Research Article

Simple Sequence Repeat (SSR) markers show greater similarity among morphologically diverse Date palm (*Phoenix dactylifera* L.) cultivars grown in Pakistan

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Abstract

Date palm is among the top three fruit crops of Pakistan which is grown throughout the country except the northern highlands. This study was conducted for evaluation of morphological, chemical and molecular diversity of date palm cultivars in Pakistan. Twenty two morphological parameters of fruit, leaf and trunk of forty five locally adapted cultivars were studied for this purpose. Proximate analysis of the date fruit was also done. Morphological traits of trunk, leaves and spines had no significant correlation with fruit traits. Seven components were found to explain 81% variability in the data set by principal component analysis. Length and width of leaf, weight, volume, diameter and length of fruit, weight of pulp, weight of seed, height of perianth, total soluble solids, % moisture, % total sugars and % reducing sugars largely contributed to variability among the cultivars. Forty six simple sequence repeat markers were used to find genetic diversity in date palm cultivars under study. Only two out of forty six SSR markers showed polymorphism with amplification of five amplicons in total. Only two of the tested markers amplified

polymorphic bands, 24 markers amplified monomorphic bands while the remaining 20 primers did not amplify. Coefficient matrices were computed to form clusters on morphological, chemical and molecular basis to assess the relationship among the studied cultivars.

Key words: Vegetative traits; Principal Component Analysis (PCA); Genetic diversity; Fruit morphology; Microsatellite markers

Introduction

Date Palm (*Phoenix dactylifera* L.), a member of the Arecaceae family is a monocotyledonous perennial woody plant which is cross breeding in nature. Almost 5000 cultivars of date palm exist in the world which are developed by date palm growers through selection [1]. Date palm covers an area of 896, 000ha in Pakistan that produces 524.6 metric tons of dates [2]. Major date producing regions in Pakistan are Khairpur, Makran, Bahawalpur and D.I. Khan [3]. Aseel, shakri, BegumJhangi, Fasli, Dhaki, Halawi and Zahidi are some of the important commercial cultivars [4].

Plants show variation in yield, vegetative traits and morphological properties of fruits and seeds in response to environmental changes [5, 6]. *Phoenix dactylifera* L. is different from other plants with respect to genetic diversity due to several reasons. Firstly it is vegetatively propagated with long life and is able to pass its genes to many generations. Secondly a single female can be selected on the basis of phenotype for further cloning. Thirdly due to cross breeding, heavy selection and transportation to long distance, its history is more complicated [7].

Adapted cultivars are a result of natural and human selection. Human selection is based mainly on fruit traits and resistance to biotic and abiotic stresses [8]. In date palm cultivars there exist a high degree of variation especially in the reproductive characters so cultivars are usually identified on the basis of fruit

characteristics [9]. Phenotypic markers have been in use for characterization of date palm germplasm by researchers in different countries [10-16]. In date palm the different naming of the cultivars in different growing regions, introduction of new varieties and their distribution in different geographical regions has made the identification of varieties difficult [17]. Long generation cycle and the time it takes in showing the reproductive characteristics is the main hindrance in date palm breeding [18]. Thus a reliable technique of characterization would be very useful for the improvement of such crops. This will make the identification of the tree very easy even at its juvenile stage [19].

Simple sequence repeats which are abundantly present throughout the higher eukaryotic genomes are highly polymorphic than other genetic markers. These are helpful in identification of cultivars, analysis of pedigree, germplasm characterization and genetic mapping study [20]. Principal component analysis, a statistical tool used for determining variation in samples, has been used for assessment of variation in apricot [21], maize [22] and date palm [11]. Little work is done on morphological characterization of date palm cultivars in Pakistan. Some cultivars were characterized morphologically and others on the basis of physico-chemical traits of their fruit. [14, 23, 24]. Date palm is prone to the loss of genetic diversity due to continuous use of offshoots from few genetic material of the gene pool. Therefore to evaluate and preserve the genetic diversity are

important for germplasm conservation of date palm [19]. This study aimed at diversity evaluation and morphological, molecular and chemical characterization of the local date palm cultivars.

