated polymorphic bands while the cultivar; Begum Jangi from Balochistan has not generated any DNA band with primer HB-12. The primer HB-15 generated DNA bands in range from 4-6 which differ from the results of Abd-Alla (2010) where the DNA bands generated with the same primer HB-15 ranged from 7-9. The primers HB-08, HB-12 and HB-15 produced DNA bands with molecular weight range of 200 to 1000 bp, the primers HB-09, HB-11 and PO-16 produced the DNA bands with molecular weight 300-350 to 1000 bp respectively. Only one primer HB-10 produced DNA bands with molecular weight of 400-900 bp.

Pair wise genetic similarity coefficient among 25 Pakistani date palm cultivars ranged from 0.608 to 0.980. This similarity index indicates that the cultivars used in the present study showed genetic diversity. The lowest pair wise similarity index was observed among the cultivars Begum Jangi and Mazawati (0.608), the highest pair wise similarity observed among Asul Kurh and Gajar (0.980) cultivars (Table 3).

The UPGMA (Unweighted Pair Group of Arithmetic Averages) analysis distributed the 25 date palm cultivars into two main clusters (Fig. 2). The data generated from seven ISSR primers were used to compute the genetic similarity index through Dice coefficient. The cluster 1 comprised 4 date palm cultivars Otaqin, Mazawati, Gulistan and Kupro. The cultivars Kupro and Gulistan showed close relationship in comparison with Otaqin and Mazawati cultivars. Otaqin was distant from other cultivars. The cluster 2 was further divided into 5 sub-clusters, the highest similarity was observed between Asul Kurh and Gajar. Aseel and Nar Aseel showed close relationship but Khar was at distant from both cultivars. The cultivars Sher Shahi Dokka, Seedless, Shakkar and Shakkri also showed close relationship in two sub-clusters but the cultivars Khar, Begum Jangi and Rabae were also at distant from all the cultivars and did not lie in any sub-cluster.

Discussion

In present study it was observed that 25 Pakistani date palm cultivars with seven ISSR primers showed 84% polymorphism level. ISSR based variation has also been reported in many previous studies like Younis *et al.* (2008) observed 87% polymorphism by using 20 ISSR primers with seven Egyptian date palm cultivars. Moghaieb *et al.* (2010) also conducted studies on genetic diversity and relatedness among six Egyptian date palm genotypes and obtained 73% polymorphism. Adawy *et al.* (2005) observed 80.4% and Karim *et al.* (2010) observed 82% polymorphism in Egyptian and Tunisain date palm cultivars respectively. Hamza *et al.* (2012) observed 80% polymorphism in date palm cultivars with seven primers these results are in harmony with the present study. Since all date palm cultivars are originated by hybridization, it may be assumed that they have a common genetic base. Nevertheless, varieties diverged from other ones by mutual events that arise during their selection mode. Farmers select date palm cultivars based on fruit quality and local adaptation. Hence, only a small part of date palm genome that concerns mainly genes encoding these agronomic traits is affected by this selection allowing maintenance of high genetic diversity among the selected genotypes (Zehdi *et al.*, 2002).

