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GENETIC CHARACTERIZATION OF DIFFERENT PAKISTANI DATE PALM VARIETIES

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Abstract

Date palm (*Phoenix dactylifera* L.) is the oldest cultivated fruit tree and it has a great socioeconomic and nutritional value. Breeding programs and conservation rely on genetic characterization and diversity in gene pool. Its genetic diversity has not been focused more in Pakistan yet, therefore the present study aimed at the evaluation of genetic relationship based on chloroplast ribosomal protein gene (*rps14*). *Rps14* gene was amplified and sequenced from selected varieties. Phylogram illustrated over all genetic distance of 0.001 representing close genetic relationship of selected *P. dactylifera* varieties. Pairwise distance was calculated for *rps14* gene and very low genetic diversity values were observed ranging 0.003-0.017. Estimates of average evolutionary divergence of overall sequence pairs and nucleotide diversity were again found very low with 0.008 and 0.007 respectively. Sequences were analyzed by MEGA6, which revealed *Pathri*, *Dhaddy, Makhi* and *Khudrawi* as recent varieties. On the basis of *rps14* genetic makeup, it can be suggested that Pakistani date palm varieties show very high degree of similarity.

Key words: Genetic evaluation; Genetic diversity; *rps14* markers.

Introduction

Date palm (*Phoenix dactylifera* L.) is one of the ancient domesticated fruit tree with a great socioeconomic importance and nutritional value (Barreveld, 1993; Elshibi, 2009). It is the major crop for agricultural income in arid and desert areas which establishes secondary crop culture (barley, alfalfa and clove as forage) providing favorable environment for both human and animal territories (Hodel & Johnson, 2007). There are almost 5000 date palm cultivars all around the world (Osman, 1984; Bashah, 1996; Jaradat & Zaid, 2004). In Pakistan, Hassan et al. (2006) reported that date palm cultivated area increased from 1990 to 2003. Khairpur district is considered as biodiversity center for date palm in Pakistan with nearly eighty five varieties. To ensure the conservation of high quality varieties, tissue culturing and direct shoot regeneration methods were suggested (Markhand *et al*., 2010; Khan & Bi Bi, 2012).

Determination of genetic relationships among date palm cultivars is of major importance for characterization of date palm germplasm, breeding programs, and conservation purposes (Haider *et al*., 2012). Fruit morphology (Sedra *et al*., 1998) biochemical markers (Herny, 1998; Gothwal *et al*., 2013) and isozymes (Al-Jibouri & Adham, 1990) used for genotype identification are found to be complex and altered by environment. Several molecular markers have been applied for genetic diversity assessment, such as RAPD (Sedra *et al*., 1998; Trifi *et al*., 2000; Al-Khalifa & Askari, 2003; Mirbahar *et al*., 2014), ISSRs (Zehdi *et al*., 2002) SSRs (Zehdi *et al*., 2004; Elmeer *et al*., 2011) RAMPO (Rhouma *et al*., 2008) and AFLP (Devanand & Chao, 2003; Bandelj *et al*., 2004; Rhouma *et al*., 2007; Khierallah *et al*., 2011). These nrDNA markers revealed high polymorphism among date palm cultivars but it remained difficult to describe cultivars.

However, cpDNA sequences can be used to estimate phylogeny (Jamil *et al*., 2014). CpDNA has high phylogenetic potential than nrDNA as it is sufficient variable but conserve to be less variable within than between species (Taberlet *et al*., 2007; Filiz, 2012). Xiong *et al*., (2009) studied that gene duplication, gene transfer and gene loss between chloroplast and nuclear genomes is a strong resource of evolution. Al-Qurainy *et al*., (2011) firstly attempted cpDNA in date palm in Saudi cultivars. There is need to generate suitable molecular markers to get a deeper and ample insight of the genetic diversity of date palm. In order to access phylogentic kinship of selected Pakistani date palm varieties, sequences of chloroplast cp gene encoding ribosomal protein for smaller subunit were computed by MEGA6 tool. Such studies have successfully been employed to elucidate relationship in various species (Sultan *et al*., 2013; Turi *et al*., 2012).