Materials and methods

Morphological analysis

Morphological data were scored for 45 date palm cultivars (Table 1) in triplicates. Each replication was represented by a single tree of the cultivar selected randomly for sampling. Twenty two parameters including leaf length and width (cm), leaf base width (cm), spine and pinnae number, midrib length (cm), length of midrib with spines (cm), length of midrib with pinnae (cm), length of the top pinnae (cm), fruit volume and dimensions (length and width), pulp weight (gm), seed weight (gm), Perianth height (mm) and trunk diameter (cm) were selected from

standard descriptors established for date palm by Rizk and Sharabasy [25]. Trunk circumference was measured and then diameter was calculated with formula:

$$\text{Diameter} = \text{Circumference} / \pi \text{ [26]}$$

Data were scored on mature leaves of selected trees for leaf length and width, midrib length, midrib length with spines, midrib length with pinnae and length of the top pinnae [12]. Twenty dates were randomly chosen from each cultivar for fruit morphological characterization. Vernier Calipers (Model: CD-6"CSX Mitutoyo Digimatic Caliper, Japan) was used to determine length, diameter and perianth height. Fruit, pulp and seed weight were measured with digital balance [27]. Water displacement method was used to determine fruit volume.

Table 1. Names of cultivars and their sampling sites

Cultivars from Jhang		Cultivars from Bahawalpur
Akhrot	Kokna	Sanduri
Dhaki	Daanda	Makhi
Aseel	Begum Jhangi	Dhady
Halawi	Peela Dora	Kur
Qantar	Shamran	Halemi
Makran	Rachna	Eedal shah
Chohara	Saib	Sufaidah
Zaidi	Zerdo	Taar wali
Berahmi	Shado	Fasli
Neelum	Peeli sunder	Basra wali
Zirin	Khudrawi	Pathri
Kohraba	Wahan wali	Kupra
Kozananbad	Angoor	Shakri
Karbaline	Champa kali	Baidhar
Jansohar		Gajjar wali
		Halwain

Proximate analysis

%Ash content and % Moisture of date fruit were determined at 'khalal' stage using method number 923.03 and 925.10 described in [28] respectively. Total soluble solids (°Brix) were measured with Abbe Refractometer (ATAGO 3T) following the method described in [28]. Lane and Eynon method was used for determination of total sugar (%) and reducing sugars (%) as described by [29].

Molecular analysis

DNA Isolation

DNA from frozen leaves of date palm was extracted using modified CTAB method [30]. The extracted DNA was quantified on 1% agarose gel as well as on Bio Spec-Nano spectrophotometer (Shimadzu analytical and measuring instruments, Japan). Polymerase chain reaction (PCR) was used to detect polymorphism by SSR markers for *Phoenix dactylifera* L., developed by [20, 31]. PCR was run in a total reaction mixture of 20ul containing 25ng DNA template. Protocol of PCR for SSR primers developed by [31] was initial denaturation of 95°C for 10 min, 35 cycles of denaturation for 30sec at 95°C, annealing at primer dependent temperature for 30sec and extension of 1min at 72°C followed by 1 cycle of final extension at 72°C for 7 min. PCR protocol for SSR primers developed by [21] was an initial denaturation of 5min at 95°C followed by 35 cycles of denaturation of 94°C for 30sec, annealing temperature of 48-60°C depending on the primer for 90 sec, extension temperature of 72°C for 90sec, a final extension temperature of 7 min and a final hold at 4°C [32]. PCR product was run on 2% agarose gel in TAE buffer for 35 min keeping current at 300 mAmp and voltage at 100V. SSR bands were

visualized with Alpha Innotech Gel Doc System using Alpha view software version 1.0.1.14 (Alpha Innotech Corporation).

Statistical analysis

The morphological and proximate data obtained were analyzed to find the characteristics contributing to the total variability. Principal component analysis and correlation tools of Minitab version 16 statistical software were used for this purpose. Score plot of the second principal component versus first principal component was also graphed (Fig 1). Score plot showing second principal component versus first principal component was also drawn using Minitab software (Figure 1). Morphological and proximate data were converted to interval data and similarity matrices were computed using Euclidean coefficient and these matrices were then used to draw dendrogram. Each polymorphic band with SSR markers on the gel was considered as an allele. Bands on gel were scored as present (1) or absent (0) to form binary data matrix. Data were then computed with NTSYSpc version 2.10j software to produce similarity matrix using Jaccard's coefficient to detect the polymorphism among the cultivars on the basis of size of alleles. The matrix was then computed to form dendrogram.

Results

Different strategies for diversity evaluation of date palm on phenotypic, molecular and proximate composition basis were used. Phenotypic data were subjected to a multivariate technique called principal component analysis using Minitab software version 16. The data obtained were used to draw dendrograms using Euclidean coefficient and Jaccard's coefficient respectively.