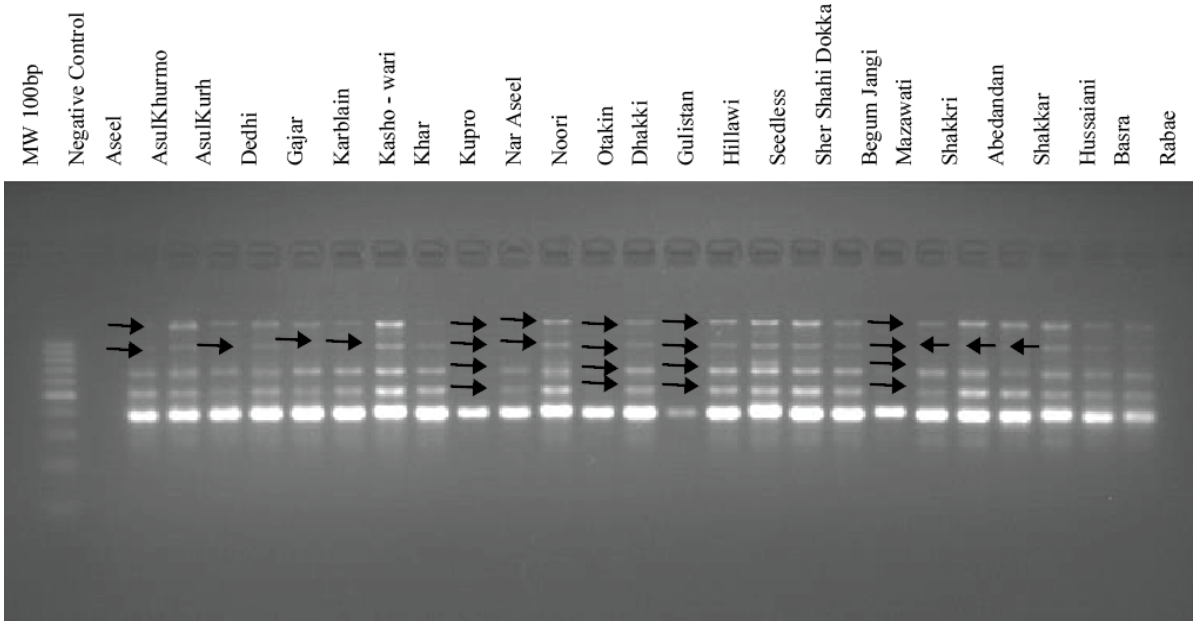

Fig. 1. DNA banding pattern, arrows indicates absence of DNA bands showing the polymorphism level with ISSR primer P016.

Table 3 .Similarity matrix based on the number of shared fragments of ISSR-PCR among 25 date palm cultivars.

S. No.	Cultivars names	Aseel	Asul Khurmo	Asul Kurh	Dedhi	Gajar	Karblain	Kasho-wari	Khar	Kupro	Nar Aseel	Noori	Otakin	Dhakki	Gulistan	Hillawi	Seedless	Sher Shahi Dokka	Begum Jangi	Mazati	Shakkri	Ab-e-Dandan	Shakkar	Hussaini	Basra	Rabae
1.	Aseel	1.000																								
2.	A.Khurmo	0.857	1.000																							
3.	A. Kurh	0.897	0.840	1.000																						
4.	Dedhi	0.808	0.833	0.875	1.000																					
5.	Gajar	0.880	0.823	0.980	0.857	1.000																				
6.	Karblain	0.830	0.777	0.888	0.846	0.872	1.000																			
7.	Kashowari	0.884	0.905	0.867	0.862	0.851	0.877	1.000																		
8.	Khar	0.846	0.830	0.867	0.823	0.851	0.807	0.857	1.000																	
9.	Kupro	0.765	0.708	0.833	0.739	0.816	0.807	0.784	0.745	1.000																
10.	N. Aseel	0.936	0.791	0.875	0.782	0.857	0.807	0.823	0.901	0.782	1.000															
11.	Noori	0.823	0.846	0.884	0.800	0.905	0.821	0.909	0.800	0.800	0.760	1.000														
12.	Otakin	0.863	0.844	0.800	0.697	0.782	0.734	0.833	0.791	0.790	0.837	0.765	1.000													
13.	Dhakki	0.791	0.857	0.816	0.851	0.800	0.830	0.884	0.769	0.723	0.723	0.823	0.772	1.000												
14.	Gulistan	0.875	0.775	0.816	0.765	0.800	0.830	0.884	0.769	0.893	0.808	0.823	0.863	0.750	1.000											
15.	Hillawi	0.846	0.830	0.867	0.823	0.851	0.807	0.892	0.821	0.745	0.784	0.836	0.750	0.846	0.807	1.000										
16.	Seedless	0.884	0.867	0.905	0.862	0.888	0.877	0.892	0.892	0.745	0.823	0.872	0.791	0.846	0.807	0.857	1.000									
17.	SS Doka	0.867	0.851	0.925	0.884	0.909	0.862	0.912	0.877	0.807	0.807	0.892	0.775	0.867	0.830	0.912	0.947	1.000								
18.	B. Jangi	0.772	0.800	0.844	0.837	0.826	0.775	0.791	0.833	0.697	0.790	0.808	0.700	0.727	0.727	0.750	0.833	0.816	1.000							
19.	Mazawati	0.720	0.666	0.745	0.693	0.769	0.836	0.740	0.740	0.857	0.734	0.716	0.739	0.760	0.800	0.703	0.777	0.763	0.608	1.000						
20.	Shakkri	0.851	0.833	0.833	0.869	0.816	0.807	0.862	0.784	0.739	0.782	0.800	0.744	0.808	0.851	0.823	0.823	0.846	0.837	0.653	1.000					
21.	Ab-e-Dandan	0.851	0.791	0.916	0.826	0.897	0.884	0.823	0.823	0.782	0.826	0.840	0.744	0.765	0.808	0.784	0.862	0.846	0.837	0.693	0.826	1.000				
22.	Shakkar	0.851	0.833	0.833	0.869	0.816	0.807	0.862	0.823	0.695	0.826	0.760	0.790	0.808	0.808	0.823	0.823	0.846	0.837	0.653	0.956	0.782	1.000			
23.	Hussaini	0.830	0.814	0.888	0.846	0.909	0.862	0.877	0.842	0.769	0.807	0.857	0.734	0.792	0.792	0.877	0.842	0.896	0.816	0.727	0.846	0.846	0.846	1.000		
24.	Basra	0.833	0.857	0.857	0.936	0.840	0.830	0.884	0.846	0.723	0.808	0.784	0.727	0.833	0.791	0.846	0.846	0.867	0.863	0.680	0.936	0.808	0.936	0.905	1.000	
25.	Rabae	0.826	0.808	0.808	0.800	0.791	0.784	0.880	0.800	0.711	0.800	0.816	0.714	0.782	0.782	0.800	0.800	0.823	0.809	0.625	0.844	0.800	0.800	0.862	0.869	1.000