Materials and Methods

Material collection: Fresh and young leaves of fifteen different varieties of date palm (*Phoenix dactylifera* L.) viz., *Pathri, Dhaddy, Aseel, Makhi, Khudrawi, Sanduri, Bedhar, Shamran, Zahidi, Kur, Haleeni, Barni, Begum jhungi, Kuhba* and *Karbalir* were selected and collected from Jamshoro (Sindh), Pakistan for present research work and leaves samples were stored at 4ºC.

DNA extraction: DNA extraction was done from the stored leaves of date palm varieties using CTAB method (Richards, 1997) with some modifications for genomic DNA extraction.

Primer designing: A pair of primer that can amplify *rps14* gene was designed from tobacco chloroplast genome (Accession No. Z00044.2) available in NIH (National Institute of Health, U.S.A) GenBank. Primers were designed form online Primer3 (version 0.4.0) software. Primer sequence is given below:
rps14 F: 5′ATGGCAAGGAAAAGTTTGATTC 3′
rps14 R: 5′ TTACCAACTTGATCTTGTTGCTCCT 3′

PCR amplification and sequencing: PCR conditions used for amplification were pre denaturation at 94˚C for 5 minutes followed by 35 cycles of denaturation at 94ºC for 1 minute, annealing in range of 57-65˚C temperature for 1 minute and extension at 72ºC for 1 minute. Final cycle was same except extension at 72ºC for 20 minutes was done to make sure that any remaining single-stranded DNA became fully extended. Following purification by JETquick (Genomed) PCR Product Purification Spin Kit, sequencing reaction was performed in 200 µl centrifuge tubes by using DTCS Quick Start Kit by Beckman and Coulter. Sequencing reaction conditions were as follow; initial PCR denaturation at 96ºC for 60 sec, then 30 cycles of denaturation at 96ºC for 25 sec, annealing at 57-65ºC for 25 sec, extension at 60ºC for 240 sec. A final extension at 60ºC for 600 sec was also performed. The samples were loaded to the available separate wells of Beckman and Coulter sequencer CEQ (8800) loading plate and a drop of light mineral oil provided with the kit was over laid on each sample. Finally, a proper sequencing program was run.

Analysis of obtained sequences: Using NCBI, *rps14* gene sequences of all fifteen date palm varieties was uploaded and BLASTn was performed one by one in query form in comparison to already reported sequences in Genbank. After BLASTn, sequence data of all fifteen varieties were submitted to Genbank in order to get accession numbers. The *rps14* gene sequences of all date palm varieties were then aligned by ClusterW software and these aligned sequences were further analyzed by MEGA6 software for phylogenetic study of date palm varieties.

Results

Sequence analysis: The sequence data of *rps14* gene obtained from selected date palm cultivars was aligned and subjected to BLASTn using NCBI. Similarity index percentage was checked with *P.dactylifera* chloroplast complete genome (Accession No. GU811709.2) and accession numbers were obtained from Genbank for all the sequences (Table 1).

Phylogenetic analysis: Phylogram was drawn with MEGA6 (Tamura *et al*., 2013) which explain the divergence among the selected Pakistani date palm varieties. Overview of dendrogram illustrated that date palm varieties indicated very little genetic distance (0.001) showing close genetic kinship among them. Apparently phylogram revealed two main clusters denoted by cluster I and cluster II. Cluster I includes nine varieties revealing a divergence pattern again sub-clustering into two groups. Group I comprises four varieties namely *Pathri, Dhaddy, Makhi and Khudrawi* clustered together with 65% bootstrap value. Branch length demonstrates that *Aseel* evolved latter as an out group with branch length value of 0.003.Group II includes four varieties in which *Sanduri* and *Sharman* allied together with 50% bootstrap similarity. Two remaining varieties *Behdar and Zahidi* clustered ingroup with 52% bootstrap value. It was observed that *Zahidi* has shown recent evolution in group II with 0.03 branch length. It was also revealed that *Aseel* and *Zahidi*have recently evolved in cluster I. In cluster II, five varieties *Karbalir, Kubha, Begum jhungi, Barni and Haleeni* appeared as earlier cultivars and have shown close clustering pattern with higher similarity (88% bootstrap value). Another variety *Kur* showed an out group pattern in cluster I (Fig. 1).