Figure 1. Score plot based on morphological and proximate data

Morphological and proximate analysis

The present study was conducted to evaluate the genetic and morphological variation in forty five cultivars of date palm in Pakistan (Table 1) and to find traits that can discriminate among the cultivars. The descriptive statistics of data showed large variation among characteristics of cultivars studied (Table 2). Thirteen traits including leaf length and width, fruit weight, volume, diameter and length, pulp weight, seed weight, perianth height, % moisture, total soluble solids, % total sugars and % reducing sugars were found to be more variable and important for characterization of date palm cultivars.

Principal component analysis

Principal component analysis of the obtained data was done. First seven components contributed 81% variability in the data. The first component had an Eigen value of 5.48 and contributed 24% variability in the data (Table 3). Five

variables including leaf length, weight, volume and length of fruit and pulp weight had more weight than other variables in this component. Leaf length, weight, volume and length of the fruit and pulp weight are the traits that mainly accounted for 24% variability in PC1 (Table 4). The second component had an Eigen value of 3.85 and explained 17 % variability in the data (Table 3). In second component the three variables including total soluble solids, % reducing sugars, and % total sugars had greater weight than other variables (Table 4). PC3 with Eigen value of 2.73 explained 12% variability in the data and represented two variables, fruit diameter and % moisture. Eigen values of the next three components were 1.78, 1.56 and 1.26 and 1.15 respectively and thus each represented one variable. These variables are leaf base, seed weight and perianth height respectively (Table 3 & 4).

Table 2. Descriptive statistics of traits of date palm cultivars studies

Variables	Mean	St. Dev	Range
Trunk diameter (cm)	52.24	7.61	39.06-83.33
Leaf length (cm)	340.97	39.21	244-420
Leaf width (cm)	82.52	11.25	54-108
Leaf base width (cm)	7.01	1.21	5-10
Spine number	19.88	5.51	9.33-35.33
Midrib length with spines (cm)	66.93	17.88	31-114
Midrib length (cm)	314.76	39.48	222.67-395.30
Pinnae number	182.10	24.81	138.33-243.00
Midrib length with pinnae (cm)	234.01	50.52	71.67-313.30
Length of top pinnae (cm)	25.71	5.37	16.66-44.33
Fruit weight (gm.)	10.97	4.04	4.76-26.03
Fruit volume (cm ³)	11.09	4.25	4-26
Fruit diameter (cm)	2.27	0.34	1.53-3.25
Fruit length (cm)	3.67	0.54	2.69-5.45
Pulp weight (gm.)	9.58	4.1	3.06-26.10
Seed weight (gm.)	1.16	0.24	0.7-1.86
Perianth height (mm)	2.60	1.2	0.53-5.61
% Moisture	65.27	8.22	45.20-83.08
TSS (Brix)	32.93	7.48	14-52
% Reducing Sugars	23.39	6.13	9.87-38.16
% Total Sugars	27.57	7.03	12.86-46.29
% Ash	3.03	0.70	0.64-4.54

Table 3. Principal components showing proportion of variability among date palm cultivars

PC	Eigen value	Percent variation	Cumulative
1	5.48	0.24	0.24
2	3.85	0.17	0.42
3	2.73	0.12	0.54
4	1.78	0.08	0.63
5	1.56	0.07	0.70
6	1.26	0.05	0.75
7	1.15	0.05	0.81

Correlation

Correlation matrix of the date palm traits studied is represented in table 5. The matrix shows weak to strong positive correlation among various vegetative

morphological traits. Similarly a positive correlation is found among the phenotypic traits of fruit and parameters of proximate composition but vegetative traits were found to have no significant correlation

with fruit traits. Total soluble solids, % reducing sugars and % total sugars were negatively correlated with % moisture content of date fruit ($r=-0.71$, $r=-0.70$ and $r=-0.79$ respectively). Reducing sugars and % total sugars were positively

correlated with total soluble solids ($r=0.90$ and $r=0.91$ respectively). Total sugars was also positively correlated with % reducing sugars ($r=0.93$).