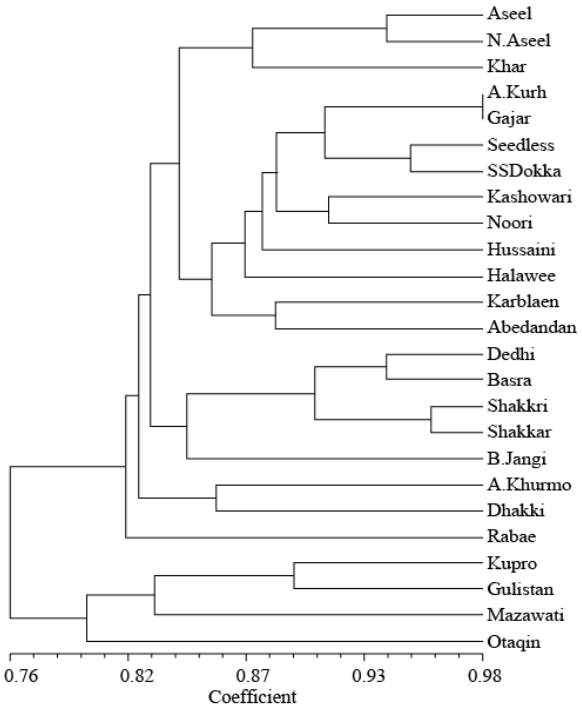

Fig. 2. Dendrogram generated using UPGMA showing genetic distances between 25 date palm cultivars based on band scores of ISSR primers.

Seven ISSR primers generated an average of 38.8 bands per primer this is much better than Zehdi *et al.* (2002) obtained 9.11 bands from 12 ISSR primers by using 18 Tunisian date palm varieties. Ahmed *et al.* (2012) obtained 48 DNA bands in Qatari date palm cultivars. All seven ISSR primers produced the DNA bands ranged from 5-8, these results are corroborated by Hamza *et al.* (2012) and Abd-Alla (2010) who applied ISSR markers to check genetic stability in field cultivated date palm cultivars from Tunisia and in micropropagated date palm plantlets from Egypt.

The ISSR primers generated multiple DNA banding patterns ranging in size from 200 to 1100bp, these results are in agreement with Zehdi *et al.* (2002) and Hamza *et al.* (2012). The present results of ISSR markers are also in agreement with Ben-Saleh & El-Helaly (2003) who have conducted the studies on similarities and dissimilarities between some Tunisian date palm cultivars.

The highest genetic similarity index (0.980) was observed between the Asul Kurh and Gajar cultivars from Sindh Province (Table 3), these results are in accordance to Abd-Alla (2010). The present results indicate high level of genetic diversity among Pakistani date palm cultivars at DNA level and these results are in harmony with Zehdi *et al.* (2002). The lowest genetic similarity was observed between Begum Jangi and Shakkri date palm cultivars. Hamza *et al.* (2012), observed highest 0.48 and 0.05 genetic distance among date palm cultivars based on ISSR data. Interestingly the highest and lowest genetic similarities have divided the date palm cultivars geographically. The cultivars having highest similarity belong to Sindh and with low similarity index belongs to Balochistan provinces respectively.

The genetic distance matrix was analyzed with the neighbor program using the UPGMA algorithm in order to cluster the accessions according to genetic similarity. The dendrogram formed 2 major clusters and five sub-clusters is an indication of the high polymorphism/genetic divergence among Pakistani date palm cultivars (Fig. 1). ISSR has proved successful for assessing genetic diversity within various plant groups for gene mapping and for germplasm identification (Santos *et al.*, 2011). It might be assured from the present study that all date palm cultivars are interconnected instead of their agronomic divergence.

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