Table 1. Accession numbers of *rps14* gene from selected Pakistani date palm varieties with similarity index.

S. No.	Date palm varieties	Accession numbers (obtained from Genbank)	Similarity indent (with *Phoenix dactylifera* chloroplast genome; accession No. GU811709.2)
1.	Pathri	KJ502000	99%
2.	Dhaddy	KJ502001	99%
3.	Aseel	KJ502002	99%
4.	Makhi	KJ502003	99%
5.	Khudrawi	KJ502004	99%
6.	Sanduri	KJ502005	99%
7.	Bedhar	KJ502006	99%
8.	Sharman	KJ502007	99%
9.	Zahidi	KJ502008	99%
10.	Kur	KJ502009	99%
11.	Haleeni	KJ502010	99%
12.	Barni	KJ502011	99%
13.	Begum jhungi	KJ502012	99%
14.	Kubha	KJ502013	99%
15.	Karbalir	KJ502014	99%

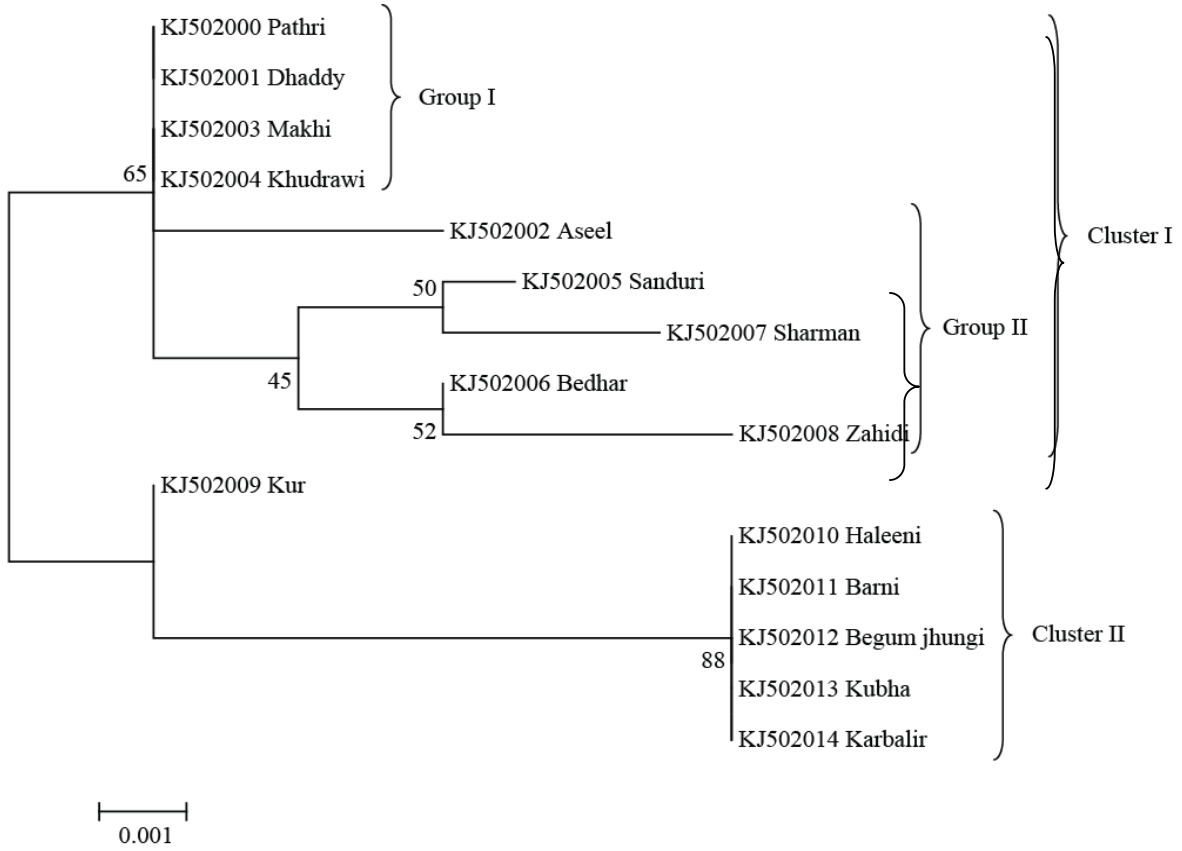

Fig. 1. Phylogram for *rps14* gene sequences indicating genetic relationship among selected date palm varieties with bootstrap values using the Neighbor-Joining method MEGA6.

Pairwise distance calculation: Pairwise distance was calculated on the basis of *rps14* gene sequences using MEGA6. The values of genetic diversity range in 0.003 to 0.017 with an overall mean distance of 0.008 for *rps14* gene (Table 2). These very low distance values indicate that all varieties are genetically closely associated to each other and there is low genetic diversity among them based on *rps14* gene.

Tajma's neutrality test: Tajma's Neutrality Test was calculated on the basis of *rps14* gene sequences of all fifteen varieties using MEGA6. Fifteen numbers of sequences (m) gave seven segregation sites (S) revealing very low nucleotide diversity (π) of 0.007 (Table 3). This low nucleotide is an indication of close genetic kinship of studied date palm varieties.

Maximum likelihood (ML) estimate of substitution matrix: Substitution pattern and rates were estimated under the Tamura-Nei (1993) model. Relative values of instantaneous r should be considered when evaluating them. For simplicity sum of r values is made equal to 100. The transitional substation rates were found higher than rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are given in italics. The nucleotide frequencies are A = 34.65%, T/U = 25.61%, C = 16.94%, and G = 22.79%. For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -469.805 (Table 4).