Table 4. Correlation among the variables studied and the first six components

Variables	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Trunk diameter (cm)	0.203	-0.108	-0.280	0.008	-0.120	0.256	-0.189
Leaf length (cm)	0.268*	-0.095	-0.391	0.080	0.015	-0.175	0.065
Leaf width (cm)	0.087	0.113	-0.202	0.380*	0.470*	0.042	0.130
Leaf base width (cm)	0.089	0.115	-0.018	0.337	0.094	-0.516	-0.282
Spine number	-0.016	-0.170	-0.194	-0.443	0.387	0.022	-0.050
Midrib length with spines (cm)	0.022	-0.217	-0.127	-0.380	0.399	-0.233	-0.063
Midrib length (cm)	0.257	-0.127	-0.058	0.039	-0.034	-0.290	0.081
Pinnae number	0.081	-0.058	0.049	-0.371	-0.252	0.037	0.289
Midrib length with pinnae (cm)	0.140	0.049	-0.301	0.129	-0.296	-0.155	-0.069
Length of top pinnae (cm)	0.070	0.041	-0.176	0.285	0.428	0.425	-0.039
Fruit weight (gm.)	0.356*	0.227	0.103	-0.054	-0.057	-0.019	0.096
Fruit volume (cm ³)	0.353*	0.236	0.105	-0.029	-0.021	-0.007	0.074
Fruit diameter (cm)	0.229	0.249	0.174*	-0.127	0.236	-0.003	0.261
Fruit length (cm)	0.269*	0.251	-0.026	-0.156	-0.091	0.129	-0.256
Pulp weight (gm.)	0.352*	0.219	0.125	-0.066	-0.004	-0.019	0.070
Seed weight (gm.)	0.029	0.259	-0.249	-0.095	-0.072	0.442*	-0.246
Perianth height (mm)	-0.088	-0.080	-0.137	0.219	-0.106	0.138	0.696*
% Moisture	0.223	-0.322	0.183*	0.035	0.007	0.052	0.064
TSS (⁰ Brix)	-0.274	0.279*	-0.173	-0.057	0.029	0.103	-0.012
% Reducing Sugars	-0.266	0.327*	-0.173	-0.037	-0.018	-0.021	0.046
% Total Sugars	-0.263	0.322*	-0.209	-0.028	-0.015	-0.151	0.027
% Ash	0.010	-0.329	-0.114	0.214	-0.161	0.149	-0.236

Table 5. Correlation matrix of the traits studied

	T. Dia	LL	LW	LBW	SN	MLS	ML	PN	MLP	LTP	F. Wt	F. Vol	F. Dia	F. Len	Pp. Wt	S. Wt	P. Ht	%M	TSS	% Red. Sugars	% T.Sugars
	0.48																				
LW	0.12	0.35																			
LBW	0.01	0.21	0.23																		
SN	0.13	0.12	0.02	-0.21																	
MLS	0.08	0.24	-0.04	-0.09	0.64																
ML	0.45	0.93	0.27	0.19	0.13	0.27															
PN	0.35	0.44	-0.21	-0.23	0.31	0.13	0.44														
MLP	0.31	0.46	0.11	0.14	-0.07	-0.11	0.48	0.20													
LTP	0.20	0.23	0.52	0.07	0.07	-0.01	0.08	-0.06	0.04												
F. Wt	0.21	0.31	0.14	0.23	-0.19	-0.17	0.27	0.10	0.21	0.03											
F. Vol	0.19	0.33	0.16	0.24	-0.21	-0.17	0.27	0.05	0.18	0.09	0.96										
F. Dia	-0.03	0.04	0.23	0.06	-0.06	-0.03	0.06	-0.05	-0.02	0.11	0.67	0.69									
F. Len	0.25	0.26	0.08	0.08	-0.10	-0.07	0.22	0.07	0.29	0.04	0.70	0.69	0.51								
Pp. Wt	0.20	0.28	0.15	0.22	-0.16	-0.13	0.24	0.06	0.17	0.06	0.96	0.96	0.69	0.68							
S. Wt	0.21	0.14	0.11	-0.05	0.01	-0.18	-0.01	0.17	0.17	0.25	0.18	0.19	0.04	0.47	0.13						
P. Ht	-0.01	0.06	0.13	-0.16	-0.08	-0.09	0.02	0.14	0.05	0.04	-0.18	-0.21	-0.15	-0.35	-0.24	-0.06					
%M	0.20	0.26	-0.11	-0.09	0.09	0.16	0.31	-0.03	-0.04	-0.01	0.17	0.20	0.14	0.01	0.21	-0.39	-0.02				
TSS	-0.24	-0.27	0.08	0.01	0.04	-0.11	-0.26	-0.01	-0.02	-0.05	-0.34	-0.30	-0.11	-0.09	-0.32	0.26	0.08	-0.71			
% R. Sugars	-0.29	-0.33	0.05	-0.01	-0.08	-0.21	-0.33	-0.00	-0.01	0.01	-0.27	-0.24	-0.05	-0.04	-0.26	0.33	0.10	-0.70	0.90		
% T.Sugars	-0.30	-0.23	0.09	0.05	-0.09	-0.16	-0.24	0.01	0.01	-0.05	-0.27	-0.24	-0.08	-0.08	-0.27	0.29	0.06	-0.79	0.91	0.93	
%Ash	0.32	0.21	-0.06	-0.07	0.06	0.07	0.25	-0.01	0.06	0.04	-0.29	-0.28	-0.47	-0.15	-0.26	-0.17	0.13	0.44	-0.21	-0.31	-0.31

Abbreviations: TD; Trunk diameter, LL; Leaf length, LBW; Leaf base width, SN; Spine number, MLS; Midrib length with spines, ML; Midrib length, PN; Pinnae number, MLP; Midrib length with pinnae, LTP; Length of top pinnae, F. Wt; Fruit weight, F. Vol; Fruit volume, F. Dia; Fruit diameter, F. Len; Fruit length, Pp Wt; Pulp weight, S. Wt; Seed weight, P. Ht; Perianth Height.

Dendrogram based on morphological and proximate data

The dendrogram (Figure 2) based on morphological data of trunk, leaves, spines, fruit and proximate composition data of date fruit shows clustering of studied cultivars into four groups. Cluster1 grouped six cultivars Aseel, Halawi, Jansohar, Dhadi, Chohara and Peelisunder together. Cluster 2 included 10 cultivars mostly paired together. Qantar and Begum Jhangi, Neelum and Zerdo, Shado and Angoor, Sanduri and Halwain were paired together and closely related to each other while distantly related to Wahanwali and Taarwali in the same cluster. Cluster 3 had 16 cultivars grouped further in 3 sub clusters. The first sub cluster had 4 cultivars including Haleni, Kupra,

Kozanabad and Zahidi. The second sub cluster also had four cultivars, Baidhar and Champakali paired together and closely related to Shamran and distantly related to Makran. The third subcluster had eight cultivars including Berahmi, Zirin, Khudrawi, Kohraba and Daanda. Rachna and Saib were paired together while Dhaki was placed in the same cluster at a distance from the rest of the cultivars. Cluster 4 was the largest cluster with two sub clusters having thirteen cultivars grouped mostly in pairs. The first sub cluster had only three cultivars Saib, Akhrot and Makhi. The second sub cluster had the remaining ten cultivars including Kerbaline, Peeladora, Kokna, Eedalshah, Basrawali, Sufaidah, Gajjarwali, Kur, Fasli and Shakri.

Figure 2. Tree plot based on combined morphological and proximate data using euclidean coefficient

Figure 3. Tree plot based on SSR data

Molecular analysis

Forty six microsatellite DNA markers were used for assessment of genetic diversity in a set of 45 date palm cultivars grown in Pakistan (Table 6). Initially fifteen samples were screened for amplification by these markers. Twenty four markers produced monomorphic bands while twenty markers did not amplify the tested samples therefore these markers were not considered for further study. Only two microsatellite markers *mpdCIRO25* and *mpdCIRO85* developed by [20] produced polymorphic bands within the expected range. Therefore only these two polymorphic markers were used for assessment of genetic relationship in all the samples (Fig 4 and 5). These two primers produced clear bands with all the genotypes except Akhrot, Dhaki, Qantar and Kohraba. SSR primer *mpdCIRO25* produced two alleles. The sizes of alleles were 190bp and 200bp respectively. 190bp

allele was scored in the cultivars Chohara, Zaidi, Berahmi, Neelum, Zirin, Jaman, Kozanabad, Kerbaine, Jansohar, Kokna, Daanda, Begum Jhangi, Peeladora, Rachna, Zerdo, Shado, Peelisunder, Khudrawi, and Wahan wali while the rest of cultivars produced band 200bp. The primer *mpdCIRO85* amplified three alleles of the sizes 185bp, 190bp and 390bp respectively in the studied cultivars. Allele of 185bp was scored in cultivar Shado while Aseel, Halawi, Makran, Chohara, Zaidi, Neelum, Zirin, Jaman, Kozanabad, Kerbaine, Jansohar, Kokna, Daanda, Begum Jhangi, Peeladora, Saib, Zerdo, Peelisunder, Wahanwali, Shamran, Sanduri, Khudrawi, Makhi, Dhady, Kur and Haminwali amplified band of 190bp. A single band of 390bp was scored in cultivars Zirin, Jaman, Kerbaine, Jansohar, Daanda, Saib and Shado while Halemi, Eedalsah, Sufaidah, Taarwali, Fasli, Basrawali, Pathri, Kupra, Shakri,