Discussion

As nrDNA molecular markers, RAPD, ISSR, AFLP, RAMPO, microsatellite as well as isozyme markers revealed high level of polymorphism so it remained problematic to effectively characterize at cultivar level in date palm (Baaziz *et al*., 2000; Zehdi *et al*., 2002; Al-Khalifa & Askari, 2003; Rhouma *et al*., 2007; Rhouma *et al*., 2008; Haider *et al*., 2012) . Cao & Chao (2002), El-Assar *et al*., (2005), and Elshibli & Kopelainen (2010) also reported similar results with different markers to characterize date palm from different geographic locations.

However, cpDNA gene sequences provide significant source for analysis of phylogeny in seed plants and have been extensively used to reconstruct the phylogeny of related species (Small *et al*., 2005; Melotto-Passarin *et al*., 2008; Rasheed *et al*., 2012). Filiz (2012) used *matK* gene along with whole cp genome and *trnL-trnF* non-coding cpDNA regions for phylogeny of some *Solanum* species. Al-Qurainy *et al*., (2011) investigated the molecular phylogeny of eight Saudi date palm cultivars utilizing cpDNA *rpoB* coding and *psbA-trnH* non-coding regions. Molecular typing of chloroplast *rpoB*and *psbA-trnH* has also been studied by many authors (Yao *et al*., 2009; Song *et al*., 2009; Feng *et al*., 2010; Chen *et al*., 2010). It has been reported that *rpoC* and ITS regions caused hindrance in amplification (Al-Qurainy *et al*., 2011) while *rpoB* and *psbA-trnH* loci showed low efficiency in *Picea* barcoding (Ran *et al*., 2010).

Table 2. Estimates of evolutionary divergence among *rps14*gene sequences from fifteen date palm varieties, calculated by pairwise distance MEGA6.