Zirin, Baidhar, Gajjarwali and Halwain amplified two alleles of 190bp and 390bp. Bands were scored either as present (1) or absent (0) to produce binary data in NTSYSpc 1.20j computer software. The binary data generated were used to produce similarity matrix using Jaccard's coefficient ranging from 0.25 to 1.00. The similarity matrix was then used to produce tree plot to show relatedness among the studied varieties. The 45 cultivars were divided into two main clusters. Cluster 1 grouped Aseel, Halawi, Makran, Halwain, Shakri, Angoor, Sanduri, Makhi, Dhady and kur clustered at one point and closely related to Haleni, Baidhar, Kupra, Fasli,

Sufaidah, Pathri, Gajjarwali, Basrawali, Eedalshah and Taarwali which were clustered at the other point. Saib and Champakali were grouped in the same cluster but at a distance from the rest of the cultivars of this group. Cluster 2 contained Chohara, Zaidi, Neelum, Kozanabad, Kokna, Wahanwali, Peelisunder, Zerdo, Shamran, Peeladora, Begum Jangi clustered at one point and closely related to Zirin, Kerbaline, Jansohar, and Daanda at another point. Berahmi, Khudrawi and Rachna were grouped in another sub cluster. Shado was grouped in the same cluster but a distance from the rest of the cultivars.

**Figure 4. PCR amplification of *mpdCIRO25* in 45 date palm cultivars on 2% agarose gel
M=100bp plus DNA ladder**

**Figure 5. PCR amplification of *mpdCIRO85* in 45 date palm cultivars on 2% agarose gel
M=100bp plus DNA ladder**

Table 6. Simple sequence repeat markers used in this study showing amplification status

Primer	Forward sequence	Reverse Sequence	Repeat motif	Expected Allele size(bp)	*Amplification status
DP150	CTGCGCAATCTAAACCATT	GCAAATTGCAACAAATCCTTG	(GAA) ₉	177	+
DP151	TTGCTGGTTGAAATGGTGTT	GCAACAGATGCTCTTGCTCA	(AC) ₃₇	168-186	-
DP152	ACGAGTTTTTGGGAGAGCAA	GCAAGTTGCCAACATTCTTGT	(TAT) ₈	224	-
DP153	TCATCACAGGCAATGGCTAA	GCAGATGGCCATTGAACC	(TCA) ₉	204	+
DP154	ACACACACACACCGCAAT	GCATGTGAGGCGCATATCTA	(AC) ₁₉	249	+
DP155	CCCCCTCTCTCTCTCTCTC	GCCAAGAGGATTGGAGATTG	(TC) ₅₁	200	-
DP156	TGTGTGTGTGTGTGTGTGA	GCCATTGTTTGTGTGGACTG	(GA) ₁₇	221	-
DP157	TGGACAATGACACCCCTTTT	GCCACACAACAACCTCTCT	(TC) ₁₉	180-244	-
DP158	TCATTGGCTAATCCACACACA	GCCTTGTGGTCATGAGAGGT	(GA) ₂₉	204	-
DP159	AGCTCCAATTTGCTGCAGAG	GCTGACCTGGAGTCCAAAAC	(TC) ₂₇	156-172	+
DP160	AAGAGCGACAATCATGACCA	GGAAATTGAAGGGCATCTTG	(GAAA) ₅	108-136	-
DP161	TGGTTGTCTGCTTATCTGCTG	GGAGGGAACCGAGAGAGAGA	(CT) ₁₃	211	-
DP162	TGGAGTTCAAGAAGTGCGAAT	GGCAGTCACATTTTGCTTCA	(TACA) ₉	183	+
DP163	GTGCGTGTGTGTGTGTGT	GGCTGTTTGGGTTCGTACTG	(GA) ₁₉	215	-
DP164	GGACCAAGAACCAGAGTTG	GGGAAGGTGAGGTGGTATGA	(ATAG) ₆	200	+
DP165	AAGCATCCTATGGCTTTGACA	GGGCTGTATGTGATGCATTG	(AATA) ₅	222	-
DP166	CAATTTCCTCTCGCTGGAG	GGGGTTTCTTTTCCTTCTGC	(GAAA) ₅	210	+
DP167	ACATCCAATGGCATCCAAAT	GGGTTTCCAGGTTTCTCTC	(GAAA) ₆	243	+
DP168	GCAGCAAAGCCCTTAGGC	GGTGTATGTGCAGCCAATG	(CAT) ₈	163-175	+
DP169	GCATGGACTTAATGTGGGTAA	GGTTTTCTCTGCCAACACAT	(AAT) ₁₂	129-223	+
DP170	TCTTTGGGCTTACGACAACC	GTATGGCCCAAGATGCAGAT	(AGGG) ₅	195-227	+
DP171	GTGGGAGTAGCGAGGTATGG	GTCCGGCACTTTAGGAAGTT	(TTC) ₁₀	197-218	-
DP172	GTGTTTGGGCTATTTCCT	GTCTCTCTCTCTCTGTCC	(AGG) ₁₁	199-235	-
DP173	GTGTTTGGGCTATTTCCT	GTGTTTGGGCTATTTCCT	(TC) ₂₇	198	-
DP174	GTGTTTGGGCTATTTCCT	GTGTTTGGGCTATTTCCT	(CGTG) ₅	187	-
DP175	GTGTTTGGGCTATTTCCT	GTGTTTGGGCTATTTCCT	(CA) ₁₉	196-274	-
DP176	GTGTTTGGGCTATTTCCT	GTGTTTGGGCTATTTCCT	(CAA) ₉	198	+
DP177	TTCCTGGGCTCACTTCAAC	TAACATGCCAGCAAAGGTGA	(AGGC) ₆	216	+
DP178	AGTTTGTGAGCCATTGTGT	TACATGTGCGTATCGGAGA	(TC) ₁₉	186	-
DP179	GGTTAGCCATCCAAAAGTGG	TATGTAGCCTCCACCGCATC	(ATTT) ₅	183	+
mPdCIR010	ACCCCGGACGTGAGGTG	CGTCGATCCTCTCTTTGTCTC	(GA) ₂₂	114-236	+
mPdCIR015	AGCTGGCTCCTCCCTTCTTA	GCTCGGTTGGACTTGTCT	(GA) ₁₅	104-150	+
mPdCIR016	AGCGGGAAATGAAAAGGTAT	ATGAAAACGTGCCAAATGTC	(GA) ₁₄	104-198	+
mPdCIR025	GCACGAGAAGGCTTATAGT	CCCCCATTAGGATTCTAC	(GA) ₂₂	192-244	++
mPdCIR032	CAAATCTTTGCCGTGAG	GGTGTGGAGTAATCATGTAGTAG	(GA) ₁₉	248-310	+
mPdCIR035	ACAAACGGCGATGGGATTAC	CCGCAGCTCACCTCTCTAT	(GA) ₁₅	154-198	+
mPdCIR044	ATGCGGACTACACTATTCTAC	GGTGATTGACTTCTTTGAG	(GA) ₁₉	250-330	-
mPdCIR048	CGAGACCTACCTCAACAAA	CCACCAACCAAATCAACAC	(GA) ₃₂	108-198	-
mPdCIR050	CTGCCATTTCTTCTGAC	CACCATGCACAAAAATG	(GA) ₂₁	114-208	+
mPdCIR057	AAGCAGCAGCCCTTCCGTAG	GTTCTCACTCGCCAAAAAATAC	(GA) ₂₀	214-284	+
mPdCIR063	CTTTTATGTGGTCTGAGAGA	TCTCTGATCTTGGGTTCTGT	(GA) ₁₇	100-216	-
mPdCIR070	CAAGACCAAGGCTAAC	GGAGGTGGCTTTGTAGTAT	(GA) ₁₇	154-230	+
mPdCIR078	TGGATTTCCATTGTGAG	CCCGAAGAGACGCTATT	(GA) ₁₃	106-184	+
mPdCIR085	GAGAGAGGGTGGTGTATT	TTCATCCAGAACCACAGTA	(GA) ₂₉	110-201	++
mPdCIR090	GCAGTCAGTCCCTCATA	TGCTTGTAGCCCTTCAG	(GA) ₂₆	108-202	-
mdCIR093	CCATTATCATTCCTCTCTTG	CTTGGTAGCTGCGTTTCTTG	(GA) ₁₆	150-188	+