Date palm varieties	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Dhaddy															
Aseel	0.003														
Makhi	0	0.003													
Sanduri	0.003	0.007	0.003												
Khudrawi	0	0.003	0	0.003											
Bedhar	0.003	0.007	0.003	0.007	0.003										
Sharman	0.007	0.01	0.007	0.003	0.007	0.003									
Zahidi	0.007	0.01	0.007	0.001	0.007	0.003	0.007								
Kur	0.003	0.007	0.003	0.007	0.003	0.007	0.01	0.01							
Haleeni	0.01	0.003	0.01	0.013	0.01	0.013	0.017	0.02	0.007						
Barni	0.01	0.003	0.01	0.013	0.01	0.013	0.017	0.02	0.007	0					
Begum jhungi	0.01	0.003	0.01	0.013	0.01	0.013	0.017	0.02	0.007	0	0				
Kubha	0.01	0.003	0.01	0.013	0.01	0.013	0.017	0.02	0.007	0	0	0			
Karbalir	0.01	0.003	0.01	0.013	0.01	0.01	0.017	0.02	0.007	0	0	0	0		
Pathri	0	0.003	0	0.003	0	0.003	0.007	0.01	0.003	0	0	0.01	0.01	0.01	

Table 3. Tajima's Neutrality Test Values based on*rps14* gene of fifteen date palm varieties MEGA6.

No. of sequences "m"	No. of segregating sites "S"	Ps= S/n	Θ=Ps/a1	Nucleotide diversity "π"	Tajma test statistic "D"
15	7	0.023102	0.007105	0.007669	0.286048

Table 4. Maximum likelihood values of transitional (bold) and transversional substitution (italics) of nucleotides of *rps14* gene for fifteen date palm varieties, calculated through MEGA6.

	A	T/U	C	G
A	-	*8.35*	*5.52*	**13.8**
T/U	*11.3*	-	**0**	*7.43*
C	*11.3*	**0**	-	*7.43*
G	**20.98**	*8.35*	*5.52*	-

The efficacy of *rps* gene sequences in molecular typing has earlier been well investigated (Hattori *et al*., 2006; Naciri *et al*., 2010) and chloroplast *rps8*, *11* and *14* gene can be used for diversity evaluation, as it is easy to amplify, less laborious and reliable for accessing genetic diversity and phylogenetic relationship (Saeed *et al*., 2011; Jabeen *et al*., 2012). Accordingly the present study was designed for chloroplast *rps14* gene that encodes for ribosomal protein S14 to evaluate genetic diversity among local date palm varieties.

After analyzing the sequence data it was found that level of polymorphism was very low in the studies date palm varieties. Current phylogenetic consequence is comparable with cp DNA sequences based phylogram of eight Saudi cultivars where all cultivars found to be genetically distant with no multiple names. The nucleotide diversity of fifteen cultivars in present study is very low than Saudi cultivars (Al-Qurainy *et al*., 2011) which might be due to high selection pressure by farmers in order to maintain pure breed or due to restricted distribution of date palm crop in specific area.

Recently Wali *et al*., (2013) investigated the *rps14* gene for accessing the phylogenetic study of selected citrus species where overall genetic diversity value was 0.02. Our results showed that *rps14* gene is more conserved genetically at intra-species level in date palm varieties indicating very higher genetic similarity (0.01 diversity value) than at inter-species level as reported earlier in phylogenetic studies of selected *Citrus* species (Wali *et al*., 2013).

Date palm has a long history of domestication with an unknown origin (Wrigley, 1995) and the nature of date palm culture may have an important role in the composition of date palm genomes. Apart from the tissue culture methods, the only way to maintain the genetic integrity of date palm cultivars is propagation by offshoots (Zaid & de Wet, 2002). Our results of low genetic diversity may also an indicative of offshoot propagation method by farmers as seeds with genetic recombinant embryo cause diversification among date palm population. Hence it is concluded that date palm showed high level of similarity and low genetic diversification among studied varieties. The high genetic similarity values lead us to the conclusion that they have been under high selection pressure.

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