Discussion

This study shows the use of phenotypic traits, proximate composition of fruit and molecular markers to evaluate diversity and characterize date palm cultivars in Pakistan. Statistical analysis revealed that the cultivars possess significant variation with respect to the studied traits. Nine phenotypic and four biochemical traits were rendered important to characterize local date palm cultivars. Leaf length [10] and spine number and spined midrib part length [9] were found to be important discriminants for date palm cultivars from Pakistan and Mauritania respectively. Fruit characteristics were found to be important for differentiation of date palm cultivars from Adrar region of Mauritania [32]. Highest fruit weight (26.50gm) and volume (26.00cm³) was measured in Dhaki. This is in accordance with [24] who recorded highest weight and volume for Dhaki among 21 date palm varieties of Pakistan. Fruit weight of Dhaki cultivar was more than the maximum fruit weight (12.78gm) recorded for Sudanese date fruit cultivar by [33]. Fruit length and diameter of date palm cultivars of Pakistan were recorded to be in the range of 2.4cm-6.0cm and 1.5cm-4.5cm respectively [14]. [23] also recorded maximum length (5.09cm), diameter (3.06cm), fruit weight (23.36g) and pulp weight (21.58g) for date palm variety Dhaki. Date fruit completes its development in terms of size at its kimri stage although there is a minor change in the fruit length that must be due to moisture loss at maturity [24]. Maximum moisture content was found to be 83.08 % for Peelisunder while [23] recorded maximum moisture content (67.38%) of date fruit at 'Khalal' stage for Dhaki variety. Total sugar and reducing sugar content of the studied varieties was recorded in the range of 12.86%-46.29%

and 9.87%-38.16% respectively. Maximum moisture content (83.08%) was found for Peeli Sunder having minimum value of TSS (14⁰ Brix). Ash content was found to range from 0.64 to 4.54%. Ash content varied from 1.82%-2.87% in eight date palm cultivars studied by [34].

Correlation study among the traits of date palm showed that vegetative traits were positively correlated with other vegetative traits but had no significant correlation with fruit traits. Similarly some fruit traits were found to be positively correlated with other fruit traits but fruit traits had no significant correlation with vegetative traits. Positive correlation between leaf length and midrib length and pinnae number and pinnated part length was also reported by [10] for Moroccan date palm cultivars and this can be explained as the long leaf can have a long midrib with greater number of pinnae thus accounting for a greater pinnated part length. The dendrogram based on combined morphological and proximate data (Fig 2) clustered Peeladora and Kerbaline along with other 9 cultivars in one cluster but these two cultivars were grouped in different clusters from the rest of the cultivars (Pathri, Makhi, Kokna, Eedal Shah, Basrawali, Sufaidah, Gajjarwali, Kur, Fasli, and Shakri) in dendrogram based on molecular data. Makran, Haleni, Baidhar and Kupra were clustered at one point with closely related Saib and distantly related Champa kali at one point in one cluster and Berahmi, Khudrawi, Zirin, Daanda, Rachna, Shamran, Zahidi, and Kozanabad in another cluster by the dendrogram based on molecular data while all these cultivars were grouped together in one cluster by combined morphological and proximate data. Begum Jhangi, Neelum, Zerdo, Shado and Wahan wali were grouped in one cluster with

Angoor, Sanduri, Halwain and Taarwali by combined morphological and proximate data while the later were grouped in different cluster by molecular data. Aseel and Halwai were placed in one cluster in both the dendrograms while Jansohar, Peelisunder and Chohara were placed in different clusters by molecular data.

Clustering of cultivars at one point on the basis of morphological traits may be due to the local naming where one cultivar is given different names or multiple cultivars may be given the same name. Unrelated cultivars may be grouped together due to the similarity in fruit characteristics. This is also observed by [33] while studying the biodiversity of dates in the Saltanate of Oman. The dendrogram formed by morphological and proximate data shows similar relationship among the cultivars as shown by the score plot of the principal component analysis.

Conclusion

The studied date palm cultivars grown in Pakistan showed great variation with respect to morphological characteristics and proximate composition of the fruit whereby some traits like length and width of leaf of date palm tree, length, diameter, weight and volume of date fruit, weight of fruit pulp and seed, height of perianth, % moisture content, total soluble solids, % total sugars and %reducing sugars were found as important parameters for characterization of date palm cultivars but microsatellite SSR markers have shown greater genetic similarity among these cultivars. Hence it is concluded from this study that although great morphological and compositional variation exists among the studied cultivars but SSR markers used in this study were unable to reflect that polymorphism correctly. The reason may be that the studied markers do not

represent those genetic regions where variation exists. Thus to look for genetic basis of these variations more robust sequence based molecular markers like single nucleotide polymorphism markers should be used to underpin the genetic regions causing these variations.

Authors' contributions

Conceived and designed the experiments: A Muhammad, GM Ali, MZ Hyder & H Rahman, Performed the experiments: N Faqir, Analyzed the data: N Faqir & A Shehzad, Contributed reagents/ materials/ analysis tools: A Muhammad, GM Ali, A Shehzad, MZ Hyder, Wrote the paper: N Faqir.

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